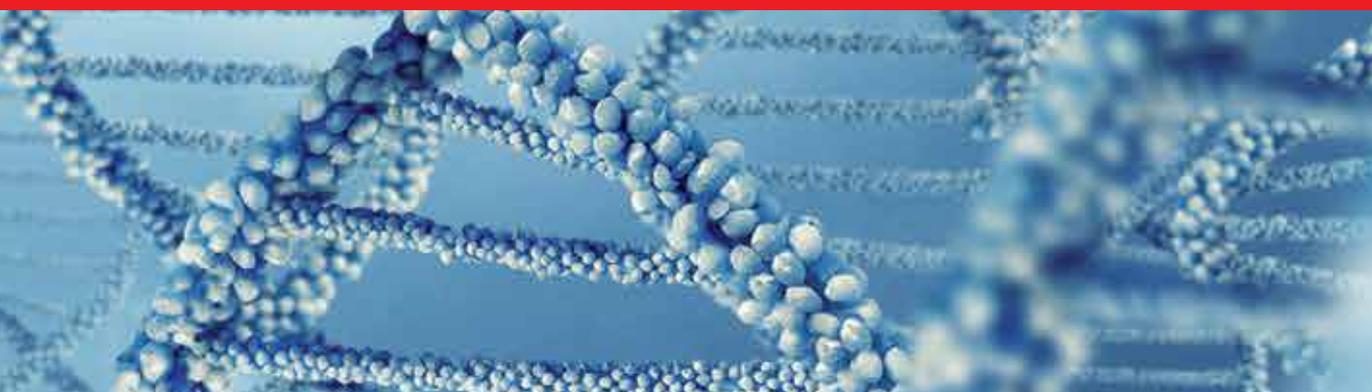




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DNA Repair and Human Health

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Meet the editor



Dr. Sonya Vengrova did her undergraduate studies in the Moscow State University in Russia. During this period, she also did a research project at the National Cancer Institute, NIH, USA, and gained her Diploma from the Moscow State University in 2000. Subsequently, she went to the Rockefeller University in New York, USA, to work in the field of developmental genetics. She moved to the UK to start a PhD program at the Imperial College, London, UK in 2009, in the field of genome stability. The research was funded by the Marie Curie Cancer Care, and was carried out in the Marie Curie Research Institute, UK. Dr. Vengrova gained her PhD in 2004 and continued post-doctoral work at the Marie Curie Research Institute. In 2009, she moved to the University of Warwick, Coventry, UK, where she currently works as a Wellcome Trust research fellow on projects addressing different aspects of genome stability.

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Preface

The genomic DNA of the cell is constantly under attack by the damaging agents from variety of sources. Ultraviolet light, ionizing radiation and environmental chemicals, as well as reactive oxygen species originating from the cellular metabolic processes, stochastic breakage of bonds within the DNA molecules and programmed cleavage of the DNA strands during differentiation all threaten the stability and integrity of the genome.

Changes in the DNA sequence, be it single nucleotide substitutions, small deletions or insertions, or rearrangements of large chromosomal fragments may disrupt the finely tuned network of interactions within the cell. Such changes can result in cell death, or in alterations in cellular growth and differentiation program. To prevent this, there is a complex network of DNA repair pathways, which exists to ensure that the damage is promptly detected and correctly repaired.

Over the last decades, great advances have been made in identifying the components and dissecting interactions within the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research into the mechanisms of DNA repair is merging with clinical research, in particular with a fast developing field of clinical genetics, which has been aided by the advancement of genome sequencing techniques. As a result, a new picture is emerging, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach provides understanding of the disease mechanisms and is invaluable for improving diagnostics and prevention, as well as designing better therapies.

The central role of DNA repair in human health and well-being is illustrated by the reviews presented in this book. Detailed descriptions of DNA repair pathways can be found in several chapters. A large body of evidence addressing the link between DNA damage repair and a number of diseases, such as cancers, Fanconi Anemia, Xeroderma pigmentosa and Alzheimer's is analysed. In addition, the role of DNA repair processes in ageing, viral infection and the link between inflammation and DNA repair is reviewed.

The chapters are tentatively assigned to sections, however, many reviews touch upon various aspects of DNA repair, so the titles of the sections by no means limit the scope of individual reviews.

This book will be of interest to a broad audience. The biochemists, geneticists and molecular biologists working on different aspects of DNA repair in any model system, will gain an insight into the place of a particular DNA repair factor or pathway in the context of the whole organism, while medical researchers will find a comprehensive overview of the molecular mechanisms of DNA repair.

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Part 1

DNA Repair Mechanisms

DNA Repair, Human Diseases and Aging

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1. Introduction

One of the most fundamental functions of a cell is the transmission of genetic information to the next generation, with high fidelity. On the face of it, this seems a challenging task given that cells in the human body are constantly exposed to thousands of DNA lesions every day both from endogenous and exogenous sources. Yet, for long periods of time, the DNA sequences are one of the most invariable and stable components of a cell, a task achieved by an arsenal of DNA damage detection and repair machinery that detects and fixes DNA lesions with high fidelity at each and every round of cell division. In this chapter, we describe how normal cells cope with DNA damage and the manner in which a defective DNA damage response can lead to human disease and aging.

2. The types and sources of DNA damage

All living cells of the human body have to constantly contend with DNA damage. Due to its chemical structure itself, the DNA is sensitive to spontaneous hydrolysis which leads to damage in the form of abasic sites and base deamination. Single strand breaks (SSBs) and oxidative damage such as 8-oxoguanine lesions in DNA are generated by endogenous by-products of metabolism like reactive oxygen species (ROS) and their highly reactive intermediates. For example, it is estimated that about 100-500 8-oxoguanine lesions form per day in a human cell (Lindahl, 1993). The formamidopyrimidine lesions, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine are also formed at similar rates as 8-oxoG after oxidative stress. Spontaneous DNA alterations may also arise due to dNTP misincorporation during DNA replication. Put together, it is estimated that each human cell can face up to 10^5 spontaneous DNA lesions per day (Maynard et al., 2009).

Apart from endogenous sources, DNA can also be damaged by exogenous agents from the environment. These include physical genotoxic stresses such as the ultraviolet light (mainly UV-B: 280-315 nm) from sunlight which can induce a variety of mutagenic and cytotoxic DNA lesions such as cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Rastogi et al., 2010). DNA damage in the form of double strand breaks (DSBs) can be incurred as a result of medical treatments like radiotherapy, ionising radiation (IR) exposure from cosmic radiation or as a result of the natural decay of radioactive compounds. For example, uranium decay produces radioactive radon gas, which accumulates in some houses to cause an increased incidence of lung cancer (Jackson and Bartek, 2009). Nuclear

disasters like the Chernobyl nuclear disaster, nuclear detonations during World War II or more recently, the radiation leakage from the Fukushima power plant in Japan are examples of other sources of severe exposure to exogenous radiation. Chemical sources of DNA damage include chemotherapeutic drugs used in cancer therapy or for other medical conditions. Alkylating agents such as methyl methanesulfonate (MMS) induce alkylation of bases, whereas drugs such as mitomycin C, cisplatin and nitrogen mustard cause DNA interstrand cross links (ICLs), and DNA intrastrand cross links. Chemotherapeutic drugs like camptothecin and etoposide are topoisomerase I and II inhibitors respectively, and give rise to SSBs or DSBs by trapping topoisomerase-DNA complexes. Other exogenous DNA-damaging sources, that are carcinogenic as well, are foods contaminated with fungal toxins such as the aflatoxin and overcooked meat products containing heterocyclic amines. Another common source of environmental mutagen is tobacco smoke, which generates DNA lesions in the form of aromatic adducts on DNA and SSBs (Jackson and Bartek, 2009).

3. DNA repair pathways

To maintain genomic integrity, a cellular machinery composed of multi-protein complexes that are capable of detecting and signalling the presence of DNA lesions and delaying cell cycle progression to promote DNA repair is activated, called as the DNA damage response (DDR) (Harper and Elledge, 2007). To accomplish DNA repair, cells utilise biochemically distinct pathways specific of the DNA lesion, which are integrated with appropriate signalling systems to delay cell division until the completion of repair (Figure.1). Small chemical alterations caused by the oxidation of bases is detected and repaired by the base excision repair system (BER), through the direct excision of the damaged base. DNA replication errors or polymerase slippage errors that commonly result in single base mismatches and insertion-deletion loops are corrected by the mismatch repair system (MMR). Lesions such as pyrimidine dimers and intrastrand cross links are corrected by the nucleotide excision repair (NER) pathway. SSBs are removed by the SSB repair pathway whereas DSBs are repaired by the DSB repair systems, which itself could be either by homologous recombination (HR) or non-homologous end joining (NHEJ) pathway (Ciccia and Elledge, 2010).

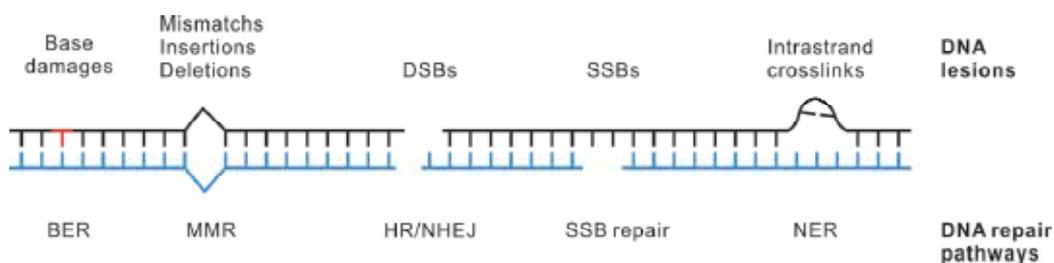


Fig. 1. Summary of DNA repair pathways

3.1 Base excision repair and single strand break repair

Base excision repair (BER) is the major pathway responsible for handling the mutagenic and cytotoxic effects of DNA damage that can arise due to spontaneous hydrolytic, oxidative, and non-enzymatic alkylation reactions. (Wilson and Bohr, 2007). This pathway focuses on

DNA lesions that do not tend to cause structural distortions of the DNA double helix. The BER target lesions can be classified as oxidised/reduced bases such as 8-oxo-G/FapyG, methylated bases, deaminated bases or bases mismatches. There are two types of BER: short patch and long patch. During short patch BER, only the damaged nucleotide is replaced, whereas in long patch BER, 2-6 new nucleotides are incorporated.

The very first step in BER involves the use of DNA glycosylases that cleave the *N*-glycosyl bond between the damaged base and sugar to generate an abasic site, called as the AP (apurinic/aprimidinic) site. Similar AP sites may also arise due to the spontaneous depurination or depyrimidination of bases. The DNA glycosylases that perform this function are classified as monofunctional or bifunctional, depending on their mode of action. Monofunctional glycosylases such as UNG only possess the glycosylase activity and therefore a second enzyme called the APE1 lyase is required to cut base-free deoxyribose to generate the 5'-deoxyribose phosphate termini (dRP). The next step involves the action of DNA polymerase β (pol β) which removes the dRP group left behind by APE1 incision in the short-patch pathway. However, if the 5' terminal is refractory to pol β activity, strand displacement synthesis is required in order to incorporate multiple nucleotides by long-patch pathway. In this case, several enzymes such as proliferating cell nuclear antigen (PCNA), FEN1 and pol β and/or pol δ/ϵ act together to remove the blocking terminus. The final step of BER consists of ligation of the remaining nick, by either Lig1 alone or Lig3-XRCC1 complex (Maynard et al., 2009).

SSB repair pathway is a major pathway responsible for the repair of SSBs that arise directly as a result of ROS-induced disintegration of oxidized deoxyribose or genotoxic stresses such as IR. SSBs may also arise spontaneously due to the erroneous activity of DNA topoisomerase 1 (TOP1). TOP1 creates a cleavage complex intermediate which contains a DNA nick to allow DNA relaxation during DNA replication and transcription. Usually, the nick generation is transient and is rapidly sealed by TOP1. However, if the nick inadvertently collides with RNA polymerase, SSBs may be generated in the process. In addition to the above scenarios where SSBs are generated directly within cells, SSBs may also arise indirectly as BER intermediates due to the lyase activity of bifunctional glycosylases such as OGG1 and NEIL1.

Single strand break repair (SSB) involves the activation of PARP (poly ADP ribose polymerase) family members PARP1 and PARP2, which act as sensors of SSBs, through the two PARP1 zinc finger motifs (Caldecott, 2008). Upon activation of PARP1 and PARP2, poly (ADP-ribose) chains are synthesized within seconds, and assembled on target proteins such as histone H1 and H2B and PARP1 itself (Schreiber et al., 2006). Histone PARylation contributes to chromatin reorganization and helps in the recruitment of DNA repair and chromatin remodelling proteins to DNA damage sites. Three types of PAR-binding motifs have been identified: the PBZ (PAR-binding zinc finger), the macrodomain and an 8 amino acid basic residue-rich cluster. Many DDR factors such as p53, XRCC1, Lig3, MRE11 and ATM have the 8 amino acid basic residue-rich cluster, whereas macrodomains containing proteins include PARP9, PARP14, PARP15, the histone variant macroH2A1.1, and the chromatin remodeling factor ALC1. PBZ motifs have been identified in the nucleases APLF and SNM1 and in the cell cycle protein CHFR.

Once SSBs are detected, they undergo end processing, where the 3' and/or 5' termini of SSBs are restored to conventional 3'-hydroxyl and 5' phosphate moieties for gap filling and DNA ligation to occur. XRCC1 has a particularly important role during DNA end

processing step, since XRCC1 directly interacts with enzymes such as pol β , PNK (polynucleotide kinase) and the nucleases APTX and indirectly with DNA ligase III α and TDP1 (tyrosyl-DNA phosphodiesterase) to facilitate end processing. Damage termini present at indirect BER-induced SSB are repaired by APE1, Pol β , PNKP and APTX. Direct sugar damage-induced SSBs are repaired by APE1, PNKP and APTX. SSBs generated by TOP1 are repaired by TDP1. Finally, end processing is followed by gap filling and ligation, and the enzymes utilized during these two steps overlap with BER (as described above). It is interesting that ~30% of all human tumors examined express a variant form of DNA polymerase β (Starcevic et al., 2004) Moreover, at least two proteins, TDP1 and APTX, involved in SSB repair are mutated in an inherited form of human neurodegenerative disease (discussed in Section 5.1).

3.2 Mismatch repair

The replication of DNA sequences in the S phase of cell cycle is subjected to a low but significant level of error that includes the inadvertent incorporation of chemically altered nucleotides in place of the normal counterparts. The cell has two main strategies to detect and remove the miscopied nucleotides. In a 'proofreading' type of monitoring executed by the DNA polymerase δ (pol- δ), the stretch of DNA that has been newly synthesized is scanned, and if misincorporation of nucleotides is detected, the enzyme uses its 3'-5' exonuclease activity to remove the aberrant nucleotide and resynthesize the DNA stretch. However, if the miscopied DNA sequence is overlooked by DNA pol- δ , the MMR pathway is activated that detects and corrects misincorporated nucleotides. The action of this pathway becomes especially critical in regions of DNA that carry repetitive sequences such as mono or dinucleotide repeats. The resulting base substitution mismatches and insertion-deletion mismatches (IDLs) may escape correction by the proofreading mechanism described above, making the repair of such defects the prime function of the MMR machinery (Kunkel and Erie, 2005).

The 'Mut' proteins are the principle active components of the mismatch repair pathway (Kolodner and Marsischky, 1999). The main function of the Mut proteins lies in the ability to detect bulges and loops in the newly formed DNA and in being able to distinguish between recently synthesized DNA from the complementary parent strand. In mammalian cells, two components of the MMR apparatus, MutS and MutL collaborate to correct mismatched DNA. MutS homologs form two major heterodimers, Msh2/Msh6 (MutS α) and Msh2/Msh3 (MutS β) which scan for the mismatched base on the newly formed daughter strand and bind directly to the mutated DNA. The main difference between the two MutS complexes is that MutS α pathway is mainly involved in the repair of base substitution and small loop mismatches, while the MutS β pathway is also involved in large loop repair. After the recruitment of MutS, MutL dimer is loaded to the mutated site through its binding to the MutS-DNA complex. MutL has three forms designated as MutL α , MutL β , and MutL γ . The MutL α complex is made of two subunits MLH1 and PMS2, the MutL β heterodimer is made of MLH1 and PMS1, while MutL γ is made of MLH1 and MLH3. MutL α is an endonuclease that facilitates strand-discrimination and nicks the discontinuous strand of the mismatched duplex in a PCNA, RFC and ATP-dependent manner. Lastly, the excision reaction is performed by the 5'-3' single-stranded exonuclease EXO1, whose exonuclease activity is increased by its direct interaction with MutS α . The resulting fragment is excised and a fresh

attempt at resynthesizing DNA using DNA pol- δ is made using the parent strand as the template (Fukui, 2010; Larrea et al., 2010).

The MMR pathway is particularly important in mammals, where the mutator phenotype conferred by loss of MMR activity contributes to the initiation and promotion of multistage-carcinogenesis (Venkatesan et al., 2006)

3.3 Nucleotide excision repair

The activation of NER pathway seems to require two distinct structural changes in DNA: a significant distortion to the native conformation of DNA and the presence of a chemically altered base. NER is accomplished by a large multisubunit complex composed of almost two dozen subunits. NER comprises of two distinct subpathways that mainly differ in the molecular mechanism used to identify the damaged base. Global genome repair (GGR) repairs DNA damage throughout the genome, whereas transcription-coupled repair (TCR) repairs lesions in regions that are undergoing transcription (Nouspikel, 2009).

Historically, the NER pathway was discovered through the study of a human syndrome involving a severe burning of the skin after only a minimal exposure to sun light. These individuals show dry, parchment like skin (Xeroderma) and freckles (pigmentosa). Individuals suffering from Xeroderma Pigmentosa (XP) have a 1000-fold greater risk of developing skin cancers and inherited defects in any of the so-called XP genes cause this syndrome. Subsequently, elegant somatic cell genetic experiments were performed and seven genetic complementation groups of the human XP, designated XPA to XPG were identified in mammalian cells. According to current understanding, two independent complexes, one composed of the DDB1/DDB2 heterodimer, and the other containing the XPC/HR23B/Centrin 2 proteins are required for the early steps of base damage recognition during NER (Guo et al., 2010; Nouspikel, 2009).

UV-damaged DNA-binding protein (UV-DDB) is a heterodimeric complex composed of DDB1 and DDB2 which upon binding to UV-damaged sites activates a UV-DDB-associated ubiquitin ligase complex that recruits XPC protein to the lesion and promotes ubiquitination of DDB2 and XPC proteins (Sugasawa et al., 2005). Upon polyubiquitination, DDB2 loses its ability to bind to UV-irradiated DNA, whereas XPC upon ubiquitination shows increased DNA binding. In turn, XPC recruits the multiprotein transcription-repair complex called as the TFIIH (transcription factor IIH). Subsequent recruitment of RPA and XPA drive detachment of cyclin-dependent kinase activating (CAK) subcomplex of TFIIH which is essential for GGR. The two helicases within the TFIIH complex (XPB and XPD) unwind the DNA by about 20 bp around the damage to form a stable complex called pre-incision complex 1 (PIC1). Localized unwinding around the damaged base then leads to the exit of XPC-HR23 and the entry of XPG to form PIC2. Finally, XPF-ERCC1 is recruited to form PIC3. At each of these steps ATP is hydrolyzed, and the free energy of ATP hydrolysis is used to unwind the helix as well as to amplify the damage recognition specificity of the enzyme system. Within PIC3, XPG and the ERCC1/XPF complex are both structure-specific endonucleases that carry out dual excision and cut the damaged strand of DNA 3' and 5' to the lesion, respectively. This generates a single-stranded oligonucleotide 24–32 nucleotides in length. The incisions are asymmetrical, such that the 3' incision occurs 2 to 8 nucleotides from the damaged base and the 5' incision occurs 15 to 24 nucleotides from the damaged base. The resulting gap is filled in by the combined actions of DNA polymerase δ or ϵ , proliferating cell nuclear antigen (PCNA), RPA, and DNA ligase I (or a complex of XRCC1 and DNA ligase III) (Hanawalt and Spivak, 2008).

Bulky lesions consisting of UV-induced pyrimidine dimers in transcribed regions can lead to RNA polymerase II stalling, evoking the activation of TCR. In TCR, the stalled RNA polymerase is removed and DNA damage is repaired, through a process initiated by the Cockayne syndrome proteins, CSA and CSB. The CSA protein belongs to the 'WD repeat' family of proteins and exhibits structural and regulatory roles while CSB protein which is an ATP-dependent chromatin remodelling factor in the SWI/SNF family. CSB, in turn recruits additional factors such as the histone acetyltransferase p300, the CSA-DDB1 E3 ubiquitin/COP9 signalosome complex (O'Connell and Harper, 2007). Similar to GGR, the subsequent steps involve the binding of TFIIH and XPA/RPA to the lesion and the nucleases XPG and XPF/ERCC1 carry out the incision 3' and 5' to the damaged lesion. The generated oligonucleotide is removed and the gap is filled in by the combined action of DNA polymerases δ or ϵ , PCNA, RPA, and DNA ligase I or XRCC1/ DNA ligase III.

3.4 DNA replication stress and ATR

Replication stress is a unifying term used to denote large unprotected regions of ssDNA generated during the course of DNA replication or formed at the resected region of DSBs (Lopez-Contreras and Fernandez-Capetillo, 2010). Both situations converge with the generation of a RPA-coated ssDNA intermediate which is the triggering signal for the DNA replication checkpoint. The ssDNA-RPA complex plays two critical roles: it recruits ATR by directly binding to the ATR partner, ATRIP. Secondly, it recruits and activates the Rad17 clamp loader which then loads the PCNA-like heterotrimeric ring 9-1-1 (Rad9-Hus1-Rad1) complex to DNA. The 911 complex binds to the ATR activator TopBP1, thus bringing it in close proximity to ATR-ATRIP, leading to ATR activation. Once activated, ATR initiates the DNA replication checkpoint through the phosphorylation of its downstream substrates. In addition to replication checkpoint signalling, ATR is also required for stabilization of stalled replication forks. In the absence of ATR, forks undergo a 'collapse' and are unable to resume replication upon the withdrawal of replication stress (Friedel et al., 2009). The absence of ATR also results in a specific type of genomic instability, named as DNA Fragile site expression. DNA fragile sites (DFS) are large (>100 Kb) genomic regions that exhibit breaks under conditions of replication stress. DFS sites are 'hot spots' for sister chromatid exchanges and are involved in gene amplification events via a breakage-fusion-bridge cycle. Breakage at DFS is associated with several cancers (Dillon et al., 2010).

3.5 Interstrand cross link repair (ICL repair)

ICLs are generated by cross linking agents like mitomycin C and these lesions covalently connect the two strands of DNA to form a barrier to replication fork progression. Important components of the ICL repair pathway are 13 genes mutated in the genetic syndrome, Fanconi anemia (FA). FA is an autosomal recessive cancer predisposition disorder characterized by progressive bone marrow failure, congenital developmental defects, chromosomal abnormalities and hypersensitivity to ICL agents (Kitao and Takata, 2011). The gene products of FA constitute a common pathway called the FA pathway, whose main role is in the repair of ICL lesions to maintain genomic integrity (Moldovan and D'Andrea, 2009). Eight of the FA proteins (FANCA, B, C, E, F, G, L and M) form the so-called FA core complex, an E3 ubiquitin ligase that monoubiquitinates downstream FANCD2-FANCD1 dimer. The core complex also incorporates FAAP100 and FAAP24 and the heterodimer MHF1/MHF2 as crucial components. Upon monoubiquitination, the ID complex

accumulates at the sites of crosslinks and co-localizes with three additional FA proteins, Brca2 (FANCD), PALB2 (FANCN), and BACH1 (FANCI). Monoubiquitinated ID complex is required for the incision and translesion synthesis steps of ICL repair by promoting the recruitment of DDR factors required for ICL repair (Moldovan and D'Andrea, 2009). Recently, another level of regulation was revealed in the FA pathway, in that ID complex is phosphorylated first by ATR and this step was shown to be critical for further monoubiquitination by the core complex. Subsequently, factors required for HR are also recruited to repair the DSBs generated during the repair process. Thus, ICL repair requires the coordinated recruitment and concerted action of several DNA repair pathways.

3.6 Double strand break repair

DNA double strand breaks are the most deleterious type of DNA damage and cells have evolved at least four types of repair pathways to detect and correct DSBs. These pathways include homologous recombination (HR), non-homologous end joining (NHEJ), alternative NHEJ pathway. The main factor influencing pathway choice is the extent of DNA processing. While NHEJ does not require DNA end resection, HR and alternative NHEJ pathways are dependent on end resection (Ciccia and Elledge, 2010).

NHEJ is an error-prone process, where broken ends are recognised and sealed together and mainly occurs in G₀, G₁ and early S phase of the cell cycle and requires the DNA-PK (DNA protein kinase) complex and the Lig4 complex (Lieber, 2010). The DNA-PK complex consists of the Ku70 and Ku80 heterodimer which recognises and binds to the DSB and recruits DNA-PKcs, the catalytic subunit of DNA-PK. Following DSB binding, DNA-PKcs autophosphorylation on the six-residue ABCDE cluster (T2609) causes destabilization of the DNA-PKcs interaction with DNA thus providing access to end processing enzymes such as ARTEMIS (Goodarzi et al., 2006). On the other hand, to prevent excessive end processing, DNA-PKcs is also autophosphorylated at the five-residue cluster called as the PQR cluster at S2056, which helps protect DNA ends. Thus, two reciprocally acting phosphorylation clusters regulate end processing by DNA-PKcs (Cui et al., 2005).

Following the recognition of DSBs, ends must be transformed into 5'phosphorylated ligatable ends to complete repair. One key enzyme required for end processing is ARTEMIS, which might be recruited to DSBs through its ability to interact with DNA-PKcs. End processing is also carried out by APLF nucleases and the PNK kinase/phosphatase prior to ligation (Mahaney et al., 2009). Next, the end processing of complex lesions might lead to the generation of gaps which are filled in by DNA polymerase μ , polymerase λ and terminal deoxyribonucleotidyltransferase. The final step involves the ligation of DNA ends, a step carried out by X4-L4 (a complex containing XRCC4, DNA ligase IV and XLF) (Hartlerode and Scully, 2009).

Interestingly, when the classical NHEJ pathway is inhibited, an alternative end-joining pathway operates in cells. This substitution is called as alternative end joining (alt-EJ), backup NHEJ (B-NHEJ) or microhomology mediated end joining (MMEJ) (Lieber and Wilson, 2010). This pathway functions even in the absence of classical NHEJ factors such as Ku, XRCC4 or DNA ligase IV. For example, the alt-NHEJ pathway is robustly activated in mice lacking X4-L4. Alt-NHEJ is mediated by the annealing of ssDNA microhomology regions followed by LIG3-dependent DNA end ligation (Ciccia and Elledge, 2010; Hartlerode and Scully, 2009). Microhomologies are short stretches of complementary "microhomology" sequences (1–10 base pairs) that often appear at repair junctions. This suggests that even limited base pairing between two ends of a double-strand break is

exploited during alt-NHEJ repair (Lieber and Wilson, 2010). Alt-NHEJ pathway is error-prone and frequently results in small deletions/insertions around the region of double strand breaks or could even result in deleterious translocations (Zhang and Jasin, 2011).

Unlike NHEJ, HR is an error-free process that involves the use of the undamaged homologous sister chromatid as the template to facilitate DNA repair of the damaged strand and is carried out in cells that are in the late S and G2 phases of cell cycle (Moynahan and Jasin, 2010). Homologous recombination (HR) provides an important mechanism to repair double strand breaks during mitosis and meiosis. Defective HR can potentially transform cells by disrupting their genomic integrity. HR involves the detection of DSB by the MRN (MRE11-Rad50-NBS1) complex which promotes activation of ATM (Ataxia-telangiectasia mutated). In addition to stabilizing DNA ends, MRE11 has endonuclease and exonuclease activities which are important to mediate 5'-3' resection along with CtIP to generate single strand DNA (ssDNA). ssDNA is coated by the ssDNA-binding protein, RPA which is a heterotrimeric complex (RPA1, RPA2, RPA3) that stabilizes ssDNA regions generated during both DNA replication, repair and recombination. At the double strand break, the RPA-coated ssDNA recruits ATR-ATRIP to chromatin, an event critical for the activation of the ATR-chk1 pathway. Similar to the trimeric RPA complex, the newly identified human ssDNA binding protein, hSSB1 is thought play a role in checkpoint activation and repair (Richard et al., 2008). The next critical step in HR is the assimilation of Rad51 to ssDNA in a Brca2 and PALB2 (Partner and localizer of Brca2) dependent manner to form the Rad51-coated nucleoprotein filament. Rad51-mediated homology search and strand invasion follows where the Rad51 recombinase utilizes the sister chromatid as the homologous template and together with Rad51 paralogs (Xrcc2, Xrcc3, Rad51L1, Rad51L2 and Rad51L3), Rad52 and Rad54 promotes strand invasion and recombination. Strand invasion and migration involve the formation of a structure termed as the Holliday junction. Following DNA polymerase δ -mediated DNA synthesis, resolution of Holliday junction occurs via Rad54/Mus81/Emc1 and Rad51C/Xrcc3 (San Filippo et al., 2008).

It is now becoming increasingly clear that the choice between the various DNA repair pathways is dictated by negative regulation of on one pathway by another. For example, DSB resection promoted by CtIP is inhibited by 53BP1 (Bunting et al., 2010). 53BP1 itself promotes NHEJ by increasing the stability of DSBs during ligation. Another example of this regulation was highlighted by a recent study where defective DSB resection in Brca1 mutant cells results in NHEJ-dependent chromosomal rearrangements which could be overcome by 53BP1 loss, suggesting that Brca1 might somehow overcome 53BP1 function at DSBs to promote HR (Bunting et al., 2010). Also, abnormal activity of alt-NHEJ in the absence of NHEJ induces chromosomal translocation in mammalian cells (Simsek and Jasin, 2010). Thus, the right choice of DSB repair pathways can be a critical determinant of genomic stability and alterations in the appropriate repair pathway choice can lead to DSB repair defects with deleterious consequences.

3.7 Double strand break signalling

Amongst the various classes of DNA repair, perhaps the best studied pathway with respect to DNA lesion sensing and signalling is the DSB repair signalling response. The response to DSBs has been characterised by the rapid localization of repair factors to DSB sites into subnuclear foci called ionizing radiation-induced foci (IRIF) (Bekker-Jensen and Mailand, 2010). At a more mechanistic level, the DDR proteins can be divided into three major classes

of proteins that act together to translate the DNA damage signal into an appropriate response. These consist of (a) sensor proteins that recognize abnormal DNA lesions and initiate the signalling cascade (b) transducers that amplify the damage signal and (c) effectors proteins that participate in a number of downstream pathways such as cell cycle, apoptosis and senescence (Figure 2: DNA damage signalling and response) (Jackson and Bartek, 2009).

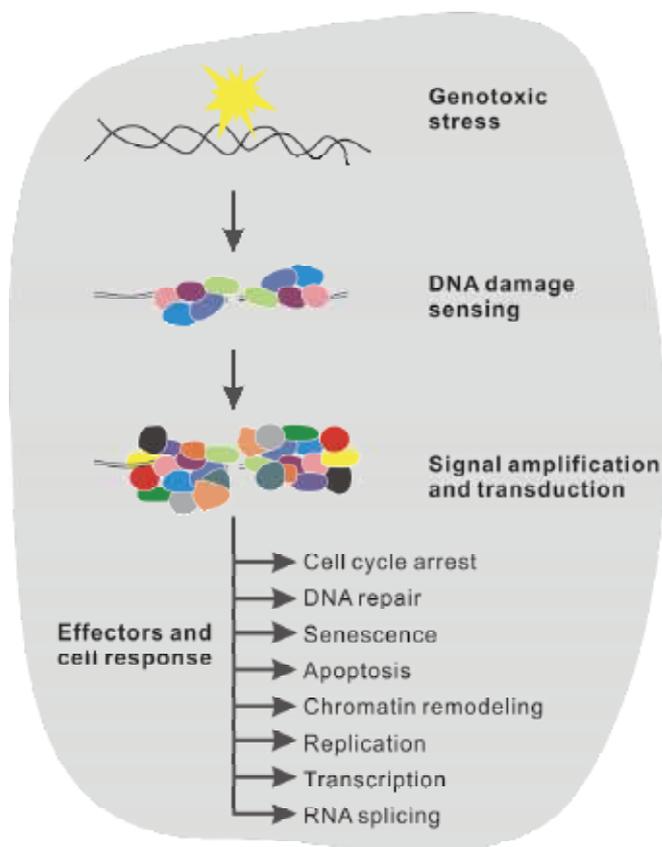


Fig. 2. Basic organization of the DNA damage response: Sensors, transducers and effectors

3.7.1 Sensing the damage

The efficient sensing of DSBs is achieved by Mre11-Rad50-Nbs1 (MRN) protein complex (Stracker and Petrini, 2011). The MRE11 (Meiotic Recombination 11) protein is an evolutionarily conserved protein that is involved in HR, NHEJ, meiosis and in the maintenance of telomeres. RAD50 is a homolog of *S.cerevisiae* Rad50 and is a member of the structural maintenance of chromosome (SMC) protein family. The third protein in the MRN complex is NBS1 (Nijmegen breakage syndrome 1) protein also known as Nibrin and p95. Structural and biochemical studies have elucidated the architecture of the MRN complex and have proposed the biochemical events that lead to the activation of the DDR. The key protein in the MRN complex is the Mre11 homodimer. This protein has the ability to bind free DSBs and also has an intrinsic 3'-5' exonuclease activity that can help in the resection of

broken ends. The Mre11 dimer can interact with two molecules of Rad50 and with the scaffolding protein Nbs1. The extended coiled-coil domains of Rad50 permit it to extend out from DNA breaks and bridge with another Rad50 protein through the Zn²⁺-binding CXXC motif, like a 'hook'. Thus, Rad50 might act as a molecular tether to bridge the DSBs.

The MRN complex interacts with the N-terminal of ATM and recruits it to DSBs (Lee and Paull, 2004). ATM belongs to the phosphatidylinositol-3-like kinase-related kinases (PIKK) family and plays an important role in the propagation of the initial DSB lesion by phosphorylating a number of downstream substrates. The PIKK family of proteins have a conserved kinase domain and three other domains: FAT (FRAP-ATM-TRRAP) domain, FATC domain (FRAP-ATM-TRRAP-C-terminal) and the PIKK regulatory domain that regulate the kinase activity of the protein. Apart from ATM, the other two PIKK proteins essential for DNA damage signalling are ATR (ATM and Rad3 related) and DNA-PKcs. ATM and DNA-PKcs are primarily involved in the signalling of DSBs and ATR is mainly required in response to stalled DNA replication forks. The interaction between the C-terminus of NBS1 and ATM leads to the recruitment of ATM to DSBs leading to its activation. In undamaged cells, ATM forms inactive dimers or multimers. However, upon the induction of DSBs, ATM is autophosphorylated at serine 1981, leading to its dissociation into activated monomers (Bakkenist and Kastan, 2003). Apart from ser1981, ATM is also autophosphorylated at serine 367 and serine 1983 and mutations at these sites reduce ATM activity. However, observations that transgenic mice carrying alanine substituted autophosphorylation serine sites 1987, 367 and 1899 of ATM (corresponding to human ATM serine 1981, 367 and 1893) display normal ATM-dependent responses, brought into question the function of ATM autophosphorylation *in vivo* (Daniel et al., 2008). This issue has been reconciled with the observation that autophosphorylation of ATM at 1981 is not needed for the initial recruitment of ATM to DSBs, but for the stable association of activated ATM with the damaged chromatin (So et al., 2009). The regulation of ATM autophosphorylation is under the control of three serine/threonine phosphatases, PP2A, PP5 and Wip1, so that ATM is not activated in an untimely manner in the absence of DSBs. Interestingly, defects in the activity of these phosphatases also lead to disease phenotypes in knock-out mice (Peng and Maller, 2010).

3.7.2 Amplification of the signal

Activated ATM rapidly phosphorylates and activates several DNA repair factors to directly promote their recruitment to sites of DNA damage. Perhaps, the most important event is the ATM-dependent phosphorylation of the histone variant, H2AX at the C-terminal of the protein, corresponding to Ser139 (γ -H2AX) (Rogakou et al., 1998). Remarkably, in mammalian cells, this phosphorylation spreads over a 2 Mb domain of chromatin surrounding the DSB. γ -H2AX flanked DSB creates a specialized chromatin compartment capable of recruiting and retaining DNA repair factors. Indeed, mice deficient for H2AX develop genomic instability and cancers (Celeste et al., 2002). A large number of proteins have been identified as substrates for activated ATM and this list includes the proteins SMC1, NBS1, CHK2, p53, BRCA1 and MDC1 (Harper and Elledge, 2007). Key amongst these substrates are the Chk2 kinase and p53 which act to reduce cyclin-dependent kinase (CDK) activity. The direct consequence of reduced Cdk activity is that cells arrest in the various stages of cell cycle to allow time of the completion of DNA repair. Amongst the various ATM substrates, MDC1, in particular, is an important mediator of DSB signalling because it

recognizes and binds γ -H2AX via its tandem BRCA1 C-terminal (BRCT) domains. The MDC1 C-terminal domain has been crystallized with γ -H2AX and the importance of this interaction was shown by experiments where mutations either in the phospho-acceptor site or in the conserved residues of BRCT domain impaired repair foci recruitment after DSB induction. Moreover, accumulation of several other repair factors such as NBS1, 53BP1 and the phosphorylation of ATM were reduced when MDC1- γ -H2AX interaction was abrogated. The serine-aspartic acid-threonine (SDT) repeats near the N-terminus of MDC1 are phosphorylated by Casein kinase 2, and this enables the interaction of MDC1 with NBS1. This interaction is not controlled by DNA damage, and MDC1 and MRN already exist as a complex in undamaged cells. Upon the generation of γ -H2AX, MDC1 together with MRN is recruited via the BRCT domain of MDC1. The concentrated binding of ATM to MDC1 and MRN further promotes the phosphorylation of H2AX, resulting in the amplification of the DDR. Thus, MDC1 is an important mediator of the DDR that regulates both the recruitment and retention of several downstream proteins (Huen and Chen, 2010).

Elegant work from the Misteli laboratory has established that the stable tethering of MDC1, MRN or ATM to DNA is sufficient to induce the DDR, even in the absence of DSBs. Upon targeted binding of MRN to a repetitive array, MDC1 and 53BP1 were recruited in an H2AX-dependent manner (Soutoglou and Misteli, 2008). These and other studies have given rise to the current model that MRN binding causes ATM recruitment and activation which initiates γ -H2AX formation. When MDC1 interacts with γ -H2AX, it provides a platform for the recruitment of MRN and ATM resulting in further propagation and spreading of γ -H2AX. Interestingly, when the kinetics of recruitment of DDR factors to DSBs was studied by live-cell imaging, NBS1 and MDC1 were the first factors to get recruited, seconds after DSB induction. The appearance of 53BP1 and BRCA1 was significantly slower than the MDC1 and NBS1 and more significantly, 53BP1 and Brca1 recruitment were abolished in MDC-null cells. This gave rise to a hierarchical model that the recruitment of 53BP1 and Brca1 was dependent on the stable recruitment of MDC1 through its interaction with γ -H2AX (Bekker-Jensen et al., 2006).

3.7.3 Recruitment of 53BP1 and Brca1

How exactly are Brca1 and 53BP1 recruited to DSB sites? A flurry of papers in recent years solved this conundrum through the identification of a ubiquitination cascade at DSB lesions. The product of the tumor suppressor gene *BRCA1* plays a central role in the maintenance of genomic integrity. Brca1 has been shown to regulate several cellular processes including transcriptional regulation, centrosome duplication, HR, NHEJ and checkpoint control. *BRCA1* encodes a large protein of 1863 amino acids and contains tandem BRCT domains in its C-terminal region (Huen et al., 2010). In its N-terminus, it harbours a RING finger domain that specifically interacts with the structurally related protein Bard1 (BRCA1-associated ring domain protein 1) to heterodimerize and form a functional E3 ubiquitin ligase which forms a complex with E2 UBCH5C to promote the formation of K6-linked ubiquitin chains, an unusual chain linkage. The Brca1-Bard1 heterodimer form three non-overlapping complexes with distinct functions. The complexes are formed between Brca1 and phosphorylated proteins through the BRCT domain. The complexes include Brca1-BRIP, Brca1-CtIP and Brca1-Abraxas-Rap80-BRCC36 complexes. The Brca1-Abraxas-Rap80 complex mainly accumulates at DSBs and promotes the G2/M checkpoint. BRCC36 is a deubiquitinating enzyme and it is speculated that it might regulate Brca1-Bard1

ubiquitination activity. The key mediator of Brca1 recruitment to DSB sites is Rap80. It was found that Rap80 depletion abolished Brca1 focus formation, whereas Brca1 depletion did not affect Rap80 focus formation. This strongly suggested that Rap80 controlled Brca1 focus formation. Upon mapping the site required for focus formation, it was found that the UIM motifs of Rap80 are required. The ubiquitin-binding function of the UIM motif is important to facilitate BRCA1-Rap80 recruitment to DNA lesions and the introduction of mutations that reduce ubiquitin binding also impair Brca1 and Rap80 focus formation. These studies led to the idea that a ubiquitin-dependent signalling system is important for Brca1 recruitment to sites of DSBs (Al-Hakim et al., 2010).

An important breakthrough in this area of research was made upon the identification of RNF8, the first of the three E3 ubiquitin ligases that catalyze regulatory ubiquitination at DNA lesions (Mailand et al., 2007). Importantly, the ubiquitin ligase activity of RNF8 was found to be absolutely essential for the recruitment of both Brca1 and 53BP1 to DSBs. RNF8 contains an FHA domain at its N-terminus and a RING finger motif at its C-terminus. The FHA domain of RNF8 recognises the ATM phosphorylated site of MDC1, leading to its recruitment to IRIF. The FHA domain of RNF8 also interacts with the HECT domain of the second E3 ligase HERC2, thus recruiting HERC2 to DSB sites. RNF8 together with HERC2 facilitates the assembly of the E2 ubiquitin conjugating enzyme Ubc13 to initiate K63-linked ubiquitin chains on H2A and its variants. The third E3 RING domain ubiquitin E3 ligase RNF168 recognizes and binds to K63-linked ubiquitin chains on H2A and H2AX through its two MIUs ((Motif Interacting with Ubiquitin). This amplifies the local concentration of K63-linked ubiquitin resulting in the recruitment and retention of 53BP1 and BRCA1 at the sites of lesions. Interestingly RNF168 was first identified as the gene mutated in RIDDLE syndrome (Stewart et al., 2009). RIDDLE (Radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties) is a novel human immunodeficiency disorder associated with defects in DSB repair and Brca1/53BP1 recruitment defects.

Recently, another layer of complexity has emerged in the scenario of post translational modifications occurring following DSB induction. DSB-induced ubiquitylation and the recruitment of BRCA1 and other repair proteins to the sites of damage are also regulated by SUMOylation, placing SUMOylation as a critical post-translational modification necessary for optimal ubiquitylation at DSBs (Tang and Greenberg, 2010). Apart of ubiquitination and sumoylation, methylation of histones is also important for 53BP1 recruitment. According to a recent study, the loading of 53BP1 to DNA lesions is enabled by a local increase in H4K20 dimethylation surrounding the DSBs and this step is catalysed by the histone methyltransferase MMSET (Pei et al., 2011).

3.8 Coordinating DDR with cell cycle transitions

One important cellular consequence of DNA damage is the activation of cell cycle checkpoints which are surveillance mechanisms that arrest the cell cycle until repair is satisfactorily accomplished. The cell cycle is regulated through oscillations in cyclin dependent kinase activity (CDK) whose activity is upregulated by cyclins and inhibited by cyclin-dependent kinase inhibitors (CKI) and inhibitory phosphorylation of CDKs (Guardavaccaro and Pagano, 2006). At the molecular level, the DNA damage checkpoint arrests cell cycle transitions by directly reducing CDK activity through various mechanisms mainly initiated by ATM and ATR. Two ATM-dependent G/S checkpoints have been described. ATM activation by DSBs in G1 leads to Chk2 phosphorylation and consequent phosphorylation of the phosphatase CDC25A. This results in the formation of a

phosphodegron which marks CDC25A for ubiquitin-mediated degradation and prevents the Thr14/Tyr15 dephosphorylation-mediated activation of CDK2. A second mechanism underlying the G1/S checkpoint involves the phosphorylation and stabilization of p53, either directly by ATM or indirectly by Chk2, which in turn acts as the transcription factor for the CKI, p21. This results in delayed G1/S transition after DNA damage. Together, CDC25A degradation and p21 upregulation form the basis for G1/S checkpoint maintenance (Bartek and Lukas, 2007).

During the S phase checkpoint, ATR is activated in response to stalled replication forks leading to the phosphorylation of Chk1 and the subsequent phosphorylation mediated degradation of Cdc25A by the SCF (Skp1-Cullin-F-box) β -TRCP ubiquitin ligase (Guardavaccaro and Pagano, 2006). This causes the inhibition of CDK2-Cyclin E/A activity preventing the initiation of new replication origins to slow down DNA replication. The failure to regulate Cdc25 leads to hyperactive Cdk activity and defective intra-S phase checkpoint. Yet another regulatory circuit to prevent DNA replication during repair involves the targeted degradation of Cdt1 which loads the replicative helicases MCM2-7 to form the pre-replication complex (Arias and Walter, 2007).

The G2/M checkpoint is initiated by the phosphorylation of checkpoint kinases, Chk1 and Chk2, which phosphorylate and inactivate CDC25C phosphatase by promoting its inhibitory sequestration by 14-3-3 proteins. This prevents the dephosphorylation-mediated activation of CDK1-Cyclin B complex required for mitotic progression (Lukas et al., 2004). Another target of the G2/M checkpoint are the Wee1 kinases. During a normal G2/M transition, Polo kinase 1(Plk1) phosphorylates Wee1 to create a phosphodegron that targets Wee1 for ubiquitin-mediated proteolysis. In the wake of G2/M checkpoint activation, Plk1 is negatively regulated by ATM/ATR and Wee1 accumulates in the cell to maintain CDKs in their inhibited form. Together, these mechanisms act in concert to halt cell cycle progression until the completion of DNA repair (Harper and Elledge, 2007).

3.9 Identification of novel ATM/ATR substrates using proteomic approaches

Recently, it has become apparent that DNA damage-activated kinases do not simply contact key individual proteins in a process, but phosphorylate multiple proteins of individual pathways. Importantly, understanding such linkages could have tremendous implications for human disease. In a large scale proteomic study, about 900 phosphorylation sites containing a consensus ATM and ATR phosphorylation motif were identified in 700 substrates that were inducibly phosphorylated after irradiation (Matsuoka et al., 2007). Based on the rationale that ATM and ATR phosphorylate substrates at the consensus SQ/TQ motifs, a phospho-antibody specific directed against this consensus site was used to immunoprecipitate peptides and mass spectrometric analyses was performed. The identified proteins were clustered into modules based on their known function. Multiple modules involved in DNA replication were identified such as the Orc module consisting of Orc3 and Or6, MCM module, including Mcm2, Mcm3, Mcm6, Mcm7 and Mcm10, RFC clamp-loader module consisting of Rfc1 and Rfc3 and the DNA polymerase module composed of the catalytic subunit of DNA polymerase epsilon, its interacting protein PolE4 and two translesion polymerases PolL and PolQ. Other modules included the mismatch repair module consisting of the proteins, Msh2, Msh3 and Msh6 and Exo1, excision repair module included XPA, XPC, RPA1, CSB, components of the transcription factor IIIH, a fanconi anaemia module and a HR module. Interestingly, three components of the COP9 signalosome involved in the regulation of SCF (Skp1-Cullin-F-box) E3 ligase function and a

novel cell cycle module comprising Cyclin E and its negative regulators FBW7, p27kip1 and CIZ1 were also identified as ATM/ATR substrates. Other proteins included components of the spindle checkpoint pathway comprising of Bub1, Mad1, Sgo1 and Mad2BP, Cdc26, separase, and cohesion subunits SMC1 and SMC3. Perhaps, the most interesting group of substrates were proteins in the insulin-IGF-1 (insulin-like growth factor)-PI3K (phosphatidylinositol 3-kinase)-AKT pathway, including the adaptor molecule IRS2 (insulin receptor substrate 2), the kinase AKT3, two regulators of AKT, HSP90 (heat shock protein 90), and PP2A (protein phosphatase 2A), and several downstream effectors of AKT such as FOXO1, and proteins involved in translation control such as TSC1 (tuberous sclerosis 1), 4E-BP1 (eIF4E binding protein 1), and p70S6K (ribosomal protein S6 kinase). This indicates that the DDR is likely to control the insulin-IGF pathway in multiple ways and further studies in this direction will shed light on the role of ATM/ATR pathway in human metabolic syndromes such as diabetes and other age-associated metabolic disorders.

4. Physiological roles of DDR

It has now become apparent that the DDR pathway is not just activated in response to genotoxic stress, but that it is essential for several physiological processes. Examples where genome alterations are induced in a programmed manner are V(D)J recombination (Bassing and Alt, 2004), class switch recombination (CSR) (Stavnezer et al., 2008) and somatic hypermutation (SHM) (Di Noia and Neuberger, 2007). These processes occur in developing T and B lymphocytes to generate T-cell receptor and immunoglobulin diversity, to allow the recognition of pathogens (Jackson and Bartek, 2009). In the meiotic cells, the DDR plays an important role when homologous chromosomes align and exchange genetic information by recombination. Meiotic HR is generated by the topoisomerase II-like enzyme Spo11, which generates DSBs. In subsequent steps that require MRN complex, HR recombination occurs which requires all mitotic HR proteins along with a Rad51-like protein, DMC (Richardson et al., 2004). In the developing nervous system, high levels of oxidative and metabolic stress are effectively repaired by the DDR. During infections with pathogens such as the avian influenza virus, the DDR proteins modulate the virulence properties of the virus through recombination (Jackson and Bartek, 2009). During bacterial infections, bacterial pathogens with defects in MMR, termed mutators or hypermutators, are overrepresented and are hypothesized to be advantageous for the establishment of chronic infections (Sundin and Weigand, 2007). DDR proteins also play important roles at normal telomeres and thus their defects cause telomere shortening and/or telomere dysfunction associated with chromosome fusions and instability (Jackson and Bartek, 2009). Telomeres are recognized by DDR proteins such as Mre11 (DSB repair), XPF/Erc1 (NER), Ku70/Ku80 (NHEJ repair), Bloom and WRN RecQ helicases and Rad51D (HR repair) (Denchi, 2009). These factors are recruited to the telomeres through their direct interaction with protein of the shelterin complex, a complex of six telomere binding proteins that promote telomere homeostasis. Recent studies have also established a relationship between DDR and another physiological phenomenon, the circadian rhythm, a process controlled by light stimuli (Sancar et al., 2010). The circadian rhythm regulator *clk-2* has been shown to affect radiation sensitivity in *C.elegans* and S phase checkpoint in response to replication stress in mammalian cells. NER has also been shown to be regulated by the circadian clock and there is strong evidence that the clock protein Cry participates in the maintenance of genomic integrity against DNA

damage induced by UV and UV mimetics. It has been proposed that such linkages between light cycle and DNA repair can allow cells to respond appropriately to environmentally-induced DNA damage ((Jackson and Bartek, 2009)). Described in greater detail below are the physiological roles of DDR in the immune system, meiosis and in the maintenance of genome integrity of stem cells.

4.1 V(D)J recombination and class switch recombination

One of the cell types where programmed DSBs are generated are B and T lymphocytes. B and T cells are the main components of the adaptive immune system and responsible for the generation of B cell receptors (BCRs, also known as immunoglobulins) and T cell receptors (TCRs), which together recognize a large repertoire of antigens. During the development of antigen receptor genes, a large number of variable (V), diversity (D), joining (J) and constant (C) segments undergo rearrangement by processes termed as V(D)J recombination and class switch recombination (CSR) (Bassing and Alt, 2004; Stavnezer et al., 2008). Immunoglobulin (Ig) contains a heavy chain and either a κ or λ light chain. TCRs are composed of either $\alpha\beta$ or $\gamma\delta$ dimers. In humans, Ig heavy chain loci contain 51 V_H , 27 D_H , 6 J_H and 9 C_H segments and Ig light chain loci contain 40 V_κ , 31 V_λ , 5 J_κ and 4 J_λ segments; TCR loci consist of 54 V_α , 61 J_α , 67 V_β , 14 J_β , 2 D_β , 14 V_γ , 5 J_γ , 3 V_δ , 3 J_δ and 3 D_δ segments. Roughly, 10^7 Igs and TCRs can be generated by V(D)J recombination. In response to antigens, antigen receptors are further diversified by CSR and somatic hypermutation (SHM). In the following section, V(D)J recombination and CSR during B cell development will be reviewed.

B cell development begins in the fetal liver during development and continues in the bone marrow shortly after birth. It is a highly ordered process, mediated by cytokines secreted by bone marrow stromal cells and lineage-specific transcription factors. In response to cytokines such as IL-7, common lymphoid progenitors are committed to B cell lineage. Subsequently, cells undergo D_H - J_H joining at the Ig heavy chain locus and begin expressing CD45 (B220) and class II MHC (major histocompatibility complex), which is followed by the joining of a V segment to the completed DJ_H . After successful V(D)J recombination, pro-B cells become pre-B cells, which undergo V-J joining on one L chain locus and further develop into mature B cells. V(D)J recombination is regulated at three levels: chromatin remodelling, DSB generation mediated by lymphoid-specific recombinases (Rag-1/2) and recombination via NHEJ and microhomology-mediated end joining (MMEJ, also called alternative NHEJ) machinery.

In eukaryotic cells, DNA is wrapped into a compact chromatic structure, which needs to be 'opened' to allow accessibility for further processing. Increasing evidence in genetically modified mouse models shows that covalent histone modifications, such as methylation on histone H3 at lysines 4, 9 and 27 (K4, K9 and K27) positions, mediate chromatin remodelling and subsequently V(D)J recombination. Enhancer of Zeste 2 (Ezh2) is a methyltransferase which trimethylates K27 of histone H3 (H3K27me3). Conditional deletion of Ezh2 in B lymphocytes leads to reduced H3K27me3 levels and defective V_H - DJ_H recombination at the most distal V segments (Su et al., 2003). Di- and tri-methylation of histone H3K4 are also found to be associated with the active segments in V(D)J recombination (Liu et al., 2007). Rag-2 binds to H3K4me3 via the PHD motif and mutations that abolish the interaction impair V(D)J recombination. In contrast, H3K9me2, a silent chromatin mark, inhibits distal V_H - DJ_H recombination (Osipovich et al., 2004), while Pax5, a transcriptional factor required for early B cell commitment, regulates the removal of H3K9me2 and promotes V_H - DJ_H recombination (Johnson et al., 2004).

The coding sequences of IgH are separated by recombination signal sequences (RSSs). RSSs are conserved heptamers (CACTGTG) and nonamers (GGTTTTGT) flanking either 12 base pairs (bp) or 23±1 bp non-conserved DNA, called the spacer. The recombination of D_H to J_H, V_L to J_L or V_H to D_{JH} occurs only between the 12 bp- and 23 bp-spacers, known as 12/23 spacer rule. Rag-1 and Rag-2 recognize RSSs and generate DSBs between the heptamers and its adjacent coding segment. Rag-1/2 first introduces a nick on one strand and then the nicked free coding end attacks the opposite strand, creating a closed hairpin structure. Disruption of either Rag-1 or Rag-2 in mice causes severely impaired V(D)J recombination, defective B cell development and immunodeficiency (Mombaerts et al., 1992; Shinkai et al., 1992).

The final step of V(D)J recombination involves NHEJ and MMEJ machineries where the two coding segments are ligated together. The proteins involved in NHEJ are not all known and new proteins are constantly being uncovered. Here, in this section we discuss those that are well-studied, including DNA-PK, Artemis endonuclease, and XLF (Xrcc4-like factor, also known as Cernunnos)-Xrcc4-DNA ligase IV complex. Deficiency in any of these proteins compromises B and/or T cell development. DNA-PK consists of catalytic subunit DNA-PKcs and regulatory subunits Ku70 and Ku80. As mentioned earlier, DNA-PKcs phosphorylates H2AX at DSB to transduce signalling, while Ku70 and Ku80 form a heterodimer to process the broken ends. DNA-PKcs deficiency is the main cause of murine severe combined immunodeficiency (SCID), where both B and T cells are depleted (Blunt et al., 1995). Any defects in Ku protein also impair V(D)J recombination (Gu et al., 1997; Taccioli et al., 1993). Artemis is thought to open the closed hairpin at the coding ends generated by Rag-1/2. Null mutants of Artemis also give rise to the severe combined immunodeficiency (SCID) phenotype (Li et al., 2005). Xrcc4 together with XLF and DNA ligase IV ligates the broken ends together. Cells deficient for either Xrcc4, XLF or DNA ligase IV are sensitive to γ -irradiation and compromised in V(D)J recombination (Ahnesorg et al., 2006; Gao et al., 1998).

Upon antigen or humoral stimulation, CSR further diversifies antibodies by switching their isotypes. Human BCR heavy chain gene contains 9 C_H segments: 1 μ (IgM), 1 δ (IgD), 4 γ (IgG), 1 ϵ (IgE) and 2 α (IgA). CSR occurs between two switch (S) regions located upstream of each C_H segment, except for C δ ; the switch between C μ (IgM) and C δ (IgD) is achieved by alternate splicing before complete maturation of B cells. Similar to V(D)J recombination, CSR also involves DSB generation and NHEJ. DSBs are created by dC deamination, BER and MMR machinery within or near S regions. In response to humoral stimulation, activation-induced cytidine deaminase (AID) deaminates dC resulting in dU bases on both strands of two transcriptionally active S regions (Chaudhuri et al., 2003). The dU is excised by the uracil DNA glycosylases (UNG) and the resultant abasic site is further cut by apurinic/apyrimidinic endonuclease 1/2 (APE-1/2), generating SSBs. Either two adjacent SSBs on opposite strands spontaneously lead to one DSB, or the MMR machinery is triggered to convert SSB to DSB (Schrader et al., 2007). Deficiency of AID, UNG, APE or any of the MMR components, including Msh2, Msh6, Mlh1, Pms2 and Exo1, leads to loss or reduction of CSR in B cells (Stavnezer et al., 2008). After DSB formation, NHEJ rather than HR pathway is activated. Components of classical NHEJ (C-NHEJ) pathway are important but not essential for CSR in B cells. Ku70-Ku80 heterodimers bind to the DNA ends and recruit necessary proteins to process the DNA ends to facilitate the ligation mediated by Xrcc4-DNA ligase IV complex (Nick McElhinny et al., 2000). CSR in *Ku70*^{-/-} and *Ku80*^{-/-} B cells is nearly ablated (Casellas et al., 1998; Manis et al., 1998). Either *Xrcc4* or *DNA ligase IV*

deficiency causes significant reduction in CSR (Soulas-Sprauel et al., 2007; Yan et al., 2007). While compatible ends are joined rapidly by canonical NHEJ components, complex lesions need substantial processing and are re-ligated slowly. In the later case, ATM, 53BP1 and MRM complex cooperate with canonical NHEJ components to mediate end-joining recombination. Disruption of ATM (Bredemeyer et al., 2006; Reina-San-Martin et al., 2004), 53BP1 (Manis et al., 2004) or MRN complex (Kracker et al., 2005) in mice leads to defects in either V(D)J recombination or CSR or both. Recent studies in mouse models deficient for NHEJ core components revealed a robust alternative NHEJ pathway (A-NHEJ) that utilizes microhomology to mediate the end joining in CSR (Soulas-Sprauel et al., 2007; Yan et al., 2007). A-NHEJ leads to Ig locus deletion and translocation. The molecular mechanisms underlying A-NHEJ are not well elucidated so far.

4.2 Meiosis

Meiosis is a form of cell division occurring in sexually reproducing organisms by which maternal and paternal chromosomes are distributed between cells to generate genetic diversity. Prior to meiosis, each chromosome duplicates and creates two sister chromatids, which stay connected at the centromere. During meiosis, the homologous chromosomes align in parallel and chromosomal crossovers are induced by recombination. DSBs are generated by meiosis-specific topoisomerase-like enzyme Spo11 (Keeney et al., 1997), together with Mei1. Mice lacking *Spo11* or *Mei1* fail in the generation of DSBs, leading to absence of Rad51 foci, faulty synapsis, meiotic failure and eventually infertility (Baudat et al., 2000; Libby et al., 2003; Munroe et al., 2000; Romanienko and Camerini-Otero, 2000). The generation of DSB on meiotic chromosomes is not entirely random but occurs preferentially on specific chromosomal locations, known as hot spots. Recombination regulator 1 (RCR1) and double strand break control 1 (DSBC1) regulate the activities of recombination hot spots (Grey et al., 2009; Parvanov et al., 2009). Although the molecular mechanisms underlying the selection of hot spots for DSB induction are still under investigation, it has been found that high-order chromatin structure could be an important factor (Buard et al., 2009). Of particular interest is the methylation of histone H3K4me3 by the methyltransferase Prdm9 (also known as Meisetz), which is enriched at meiotic recombination hot spots (Baudat et al., 2010; Borde et al., 2009; Buard et al., 2009). *Prdm9* null mice are sterile owing to defective chromosome pairing and impaired sex body formation (Hayashi et al., 2005; Mihola et al., 2009).

Many components of the HR pathway are of particular importance for proper strand exchange and meiosis. As mentioned earlier, MRN complex is recruited to the DSBs to remove Spo11 and degrade the 5' of DNA, generating long 3' ssDNA overhangs. ATM is activated by MRN and further amplifies the signaling via phosphorylation of many downstream transducers and effectors, such as H2AX and Chk2 (Shiloh, 2003). Finally, ubiquitously expressed Rad51 and meiosis-specific Dmc1 recognize and bind to the resected 3' ssDNA overhangs and form nucleoprotein filaments, which mediate the search for homologous template and subsequent strand exchange. As they are all essential for development, disruption of either Rad51 or any component of MRN complex causes embryonic lethality (Buis et al., 2008; Luo et al., 1999; Zhu et al., 2001). Loss of ATM in mice causes general defects in DSB repair and mislocalization of Rad51 and Dmc1 in spermatocytes (Barlow et al., 1996; Barlow et al., 1998). During meiosis, *Dmc1*^{-/-} germ cells arrest at the early zygotene stage due to the failure of homologous chromosome synapsis (Yoshida et al., 1998).

In contrast to mitotic recombination in which Rad51-mediated HR utilizes the identical sister chromatid as homologous template (reviewed earlier in Section 2.6), Rad51 and Dmc1-mediated strand invasion prefers the chromatids from the homologous chromosome in meiotic recombination. This preference is achieved with the help of other factors such as chromosome-associated kinase Mek1, which excludes identical sister chromatid from being selected as a homologous template for further meiotic recombination. As a byproduct, this generates a heteroduplex DNA (hDNA) if there are heterologies between the two homologous chromosomes (maternal and paternal). In this case, MMR pathway is activated to repair the hDNA, resulting in either gene conversion (GC) or restoration of original sequences (Kramer et al., 1989). Two recombination pathways are employed, i.e. cross-over (CO) and non-cross-over (NCO). CO occurs when double Holiday junctions (dHJs), which are visualized as chiasmata, are formed and subsequently cut by resolvases. NCO is also known as synthesis-dependent strand annealing (SDSA), leading to gene conversion. MMR pathways are not only restricted to resolving hDNA because *Mlh1*^{-/-} mice exhibit defective gametogenesis due to reduction of chiasmata, recombination and COs, and deficiency in *Pms2* leads to disrupted synapsis (Baker et al., 1995; Baker et al., 1996; Edelman et al., 1996). Msh4 and Msh5 are two meiosis-specific homologues of MutS, which interact with Rad51 to stabilize the synaptonemal complex (SC) mediated chromosome pairing. Targeted mutations of *Msh4* and *Msh5* in mice give rise to meiotic-specific phenotypes, including meiotic arrest at zygotene stage and defective synapsis (de Vries et al., 1999; Edelman et al., 1999; Kneitz et al., 2000). *Mlh3* and *Exo1* null mice are also infertile, attributed to meiotic failure and apoptosis (Lipkin et al., 2002; Wei et al., 2003).

4.3 DNA damage signaling in stem cells

Recently, it has become apparent that tissue stem cells possess an elaborate DDR to maintain organ homeostasis, although the mechanistic details seem to vary greatly between different tissues. The DDR in response to radiation exposure has been studied in detail in at least four adult stem cell types: epidermal stem cells, hematopoietic stem cells, mammary stem cells and intestinal stem cells. By comparing the response of haematopoietic stem cells (HSCs) with their differentiated progeny at low doses of IR, it has become clear that different DNA repair and signalling mechanisms exist within the stem cell compartment. Using fetal human umbilical cord blood derived HSCs and by comparing them with their more mature progenitors, it was found that stem cells had greater level of apoptosis, due to the ASPP1 protein, and the phenotype could be rescued by the down regulation of p53 (Milyavsky et al., 2010). In contrast, when a similar experiment was performed using adult mouse HSCs, low doses of IR showed a greater degree of protection in stem cells as compared to their differentiated counterparts. The underlying mechanism was proposed to be the increased expression of anti-apoptotic Bcl2, Bcl-xl which inhibited p53-mediated cell death, while allowing p53-dependent increase in p21 expression (Mohrin et al., 2010). Interestingly, quiescent HSCs mostly preferred NHEJ pathway for DNA repair, and as a consequence their progeny often showed increased levels of genomic instability due to misrepaired DNA. Recent investigations using multipotent hair follicle bulge stem cells (BSC) also revealed that BSCs were more resistant to DNA damage-induced cell death as compared to other cells of the epidermis (Blanpain and Fuchs, 2009). The underlying mechanism was shown to be the increased expression levels of anti-apoptotic Bcl2 and the transient and reduced duration of

p53 up regulation. BSCs also displayed accelerated repair by the error-prone NHEJ pathway and this result suggested that both HSCs and BSCs show short term survival in the wake of DNA damage at the expense of a compromise on their genomic integrity.

Irradiation experiments have also been performed on intestinal stem cells (ISC) to understand their DDR. The intestinal stem cells are localized at the bottom of the crypt, where they proliferate and generate the transit amplifying cells, which divide and migrate to the upper part of the crypt. At least two distinct stem cell populations have been isolated from the intestine corresponding to the +4 position from the bottom of the crypt, which are positive for the stem cell marker Bmi1+ and quiescent and the cycling Lgr5+ fraction located in between the paneth cells at the base of the intestinal crypt (Barker et al., 2010). Radiation sensitivity experiments have revealed that the quiescent stem cells at the +4 position are extremely radiosensitive, followed by the more active Lgr5+ve cells, whereas the rapidly cycling transit amplifying cells were the most radioresistant. Different mechanisms have been proposed for the extreme sensitivity of the Bmi1+ve ISCs to DNA damage, such as enhanced activation of the p53 pathway, lower expression of anti-apoptotic protein Bcl2. However, based on the observation that IR-induced stem cell apoptosis is blocked in PUMA-deficient mice, it is accepted that Puma is the main mediator of DDR in ISCs (Qiu et al., 2008). The DDR has also been studied in germ stem cells (GSC) since the inability to repair DNA damage in the germ line can be extremely dangerous and can directly lead to infertility or the transmission of genetic diseases. The main source of DNA damage in the germ-line could be from teratogenic chemicals or from normal metabolic activity and ROS production. In studies using human male GSCs, it was found that these cells are mostly kept in the G0/G1 phase of the cell cycle, and preferentially use NHEJ as their repair of choice. On the other hand, the female GSCs are located in the oogonia where the homologous chromosomes are close to each other and hence HR is the preferred pathway for DNA repair (Forand et al., 2009). Interestingly, in contrast to other stem cell populations, the female GSCs do not depend on p53 for their genomic integrity. Instead, TAp63, an isoform of the p63 gene, is constitutively expressed in oocytes and is the primary mediator of DDR. Consistently, TAp63 deletion in mice results in an increase in oocyte radioresistance (Suh et al., 2006). The study of DNA repair in another stem cell type, namely the mammary stem cells (MSCs), is clinically very relevant because, mutations in Brca1 and Brca2 are found in a majority of patients with hereditary breast cancers, demonstrating the importance of HR in preventing the onset of mammary tumors. The MSCs are responsible for the homeostasis of the breast tissue, and represent multipotent stem cells that self-renew and differentiate into the various lineages. Mouse MSCs are more radioresistant than their differentiated progeny. MSCs present less DNA damage and following IR exposure, activate the Wnt/beta-catenin pathway and increase the survival of MSCs through the upregulation of survivin, a direct Wnt target gene (Woodward et al., 2007).

5. DNA damage response and human diseases

Congenital or acquired defects in genes involved in the DDR give rise to disease phenotypes such as neurodegeneration, infertility, immune deficiencies, growth retardation, cancer and premature aging. In the following sections, human diseases caused due to impaired DDR are described in greater detail and summarized in Figure 3 and Table 1.

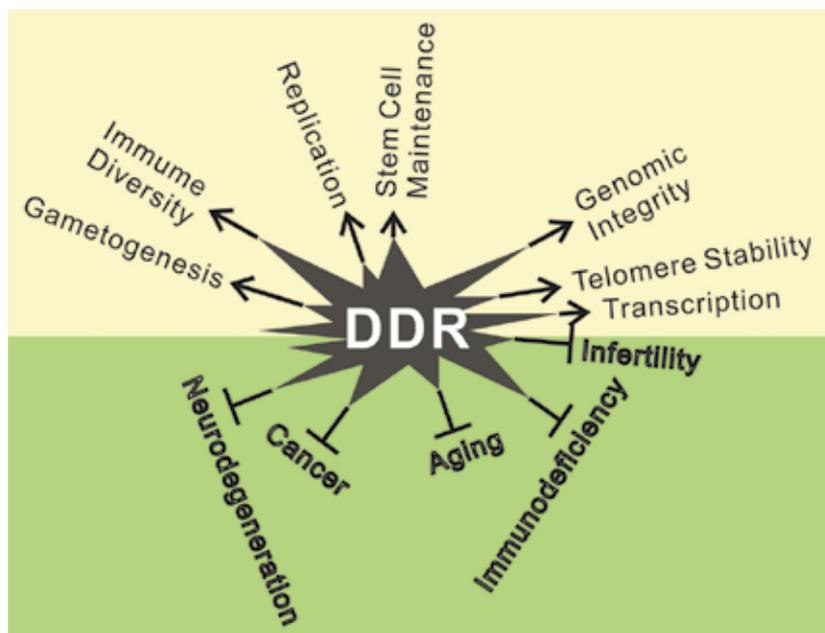


Fig. 3. Physiological roles of DDR and disease phenotypes caused by defective DNA repair

5.1 Neurodegeneration

The mammalian nervous system is generated through consecutive cycles of proliferation, differentiation and maturation that eventually give rise to the various cell types of the brain. Two main classes of cells make up the nervous system: the glia and the neurons, and these contain many specialized sub-types. These cells exit the cell cycle, migrate and differentiate to form the nervous system. In general, neurons display high rates of transcription and translation which are associated with high rates of mitochondrial and metabolic activity. Thus, the neural cells are more susceptible to DNA damage than other adult tissue cell types because they face high levels of oxidative and metabolic stress during their life time. The DNA damage takes the form of both SSBs and DSBs that have to be efficiently repaired to maintain neural homeostasis. Indeed, one of the most commonly observed symptoms in both DNA repair-deficient mice and in humans is that of neurodegeneration. This is mainly because individuals possessing mutations in DNA repair pathways are incapable of handling DNA damage in the neurons, resulting in neuronal cell death that is manifested as neurodegeneration (Katyal and McKinnon, 2008).

The DNA strand breaks in the nervous system may arise due to reactive oxygen species (ROS) that arise as a by-product of cellular metabolism. ROS, in turn carries out nucleophilic attack on DNA to generate single strand breaks. Due to the high levels of oxygen consumption in the brain, ROS levels are very high in the neurons compared to other adult cell types. In fact, tens to thousands of SSBs are generated by ROS in neuronal cells, and these constitute the single largest source of DNA damage in the nervous system (Katyal and McKinnon, 2008). As discussed earlier (Section 3.1), SSBs are efficiently detected by PARP which recruits the scaffolding protein XRCC1 so that the DNA lesions can be resolved by enzymes involved in DNA processing. Unrepaired SSBs can cause a block in transcription, or replication forks can collide with SSBs resulting in DSBs that have to be then repaired by the DSB repair pathway.

Human monogenic neurodegenerative defects associated with DDR defects can be broadly classified as those associated with DSB repair or SSB repair. Defective DSB repair associated neuropathologies include A-T, Nijmegen breakage syndrome (NBS), ATLD (A-T like disorder), Seckel syndrome, Primary microcephaly (PM), and the Lig4 syndrome. In most DNA repair-associated syndromes, cerebellum is often the primary target of neurodegeneration which then spreads to the remaining parts of the brain. A-T syndrome arises as a result of mutations in ATM. Since cerebellum is mainly responsible for sensory motor coordination, individuals with A-T often present with profound ataxia (defective motor coordination) and are wheelchair-bound within the first decade of life. The DDR pathway in A-T strongly affects the cerebellum and widespread loss of cerebellar purkinje cells and granule neurons is seen in A-T brains and is later accompanied by widespread cerebral and spinal defects. Additionally, A-T patients develop other neurological defects such as defective eye movement, speech defects (dysarthria) and non-neurological symptoms such as the absence or the rudimentary appearance of a thymus, immunodeficiency, insulin-resistant diabetes, clinical and cellular radiosensitivity, cell cycle checkpoint defects, chromosomal instability and predisposition to lymphoid malignancies (Biton et al., 2008). Because of the central role of the MRN complex in the ATM-dependent DDR, human syndromes caused by defects in the MRN complex have been regarded as AT-like disorders. Very similar to AT, NBS is characterized by growth retardation, frequent infections, microcephaly (described below), ovarian dysgenesis (defective development), primary amenorrhoea and lymphoma predisposition. However, NBS cells also appear to be defective in some aspects of the ATR pathway and exhibit phenotypes like microcephaly (described below), not usually seen in A-T patients. Patients suffering from ATLD (A-T like disorder) have hypomorphic mutations of MRE11 and show neurological symptoms very similar to A-T such as dysarthria, oculomotor apraxia and ataxia. Interestingly, a cancer predisposition phenotype has not been observed with ATLD. Also, in contrast to NBS patients, ATLD subjects do not have microcephaly (Katyal and McKinnon, 2008).

In addition to neurodegeneration, defective DSB repair can cause developmental defects in the brain, resulting in the development of a smaller brain, called microcephaly where the brain size is at least two standard deviations smaller than the normal brain. This phenotype probably arises as a result of brain cell loss due to the inability to cope with DNA damage in the developing brain. For example, patients harbouring germ-line mutations in ATR, suffer from the Seckel syndrome, an autosomal recessive disorder characterized by severe intrauterine growth retardation, profound microcephaly, a 'bird-like' facial profile, mental retardation and isolated skeletal abnormalities (O'Driscoll et al., 2009). Patients harbouring mutations in the centrosomal protein pericentrin also show pronounced microcephaly as a result of DNA replication fork defects and defective DNA repair. Patients bearing hypomorphic mutations in Lig4, the DNA ligase in the NHEJ pathway also present with microcephaly, developmental and growth delay, immunodeficiency and lymphoid malignancies. Mutation in another NHEJ protein XLF, causes a disorder called as HIM (Human immunodeficiency and microcephaly), primarily characterised by growth delay, recurrent infections, autoimmunity and microcephaly (Katyal and McKinnon, 2008).

Unlike defective DSB pathway, where the associated defects are more wide spread across tissues, defective SSB repair pathway often results in phenotypes restricted to the nervous system. For example, defective SSB repair pathway result in spinocerebellar ataxia and axonal neuropathy (SCAN1) and ataxia with oculomotor apraxia (AOA1), which are caused

by mutations in the 3'-end-processing enzyme tyrosyl DNA phosphodiesterase 1 (TDP1) and the 5'-end-processing enzyme aprataxin (APTX), respectively (McKinnon, 2009). As mentioned earlier, TDP1 is an enzyme involved in the processing of damaged DNA ends, such as 3'-phosphoglycolate and 3' Top1 and other non-ligatable termini generated after DNA oxidation, DNA replication and other types of genotoxic stress. APTX, on the other hand, is a nucleotide hydrolase that cleaves the 5' adenylate intermediate prior to sealing the nick after the generation of SSB. Similar to A-T patients, SCAN1 and AOA1 patients show ataxia, oculomotor apraxia, cerebellar atrophy and dysarthria. Cockayne's syndrome and XP/CS are associated with defects in CSA, CSB, XPB, XPD and is associated with neurological symptoms such as microcephaly, progressive neurodegeneration, cerebral and cerebellar atrophy and sensorineural deafness (Weidenheim et al., 2009). In another SSR-associated syndrome called as trichothiodystrophy (TTD), the proteins XPB, XPD, TTDA are found defective (Stefanini et al., 2010). This syndrome is associated with cerebral cortex microcephaly, hypomyelination and psychomotoric abnormalities.

Recent studies have also highlighted the role of defective DNA repair in late-onset chronic neuropathologies such as Alzheimer's disease and Parkinson's disease. For instance, decreased levels of NHEJ factor DNA-PKcs and of MRN proteins and reduced BER capacity has been documented in Alzheimer's disease patients, whereas increased DNA damage accumulation has been observed in the substantia nigra neurons, a population of neurons often depleted in Parkinson's disease patients (Katyal and McKinnon, 2008).

5.2 Infertility

Human infertility is primarily defined as the inability to conceive after 12 months' intercourse and is often underdiagnosed (Thonneau et al., 1991). However the investigation of human infertility is hampered by the fact that defective meiotic recombination usually leads to either germ cell arrest or abnormal gametes. Some candidate genes such as PRDM9 and MEI1 have been found to contain mutations/polymorphisms in patients suffering from infertility (Miyamoto et al., 2008; Sato et al., 2006). MSH4 and DMC1 mutations have also been found in patients suffering from testis vanishing syndrome and premature ovarian failure (Mandon-Pepin et al., 2002).

5.3 Immune deficiency

As reviewed earlier in this chapter (Section 4.1), NHEJ machinery is used for V(D)J recombination, CSR and SHM in order to generate antigen receptor diversity. Defects in DSB generation and NHEJ components usually compromise V(D)J recombination, CSR and/or SHM, thereby leading to primary inherited immunodeficiency. Severe combined immunodeficiency (SCID) is a severe form of inheritable immunodeficiency. In SCID, both B and T adaptive immune systems are impaired and the patients are extremely vulnerable to various infectious diseases. Defects in V(D)J recombination usually lead to SCID in humans because it is required for both B and T antigen receptor generation. During V(D)J recombination, RAG-1 and RAG-2 are responsible for DSB generation and mutations of either of them in humans are reported in some cases of SCID (Schwarz et al., 1996; Villa et al., 1998; Villa et al., 2001). Recently the first missense mutation of DNA-PKcs was reported in a human patient suffering from pronounced immunodeficiency. Strikingly, the mutation doesn't affect the kinase activity of DNA-PKcs but rather compromises the activity of Artemis (van der Burg et al., 2009). After DSB generation, Artemis processes the hairpin

intermediates for end-joining. Mutations in Artemis arrest B and T cells at very early stage in some cases of SCID, owing to the defective V(D)J recombination (Ege et al., 2005; Moshous et al., 2001). Hypomorphic mutations of DNA ligase IV have been identified in individuals with SCID or combined immunodeficiency (CID) displaying microcephaly and delayed growth (Enders et al., 2006; O'Driscoll et al., 2001; Riballo et al., 1999; van der Burg et al., 2006). Similarly, patients with XLF mutations develop severe B-T lymphocytopenia, microcephaly and growth retardation (Ahnesorg et al., 2006; Buck et al., 2006).

In addition to the essential components of V(D)J recombination, mutations in other DSB repair factors also cause a less profound immunodeficiency characterized by defective lymphocyte development and IgG CSR defects. As described earlier (Section 5.1), Ataxia telangiectasia (AT) is a progressive genetic disorder caused by mutated ATM and is characterized with cerebellar ataxia, oculocutaneous telangiectasia, growth retardation, infertility, immunodeficiency and increased carcinogenesis mainly lymphoid tumours (Chun and Gatti, 2004). In AT patients, V(D)J recombination is not affected, but both B and T lymphocytes are restricted and skewed by diffused oligoclonal expansions (Giovannetti et al., 2002). While dispensable for SHM, ATM-deficient cells are compromised in diversifying IgM to other classes (Reina-San-Martin et al., 2004). Immunodeficiency is also manifested in NBS patients who are found more susceptible to infectious diseases (Digweed and Sperling, 2004). Similar to ATM, deficiency of either NBS1 or MRE11 doesn't affect normal V(D)J recombination although CSR is impaired (Lahdesmaki et al., 2004; van Engelen et al., 2001).

5.4 Cancer

Loss of genomic integrity and accumulation of mutations is a fundamental property of cancerous cells. In that context, the DDR influences cancer progression in multiple ways, as discussed below.

5.4.1 DNA repair defect and familial cancers

The concept that cancers could be acquired by DNA repair defects was first obtained by the study of patients suffering from familial hereditary cancers. Xeroderma pigmentosa (XP) was one of the first of many human cancers discovered to be acquired due to defective DNA repair (Cleaver, 2005). XP patients are defective in the NER of UV-induced DNA damage and have a 1000-fold greater risk of developing skin cancers and a 100,000 fold greater risk for developing squamous cell carcinoma of the tip of the tongue when compared to the general population. HNPCC (Hereditary non-polyposis colon cancer, also called as Lynch syndrome) is another inherited cancer syndrome where patients show an increased susceptibility to colon cancers. While the adenoma to carcinoma progression requires 8-10 years in the general population, this process is accelerated to 2-3 years in HNPCC patients. The majority of HNPCC cases result from germ-line mutations in genes encoding for the MMR proteins MSH2 and MLH1 (Fishel et al., 1993; Leach et al., 1993). Mutations in two other MMR genes *MSH6* and *PMS2* were also detected in a small proportion of cases. The resulting inability to detect and repair mismatches can lead to mutations of genes possessing microsatellite repeats in their sequences, causing a phenotype referred to as microsatellite instability. In addition to hereditary cases, MMR defects have also been detected in about 15% of sporadic gastric, endometrial and colon tumors owing to promoter hypermethylation and the consequent silencing of *MLH1*. In many of the tumors showing MMR defects and microsatellite instability, it is likely that hundreds of genes are

concomitantly mutated and some of them such as the commonly studied type II TGF- β receptor may confer a proliferative advantage to incipient cancer cells and drive tumor progression. Another example is hereditary MYH-associated polyposis, in which biallelic germ-line mutations in *MYH*, a BER gene, result in increased GC to TA transversion and predisposition to colon cancer (Al-Tassan et al., 2002). Defective DSB repair attributed to germ-line transmission of mutant *BRCA1* and *BRCA2* alleles also confer enhanced susceptibility to breast and ovarian cancers. It is estimated that about 70-80% of all familial ovarian cancers is due to germ-line transmission of *BRCA1* or *BRCA2* (Scully and Livingston, 2000). Germ-line mutations in DNA repair genes *NBS1*, *ATM*, Werner syndrome helicase (*WRN*), Bloom syndrome helicase (*BLM*), RecQ protein-like 4 helicase (*RECQL4*) has also been linked to increased tumor susceptibility. Mutations in *p53* are present in cancer-prone families with Li-Fraumeni syndrome (Brown et al., 2009). As discussed in section 2.5, mutations in 14 FANC genes have been identified in Fanconi anaemia, which is an autosomal recessive hereditary disorder characterized by progressive bone marrow failure, congenital developmental abnormalities, and early onset of cancers such as acute myelogenous leukemia (AML) and squamous cell carcinomas of the head and neck. Together, these observations support the 'mutator hypothesis' which postulates that inherited defects in DDR contribute to the 'mutator' phenotype and increased mutation rate in many malignancies which might allow tumor cell survival and proliferation.

5.4.2 DDR and sporadic cancers

Apart from inherited mutations in proteins involved in DNA repair, recent large scale genome sequencing of cancer genomes have also identified mutational inactivation of components of the DDR machinery in sporadic cancers. Of these, mutations that perturb p53 functions, often in its DNA binding domain, or defects in p53's upstream or downstream regulatory network have been identified in more than half of all human cancer samples. Recently, coding sequences of about 20,000 genes in carcinomas of the colon, breast, pancreas and glioblastomas were sequenced. In these sequencing studies representing 68 cancers in the discovery screen and 221 cancers in the follow-up validation screen, one of the most frequently mutated gene was Cockayne syndrome B (*CSB*), found mutated in 6 cancers (Sequencing data compiled by Negrini et al, 2010 (Negrini et al., 2010)). Four genes involved in the DSB repair pathway, *BRCA1*, *BRCA2*, *MRE11* and *PRDKC* (gene encoding DNA-PKcs) were each mutated in two cancers and one mutation each in the genes encoding *FANCA*, *FANCG* and *FANCM*, *PABL2*, *WRN*, *XRCC 1*, *XPB*, *XPF*, *XPG*, *RAD23A* was identified. In a more controlled study involving the analysis of a limited set of DNA repair and cell cycle checkpoint genes in 188 lung adenocarcinomas and 91 glioblastomas, the NHEJ mutation in *PRDKC* and mismatch repair gene *MSH6* were each mutated in six and four cases respectively. The HR repair genes *BRCA2*, *BAP1* and *BARD1* were mutated in two cases each. Mutations in *ATM* were also identified in a few cases of lung adenocarcinomas. Notably, although large scale genome sequencing projects identified mutations in the genes involved in DNA repair, such mutations were detected only in a small proportion of human cancers (Negrini et al., 2010).

5.4.3 DDR and oncogene-induced senescence

An alternative model has been proposed that postulates that DDR, in fact, acts as a barrier to cellular transformation in the early stages of tumor progression by preventing the

accumulation of mutations in the face of activated oncogenes (Bartek et al., 2007). According to this idea, the first hallmark to be acquired in sporadic cancer might be activated growth factor signalling owing to mutations in oncogenes and loss of tumor suppressor genes (Negrini et al., 2010). This results in an increased cell proliferation rate which generates a DNA replication stress, as shown for several oncogenes like Ras, Myc, Stat3 and E2FF1 (Bartkova et al., 2006). DNA replication stress, in turn, creates a high level of DNA damage, which results in the persistent activation of the DDR in the form of activated ATM/ATR signalling cascade causing cell death or senescence of incipient cancer cells. The senescence pathway evoked by oncogenes upon hyper-activation is now commonly referred to as the oncogene-induced senescence and is recognised as an important barrier to tumor progression (Gorgoulis and Halazonetis, 2010). Consistent with this idea, human lung, skin and colon precancerous lesions, show both apoptosis and senescence at the early stages of tumor development, whereas these processes are actively suppressed during cancer progression. Endogenous oncogenic K-Ras (K-Ras G12V) was shown to trigger senescence during the early stages of lung and pancreatic tumorigenesis. Melanocyte senescence has also been associated with the presence of oncogenic BRAFV60E, an oncogenic form of BRAF, *in vivo* (Michaloglou et al., 2005). Similarly, human prostate intraepithelial neoplasia (PIN) lesions and premalignant human colon adenomas display features of cellular senescence. Loss of the tumor suppressor neurofibromin 1 (NF 1), a Ras GTPase-activating protein that negatively regulates Ras, also leads to cellular senescence. By sharp contrast, senescence was absent in the corresponding malignant stages of human lung adenocarcinomas, pancreatic ductal adenocarcinomas, prostate adenocarcinoma and melanomas, suggesting that senescence acts as a barrier to tumor development. It is proposed that breaches to this anti-cancer barrier, arising due to mutational or epigenetic inactivation of DDR components help in the evasion of senescence and are subsequently selected for during tumor development.

5.4.4 DDR in cancer stem cells

Gaining a better understanding of the role of DDR in cancer cells is also important from a standpoint of therapy, since most chemotherapeutic compounds are DNA damaging agents. Many human cancers including leukemia, glioblastoma, breast and skin cancers contain a small proportion of cells which are functionally similar to tissue-stem cells, but have aberrant self-renewal and differentiation characteristics and these have been called as cancer stem cells (CSCs) (Clarke and Fuller, 2006). Recent studies have suggested that CSCs are responsible for disease progression and tumor relapse after therapy, since they may take advantage of the DNA repair systems used by tissue-stem cells to achieve resistance to chemotherapy/radiotherapy (Blanpain et al., 2011). Here, we discuss three instances where the DDR has been demonstrated to have an effect on the outcome of cancer therapy. In the first case, the CSCs of leukemias, which exist in both acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML) have been shown to be more resistant to cancer therapies as compared to the bulk of the leukemia cells. Leukemia CSCs have been shown to use protective mechanisms similar to HSCs, such as cell cycle quiescence, and DDR mechanisms to escape chemotherapy. For example, p53-dependent induction of p21 and the resulting growth arrest has been found to be critical in protecting adult HSCs from IR (Mohrin et al., 2010) and CSCs from leukemia co-opt similar protective mechanisms to evade apoptosis and during chemotherapy (Viale et al., 2009). There is also evidence that CSCs isolated from breast cancers (CD44 high, CD24 low cells) show resistance to chemo/and or radiotherapy. Transcriptional profiling of murine mammary CSCs also

showed increased expression of several DDR and DNA repair genes (Zhang et al., 2008), suggesting that mammary gland CSCs may also be more resistant to therapy. Indeed, a comparison of tumor biopsies before and after neoadjuvant chemotherapy showed increased proportion of CSCs following chemotherapy. One possible mechanism seems to be that like normal mammary stem cells, CSCs from mammary gland have increased levels of genes regulating free radical scavenging systems, like those of glutathione metabolism (Diehn et al., 2009). Lastly, glioblastoma multiforme (GBM) represents the most aggressive form of brain tumor and CSCs are isolated from these tumors based on the expression of prominin (CD133-positive cells). Upon irradiation, the proportion of CD133+ cells increased, suggesting that CSCs may be responsible for tumor relapse after radiotherapy (Bao et al., 2006). It was found that CSCs from GBM exhibited a more robust activation of DNA damage checkpoint and increased phosphorylation of ATM, Chk1 and Chk2 were observed in the CSCs as compared to the non-CSC counterparts. Consistently, treatment with Chk1/Chk2 inhibitors sensitized the CSC to IR-induced cell death.

6. DNA repair and aging

Aging involves a gradual deterioration of several physiological functions, resulting in the reduced capacity to repair injured organs, increased propensity to infections, cancer predisposition and decreased fecundity (DiGiovanna, 2000; Partridge and Mangel, 1999). Many hypotheses have been proposed to understand the underlying mechanisms of the aging process, and these include the disease theory, free radical theory and DNA damage accumulation theory. In this section, we only discuss evidences that support the relationship between DNA damage accumulation and aging, a concept proposed more than half century ago by Leo Szilard (Szilard, 1959). Throughout the life-span, cells are constantly exposed to different endogenous or exogenous conditions that lead to DNA lesions which trigger DNA damage checkpoint response and DNA repair signalling. The accumulation of unrepaired/unrepairable DNA damage within cells leads to a sustained DNA damage checkpoint response and induces a state called cellular senescence, wherein cells permanently exit from the cell cycle. Consistent with this idea, it has been documented that DNA damage in the form of DSB-specific foci containing γ -H2AX accumulate in senescent human cells, germ and somatic cells of aged mice, and in dermal fibroblasts from aged primates (Maslov and Vijg, 2009). Mouse models harbouring deficiency in DNA repair proteins, such as ATM, Ku70, Ku80, DNA ligase IV or Ercc1 also show premature aging phenotypes, providing evidence of a direct correlation between impaired DDR and premature aging (Hasty, 2005; Hoeijmakers, 2009).

So far, the relationship between DNA damage accumulation and aging has gained maximum credibility through studies conducted on various human progeria syndromes. Progeria syndromes are genetic disorders where patients precociously develop features resembling natural aging. Interestingly, most of the reported progeria syndromes, including Werner syndrome (WS), Bloom's syndrome (BS), Rothmund-Thomson syndrome (RTS), Cockayne syndrome type A and type B (CSA and CSB), Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD) and Hutchinson-Gilford progeria syndrome (HGPS) were caused by mutations of genes that were directly or indirectly involved in DNA repair pathways. WS, BS and RTS are associated with defects in RecQ helicases, i.e. RECQL2 (WRN), RECQL3 (BLM) and RECQL4 respectively, whereas CS, XP and TTD shared similar defects in NER pathway. RecQ helicases are a group of highly conserved proteins from

bacteria to humans. The roles of RecQ helicases in DNA metabolism, including DNA replication (Lebel et al., 1999), transcription (Balajee et al., 1999), repair (Cooper et al., 2000; Li and Comai, 2000) and recombination, have been extensively investigated and are demonstrated to be the underlying pathological basis of WS, BS and RTS. Most recently, delayed DNA damage checkpoint response and defective DNA repair were found to contribute to the progeria phenotypes in HGPS as well (Liu et al., 2005).

Since WS closely resembles physiological aging, WS cells have been the subject of intense investigation to understand the biology and molecular mechanism of normal aging. WS is an autosomal recessive genetic disorder of "progeria in adulthood", affecting about 10 in one million (Multani and Chang, 2007). Patients suffering from WS are usually born healthy, with obvious growth retardation from the second decade and other ageing-related features, including short stature, premature cataract, beaked nose, skin atrophy and alopecia, loss of adipose tissues, type II diabetes, osteoporosis, arteriosclerosis, hypogonadism and predisposition to cancer. WS patients typically die of early onset cardiovascular diseases or neoplasia in the fourth decade of life and have an average life expectancy of 47 years. Skin fibroblasts cultured from affected individuals develop accelerated senescence with increased chromosome aberrations (Melcher et al., 2000; Salk et al., 1981). By positional cloning, WRN was firstly linked to WS (Yu et al., 1996). Before the identification of *LMNA* mutations (see below) in atypical WS, WRN was the only protein implicated in WS. WRN belongs to the family of RecQ helicases, and is the only member with a specific exonuclease domain within the N-terminus (Gray et al., 1997; Huang et al., 1998). Physiological and functional interactions between WRN and other proteins suggest that it has crucial roles in DNA replication and repair. WRN interacts with proteins required for DNA replication, such as RPA (Brosh et al., 1999), PCNA (Lebel et al., 1999), FEN1 (Brosh et al., 2001) and DNA polymerase ($\text{Pol}\delta$) (Kamath-Loeb et al., 2001). Studies from Lebel and colleagues (1999) indicated that WRN was involved in restoration of stalled replication forks. WRN also interacts with heterodimer of Ku70-Ku80, which is involved in NHEJ pathway, indicating its potential role in regulating DSB repair (Cooper et al., 2000; Li and Comai, 2000). WRN also plays an important role in maintaining telomere integrity. It has been reported that WRN associates with three of the six members of telomere complex, including telomeric repeat binding factor 1/2 (TRF1/2) and POT1, to modulate exonuclease and helicase activities of WRN during telomeric metabolism (Machwe et al., 2004; Multani and Chang, 2007). Recently, lamin A/C mutations (A57P, R133L, L140R, and E578V) were also reported in autosomal dominant atypical WS where patients presented with more severe phenotypes compared to those associated with WRN (Chen et al., 2003; Csoka et al., 2004; Fukuchi et al., 2004).

Bloom's syndrome is another rare genetic disease characterized by dwarfism, sun-induced erythema, type II diabetes, narrow face and prominent ears, infertility, benign and malignant tumors. Deaths usually result from neoplasia before the age of 30. RecQ helicase BLM is associated with BS, which is shown to be capable of regulating HR. Upon DNA damage, BLM forms discrete nucleoplasmic foci that co-localize with RAD51 as well as BRCA1-associated genome surveillance complex (BASC) containing BRCA1, MLH1, MRN complex and ATM in mammalian cells (Hickson, 2003). BLM is also involved in the correct localization and activation of topoisomerase III α (Wu et al., 2000). Deletion of BLM in mice results in early embryonic death by E13.5. BLM mutant embryos show growth retardation and *Blm*^{-/-} ES cells have an elevated HR between sister chromatids (Chester et al., 1998; Guo et al., 2004). Goss et al (Goss et al., 2002) showed that haploinsufficiency of BLM caused

early development of lymphoma. In another example involving the XPF-XRCC1 endonuclease, a patient bearing a severe XPF mutation presented with dramatic progeroid symptoms. A mouse model for this mutation was generated and expression data from this mouse indicated a shift towards reduced growth hormone/insulin-like growth factor 1 (IGF1) signalling, a known regulator of lifespan. It was proposed that DNA damage accumulation re-allocates resources from growth to life extension by suppressing the somatotroph axis (Niedernhofer et al., 2006).

Hutchinson-Gilford progeria syndrome (HGPS) is an extremely rare severe genetic disorder of early onset premature aging, also referred to as "progeria in childhood". The prevalence is one out of 8 million. So far only about 100 patients have been reported, mainly in western world. Patients with HGPS can only survive for 12-16 years with a mean age of 13.4 years and are clinically characterized with early growth retardation, short stature, lipodystrophy, alopecia, stiff joints, osteolysis, dilated cardiomyopathy and atherosclerosis (Hennekam, 2006; Pollex and Hegele, 2004). A recurrent, *de novo*, dominant point mutation (1824 C→T) of *LMNA* gene was identified to be responsible for about 76% reported cases of HGPS. This mutation (G608G) activates a cryptic splicing donor signal in exon 11, leading to 150 nucleotides deletion in mutant transcript and a 50-residue truncation in lamin A protein (Eriksson et al., 2003; Reddel and Weiss, 2004). The 50-residue truncation in lamin A removes the second proteolytic cleavage site of ZMPSTE24 but leaving the CAAX motif unaffected. A detailed study demonstrated that the mutant allele only expressed about 80% transcripts of total lamin A from the same allele and ~40% of total lamin A from both alleles (Reddel and Weiss, 2004). Studies in HGPS cells and mice lacking *Zmpste24*, a metalloprotease processing prelamin A to its mature form, reveal that accumulation of progerin and unprocessed prelamin A leads to either delayed or reduced recruitment of necessary DNA repair proteins, such as 53BP1 and Rad51 to sites of double strand breaks (Liu et al., 2005).

Cockayne syndrome is an autosomal recessive disorder with growth retardation, skin atrophy, sparse hair, cataract, neural system deterioration, but without cancer susceptibility. As described earlier (section 3.3), Cockayne syndrome proteins are involved in TCR.

Human Syndrome	Mutated Gene	Phenotypes	Disrupted DNA repair pathway
Ataxia Telangiectasia (AT)	<i>ATM</i>	Cerebellar ataxia, telangiectases, oculomotor apraxia, predisposition to lymphoid malignancies, leukemias, immune defects, dilated blood vessel, infertility, metabolic defects, growth defects	DSB repair, DNA damage signalling
Ataxia Telangiectasia-like disorder (A-TLD)	<i>MRE11</i>	Ataxia, oculomotor apraxia, immunodeficiency	DSB repair, DNA damage signalling
Nijmegen break syndrome (NBS)	<i>NBS1</i>	Microcephaly, immunodeficiency, growth defects, mental retardation, B cell lymphoma, facial dysmorphism	DSB repair, DNA damage signalling
NBS-like syndrome	<i>RAD50</i>	Microcephaly, facial dysmorphism, growth defects	DSB repair, DNA damage signalling

Human Syndrome	Mutated Gene	Phenotypes	Disrupted DNA repair pathway
RIDDLE syndrome	<i>RNF168</i>	Radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties	DSB repair, DNA damage signalling
Primary microcephaly 1	<i>MCPH1/BRIT1</i>	Microcephaly and mental retardation	DSB repair, DNA damage signalling
Seckel Syndrome	<i>ATR, PCTN, SCKL2, SCKL3</i>	Severe intrauterine growth retardation, profound microcephaly, a 'bird-like' facial profile, mental retardation and isolated skeletal abnormalities dysmorphic facial features	DSB repair, DNA damage signalling
Restrictive dermopathy (RD)	<i>LMNA, ZMPSTE24</i>	Tight adherent skin, joint contractures and respiratory insufficiency, features of progeroid syndromes and premature death during gestation	DSB repair
Hutchinson-Gilford progeria syndrome	<i>LMNA</i>	Progeria (early growth retardation, short stature, lipodystrophy, alopecia, stiff joints, osteolysis, dilated cardiomyopathy and atherosclerosis)	DSB repair, DNA damage signalling
Li-Fraumeni syndrome	<i>TP53</i>	Brain, breast cancer, sarcomas, leukemias, melanomas and gastrointestinal cancers	DNA damage signalling
Xeroderma pigmentosum	<i>XPA-XPG, POL H</i>	Skin cancer, photosensitivity, neurodegeneration and microcephaly	NER
Trichothiodystrophy	<i>XPB, XPD, TTDA</i>	Neurodegeneration, hypomyelination, progeria (cachexia, cataracts, osteoporosis), microcephaly, and psychomotoric abnormalities.	NER
Cockayne syndrome	<i>CSA, CSB, XPB, XPD, XPG</i>	Microcephaly, neurodegeneration, neuronal demyelination, microcephaly, progeria (skin atrophy, sparse hair cachexia, cataracts, hearing loss, retinopathy), photosensitivity, growth defects	NER
Cerebro-oculo-facio-skeletal (COFS) syndrome	<i>XPD, XPG, CSB, ERCC1</i>	Neuronal demyelination and dysmyelination, brain calcification, microcephaly, neurodegeneration, progeria (cataracts, hearing loss, retinopathy), photosensitivity, growth defects and facial dysmorphism	NER
Ataxia with oculomotor apraxia 1 (AOA1)	<i>APTX</i>	Ataxia, neurodegeneration, oculomotor apraxia, hypercholesterolemia and dysarthria	SSB repair
Spinocerebellar ataxia with axonal neuropathy (SCAN1)	<i>TDP1</i>	Ataxia, oculomotor apraxia, cerebellar atrophy, dysarthria, hypercholesterolemia, muscle weakness, sensory neuropathy	SSB repair
Immunodeficiency with microcephaly	<i>XLF</i>	Growth delay, recurrent infections, autoimmunity, hypoglobulinemia, lymphopenia and microcephaly	NHEJ

Human Syndrome	Mutated Gene	Phenotypes	Disrupted DNA repair pathway
Ligase IV syndrome	<i>LIG4</i>	Microcephaly, developmental and growth delay, immunodeficiency (Aggammaglobulinemia, lymphopenia) and lymphoid malignancies	NHEJ
Radiosensitive severe combined immunodeficiency (RS-SCID)	<i>ARTEMIS</i>	Aggammaglobulinemia, lymphopenia, growth defects	NHEJ
Severe combined immunodeficiency (SCID)	<i>RAG1</i> , <i>RAG2</i>	Aggammaglobulinemia, lymphopenia, growth defects	NHEJ
Bloom syndrome	<i>BLM</i>	Microcephaly, short stature, dysmorphic feature, elevated predisposition to all cancers, mild/moderate mental retardation, immunoglobulin deficiency, infertility, growth defects	HR
Werner syndrome	<i>WRN</i>	Premature aging (short stature, premature cataract, beaked nose, skin atrophy and alopecia, loss of adipose tissues) type II diabetes, osteoporosis, arteriosclerosis, hypogonadism and predisposition to cancer	HR, BER, telomere maintenance
Rothmund Thomson syndrome (RTS)	<i>RECQL4</i>	Stunted growth, premature aging (cataracts, grey hair), osteosarcomas, skin cancers, skin and skeletal abnormalities	BER
Early onset breast cancer	<i>BRCA1</i>	Breast and ovarian cancer	HR
Early onset breast cancer	<i>BRCA2</i>	Breast and ovarian cancer, increased predisposition to prostate, gastric, pancreatic cancers and melanoma	HR
Fanconi anemia	<i>FANCA</i> - <i>FANCL</i> , <i>BRCA2</i> (<i>FANCD1</i>)	Congenital abnormalities, pancytopenia, microcephaly, AML, myelodysplasia, squamous cell carcinoma of head and neck, abnormal skin pigmentation, infertility, limb deformities, renal dysfunction	ICL repair and HR
Hereditary non-polyposis colorectal cancer (HNPCC)	<i>MSH2</i> , <i>MSH3</i> , <i>MSH6</i> , <i>MLH1</i> , <i>PMS2</i>	Colon and gynaecological cancers	MMR
Dyskeratosis congenita (DKC)	<i>DKC1</i> , <i>TERC</i>	Progeria (progressive bone marrow failure, pancytopenia, growth defects, osteoporosis), abnormal skin pigmentation, microcephaly, mental retardation carcinomas	Telomere maintenance

Table 1. Human diseases and syndromes caused by defective DNA repair

7. Conclusions and future directions

Much of our current understanding of mammalian DNA damage signalling, checkpoint and repair is based on elegant experiments done using yeast and *Xenopus* as models. During the past decade, greater insights into the mechanism/regulation of the DDR were obtained using mouse models and human congenital disorders. It is now clear that the DDR pathway is critical for both repairing DNA lesions arising from genotoxic stress, as well as for several developmental processes like VDJ recombination, CSR, SHM and meiosis. Through extensive studies conducted in mouse models and human syndromes, it is now known that the principle phenotypes manifested in diseases associated with defective DNA repair are immunodeficiency, infertility, growth retardation, neurodegeneration, microcephaly, increased cancer predisposition and premature aging. Even so, some caveats in our knowledge exist that need further research. The molecular basis for certain phenotypes associated with DNA repair-related human diseases are not clear. For example, the learning difficulty in RIDDLE patients harbouring mutations in RNF168, skeletal abnormalities in ATR-defective Seckel syndrome and Fanconi anemia patients or insulin resistance and glucose intolerance in ATM-defective patients are some of the phenotypes not fully understood. Perhaps, some of these outcomes are related to tissue-specific novel effectors of the DDR pathway, and future studies are required in this direction. In this regard, it is really interesting that some unexpected substrates of the ATM/ATR pathway were uncovered in an unbiased genome-wide proteomic screen and studies on these substrates are likely to shed light on how the DRR can profoundly influence certain tissue-specific phenotypes. Likewise, recent studies have revealed an amazing complexity in the DDR pathway of stem cells and it seems the mechanistic details of how tissue stem cells respond to DNA damage vary between different stem cells populations. Since tissue stem cells have a critical role in the maintenance of organ homeostasis, any disruption in the genomic integrity of stem cells is likely to elicit diseased states such neurodegeneration, cancer or premature aging, or other specific phenotypes depending on the stem cell from the tissue of origin. In this context, further studies are clearly required to gain a total understanding of the genome maintenance pathways in stem cells.

Apart from providing an in-depth understanding of human diseases, understanding the complexity of the DDR pathway has been harnessed for therapeutic benefit against certain cancer subtypes. The proposed rationale is that the DNA repair pathways and their downstream cellular components have undergone genetic alterations in cancer cells resulting in drug resistance. Hence abrogating or modulating the DDR through the use of DDR inhibitors can sensitize cancer cells to chemotherapy (Al-Ejeh et al., 2010). The most successful example in support of this concept has been the use of PARP inhibitors to sensitize Brca-deficient tumors to chemotherapy by about 1000 fold due to excessive DSBs generated as a result of compromised HR (Bryant et al., 2005). The idea being that the enzyme PARP-1 binds SSBs and BER intermediates to facilitate these repair processes. But in HR-defective Brca1 or Brca2 tumors, PARP inhibitors become particularly toxic due to the accumulation of unrepaired DSBs, while PARP inhibition remains nontoxic to normal cells, due to availability of backup mechanisms. PARP inhibitors are currently undergoing clinical trials in Brca-deficient breast and ovarian cancers. Along similar lines, Chk1 inhibition sensitizes p53-deficient tumors to DNA damaging agents better than p53-wild type cells. Thus, both DNA damaging therapies and DDR-inhibitor therapies can be tailored to the needs of each patient, based on the genetic alteration detected in their tumors.

In conclusion, great strides have been made towards understanding the DDR and its regulation in the past decade. We now have a detailed understanding of how exactly normal cells maintain their genomic integrity when faced with DNA damaging agents. It is now known that the DDR network is critical for several important physiological processes and for human health as evident by the growing list of human syndromes known to be associated with defective DNA repair. The real future challenge lies in developing a more thorough understanding of the functioning and regulation of DDR across various cell/tissue types and then harnessing this knowledge to develop therapeutics for the better management of human health or, perhaps, even extend life span.

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9. References

- Ahnesorg, P., P. Smith, and S.P. Jackson. 2006. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell* 124:301-313.
- Al-Ejeh, F., R. Kumar, A. Wiegman, S.R. Lakhani, M.P. Brown, and K.K. Khanna. 2010. Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes. *Oncogene* 29:6085-6098.
- Al-Hakim, A., C. Escribano-Diaz, M.C. Landry, L. O'Donnell, S. Panier, R.K. Szilard, and D. Durocher. 2010. The ubiquitous role of ubiquitin in the DNA damage response. *DNA Repair (Amst)* 9:1229-1240.
- Al-Tassan, N., N.H. Chmiel, J. Maynard, N. Fleming, A.L. Livingston, G.T. Williams, A.K. Hodges, D.R. Davies, S.S. David, J.R. Sampson, and J.P. Cheadle. 2002. Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors. *Nature genetics* 30:227-232.
- Arias EE, Walter JC. Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes Dev.* 2007 Mar 1;21(5):497-518
- Baker, S.M., C.E. Bronner, L. Zhang, A.W. Plug, M. Robatzek, G. Warren, E.A. Elliott, J. Yu, T. Ashley, N. Arnheim, R.A. Flavell, and R.M. Liskay. 1995. Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* 82:309-319.
- Baker, S.M., A.W. Plug, T.A. Prolla, C.E. Bronner, A.C. Harris, X. Yao, D.M. Christie, C. Monell, N. Arnheim, A. Bradley, T. Ashley, and R.M. Liskay. 1996. Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nat Genet* 13:336-342.
- Bakkenist, C.J., and M.B. Kastan. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421:499-506.
- Balajee, A.S., A. Machwe, A. May, M.D. Gray, J. Oshima, G.M. Martin, J.O. Nehlin, R. Brosh, D.K. Orren, and V.A. Bohr. 1999. The Werner syndrome protein is involved in RNA polymerase II transcription. *Mol Biol Cell* 10:2655-2668.

- Bao, S., Q. Wu, R.E. McLendon, Y. Hao, Q. Shi, A.B. Hjelmeland, M.W. Dewhirst, D.D. Bigner, and J.N. Rich. 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444:756-760.
- Barker, N., S. Bartfeld, and H. Clevers. 2010. Tissue-resident adult stem cell populations of rapidly self-renewing organs. *Cell Stem Cell* 7:656-670.
- Barlow, C., S. Hirotsune, R. Paylor, M. Liyanage, M. Eckhaus, F. Collins, Y. Shiloh, J.N. Crawley, T. Ried, D. Tagle, and A. Wynshaw-Boris. 1996. Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* 86:159-171.
- Barlow, C., M. Liyanage, P.B. Moens, M. Tarsounas, K. Nagashima, K. Brown, S. Rottinghaus, S.P. Jackson, D. Tagle, T. Ried, and A. Wynshaw-Boris. 1998. Atm deficiency results in severe meiotic disruption as early as leptotema of prophase I. *Development* 125:4007-4017.
- Bartek, J., J. Bartkova, and J. Lukas. 2007. DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene* 26:7773-7779.
- Bartek, J., and J. Lukas. 2007. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol* 19:238-245.
- Bartkova, J., N. Rezaei, M. Lontos, P. Karakaidos, D. Kletsas, N. Issaeva, L.V. Vassiliou, E. Kolettas, K. Niforou, V.C. Zoumpourlis, M. Takaoka, H. Nakagawa, F. Tort, K. Fugger, F. Johansson, M. Sehested, C.L. Andersen, L. Dyrskjot, T. Orntoft, J. Lukas, C. Kittas, T. Helleday, T.D. Halazonetis, J. Bartek, and V.G. Gorgoulis. 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444:633-637.
- Bassing, C.H., and F.W. Alt. 2004. The cellular response to general and programmed DNA double strand breaks. *DNA Repair (Amst)* 3:781-796.
- Baudat, F., J. Buard, C. Grey, A. Fledel-Alon, C. Ober, M. Przeworski, G. Coop, and B. de Massy. 2010. PRDM9 is a major determinant of meiotic recombination hotspots in humans and mice. *Science* 327:836-840.
- Baudat, F., K. Manova, J.P. Yuen, M. Jasin, and S. Keeney. 2000. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Mol Cell* 6:989-998.
- Bekker-Jensen, S., C. Lukas, R. Kitagawa, F. Melander, M.B. Kastan, J. Bartek, and J. Lukas. 2006. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol* 173:195-206.
- Bekker-Jensen, S., and N. Mailand. 2010. Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair (Amst)* 9:1219-1228.
- Biton, S., A. Barzilai, and Y. Shiloh. 2008. The neurological phenotype of ataxia-telangiectasia: solving a persistent puzzle. *DNA repair* 7:1028-1038.
- Blanpain, C., and E. Fuchs. 2009. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nature reviews. Molecular cell biology* 10:207-217.
- Blanpain, C., M. Mohrin, P.A. Sotiropoulou, and E. Passegue. 2011. DNA-damage response in tissue-specific and cancer stem cells. *Cell Stem Cell* 8:16-29.
- Blunt, T., N.J. Finnie, G.E. Taccioli, G.C. Smith, J. Demengeot, T.M. Gottlieb, R. Mizuta, A.J. Varghese, F.W. Alt, P.A. Jeggo, and et al. 1995. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell* 80:813-823.

- Borde, V., N. Robine, W. Lin, S. Bonfils, V. Geli, and A. Nicolas. 2009. Histone H3 lysine 4 trimethylation marks meiotic recombination initiation sites. *EMBO J* 28:99-111.
- Bredemeyer, A.L., G.G. Sharma, C.Y. Huang, B.A. Helmink, L.M. Walker, K.C. Khor, B. Nuskey, K.E. Sullivan, T.K. Pandita, C.H. Bassing, and B.P. Sleckman. 2006. ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature* 442:466-470.
- Brosh, R.M., Jr., D.K. Orren, J.O. Nehlin, P.H. Ravn, M.K. Kenny, A. Machwe, and V.A. Bohr. 1999. Functional and physical interaction between WRN helicase and human replication protein A. *J Biol Chem* 274:18341-18350.
- Brosh, R.M., Jr., C. von Kobbe, J.A. Sommers, P. Karmakar, P.L. Opresko, J. Piotrowski, I. Dianova, G.L. Dianov, and V.A. Bohr. 2001. Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity. *Embo J* 20:5791-5801.
- Brown, C.J., S. Lain, C.S. Verma, A.R. Fersht, and D.P. Lane. 2009. Awakening guardian angels: drugging the p53 pathway. *Nature reviews. Cancer* 9:862-873.
- Bryant, H.E., N. Schultz, H.D. Thomas, K.M. Parker, D. Flower, E. Lopez, S. Kyle, M. Meuth, N.J. Curtin, and T. Helleday. 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434:913-917.
- Buard, J., P. Barthes, C. Grey, and B. de Massy. 2009. Distinct histone modifications define initiation and repair of meiotic recombination in the mouse. *EMBO J* 28:2616-2624.
- Buck, D., L. Malivert, R. de Chasseval, A. Barraud, M.C. Fondaneche, O. Sanal, A. Plebani, J.L. Stephan, M. Hufnagel, F. le Deist, A. Fischer, A. Durandy, J.P. de Villartay, and P. Revy. 2006. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell* 124:287-299.
- Buis, J., Y. Wu, Y. Deng, J. Leddon, G. Westfield, M. Eckersdorff, J.M. Sekiguchi, S. Chang, and D.O. Ferguson. 2008. Mre11 nuclease activity has essential roles in DNA repair and genomic stability distinct from ATM activation. *Cell* 135:85-96.
- Bunting, S.F., E. Callen, N. Wong, H.T. Chen, F. Polato, A. Gunn, A. Bothmer, N. Feldhahn, O. Fernandez-Capetillo, L. Cao, X. Xu, C.X. Deng, T. Finkel, M. Nussenzweig, J.M. Stark, and A. Nussenzweig. 2010. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141:243-254.
- Caldecott, K.W. 2008. Single-strand break repair and genetic disease. *Nat Rev Genet* 9:619-631.
- Casellas, R., A. Nussenzweig, R. Wuerffel, R. Pelanda, A. Reichlin, H. Suh, X.F. Qin, E. Besmer, A. Kenter, K. Rajewsky, and M.C. Nussenzweig. 1998. Ku80 is required for immunoglobulin isotype switching. *EMBO J* 17:2404-2411.
- Celeste, A., S. Petersen, P.J. Romanienko, O. Fernandez-Capetillo, H.T. Chen, O.A. Sedelnikova, B. Reina-San-Martin, V. Coppola, E. Meffre, M.J. Difilippantonio, C. Redon, D.R. Pilch, A. Oлару, M. Eckhaus, R.D. Camerini-Otero, L. Tessarollo, F. Livak, K. Manova, W.M. Bonner, M.C. Nussenzweig, and A. Nussenzweig. 2002. Genomic instability in mice lacking histone H2AX. *Science* 296:922-927.
- Chaudhuri, J., M. Tian, C. Khuong, K. Chua, E. Pinaud, and F.W. Alt. 2003. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* 422:726-730.

- Chen, L., L. Lee, B.A. Kudlow, H.G. Dos Santos, O. Sletvold, Y. Shafeghati, E.G. Botha, A. Garg, N.B. Hanson, G.M. Martin, I.S. Mian, B.K. Kennedy, and J. Oshima. 2003. LMNA mutations in atypical Werner's syndrome. *Lancet* 362:440-445.
- Chester, N., F. Kuo, C. Kozak, C.D. O'Hara, and P. Leder. 1998. Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom's syndrome gene. *Genes Dev* 12:3382-3393.
- Chun, H.H., and R.A. Gatti. 2004. Ataxia-telangiectasia, an evolving phenotype. *DNA Repair (Amst)* 3:1187-1196.
- Ciccio, A., and S.J. Elledge. 2010. The DNA damage response: making it safe to play with knives. *Mol Cell* 40:179-204.
- Clarke, M.F., and M. Fuller. 2006. Stem cells and cancer: two faces of eve. *Cell* 124:1111-1115.
- Cleaver, J.E. 2005. Cancer in xeroderma pigmentosum and related disorders of DNA repair. *Nature reviews. Cancer* 5:564-573.
- Cooper, M.P., A. Machwe, D.K. Orren, R.M. Brosh, D. Ramsden, and V.A. Bohr. 2000. Ku complex interacts with and stimulates the Werner protein. *Genes Dev* 14:907-912.
- Csoka, A.B., H. Cao, P.J. Sammak, D. Constantinescu, G.P. Schatten, and R.A. Hegele. 2004. Novel lamin A/C gene (LMNA) mutations in atypical progeroid syndromes. *J Med Genet* 41:304-308.
- Cui, X., Y. Yu, S. Gupta, Y.M. Cho, S.P. Lees-Miller, and K. Meek. 2005. Autophosphorylation of DNA-dependent protein kinase regulates DNA end processing and may also alter double-strand break repair pathway choice. *Mol Cell Biol* 25:10842-10852.
- Daniel, J.A., M. Pellegrini, J.H. Lee, T.T. Paull, L. Feigenbaum, and A. Nussenzweig. 2008. Multiple autophosphorylation sites are dispensable for murine ATM activation in vivo. *J Cell Biol* 183:777-783.
- de Vries, S.S., E.B. Baart, M. Dekker, A. Siezen, D.G. de Rooij, P. de Boer, and H. te Riele. 1999. Mouse MutS-like protein Msh5 is required for proper chromosome synapsis in male and female meiosis. *Genes Dev* 13:523-531.
- Denchi, E.L. 2009. Give me a break: how telomeres suppress the DNA damage response. *DNA repair* 8:1118-1126.
- Di Noia, J.M., and M.S. Neuberger. 2007. Molecular mechanisms of antibody somatic hypermutation. *Annual review of biochemistry* 76:1-22.
- Diehn, M., R.W. Cho, N.A. Lobo, T. Kalisky, M.J. Dorie, A.N. Kulp, D. Qian, J.S. Lam, L.E. Ailles, M. Wong, B. Joshua, M.J. Kaplan, I. Wapnir, F.M. Dirbas, G. Somlo, C. Garberoglio, B. Paz, J. Shen, S.K. Lau, S.R. Quake, J.M. Brown, I.L. Weissman, and M.F. Clarke. 2009. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* 458:780-783.
- DiGiovanna, A.G. 2000. Human Aging: Biological Perspectives. McGraw-Hill, New York.
- Digweed, M., and K. Sperling. 2004. Nijmegen breakage syndrome: clinical manifestation of defective response to DNA double-strand breaks. *DNA Repair (Amst)* 3:1207-1217.
- Dillon, L.W., A.A. Burrow, and Y.H. Wang. 2010. DNA instability at chromosomal fragile sites in cancer. *Curr Genomics* 11:326-337.
- Edelmann, W., P.E. Cohen, M. Kane, K. Lau, B. Morrow, S. Bennett, A. Umar, T. Kunkel, G. Cattoretti, R. Chaganti, J.W. Pollard, R.D. Kolodner, and R. Kucherlapati. 1996. Meiotic pachytene arrest in MLH1-deficient mice. *Cell* 85:1125-1134.

- Edelmann, W., P.E. Cohen, B. Kneitz, N. Winand, M. Lia, J. Heyer, R. Kolodner, J.W. Pollard, and R. Kucherlapati. 1999. Mammalian MutS homologue 5 is required for chromosome pairing in meiosis. *Nat Genet* 21:123-127.
- Ege, M., Y. Ma, B. Manfras, K. Kalwak, H. Lu, M.R. Lieber, K. Schwarz, and U. Pannicke. 2005. Omenn syndrome due to ARTEMIS mutations. *Blood* 105:4179-4186.
- Enders, A., P. Fisch, K. Schwarz, U. Duffner, U. Pannicke, E. Nikolopoulos, A. Peters, M. Orłowska-Volk, D. Schindler, W. Friedrich, B. Selle, C. Niemeyer, and S. Ehl. 2006. A severe form of human combined immunodeficiency due to mutations in DNA ligase IV. *J Immunol* 176:5060-5068.
- Eriksson, M., W.T. Brown, L.B. Gordon, M.W. Glynn, J. Singer, L. Scott, M.R. Erdos, C.M. Robbins, T.Y. Moses, P. Berglund, A. Dutra, E. Pak, S. Durkin, A.B. Csoka, M. Boehnke, T.W. Glover, and F.S. Collins. 2003. Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* 423:293-298.
- Fishel, R., M.K. Lescoe, M.R. Rao, N.G. Copeland, N.A. Jenkins, J. Garber, M. Kane, and R. Kolodner. 1993. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 75:1027-1038.
- Forand, A., P. Fouchet, J.B. Lahaye, A. Chicheportiche, R. Habert, and J. Bernardino-Sgherri. 2009. Similarities and differences in the in vivo response of mouse neonatal gonocytes and spermatogonia to genotoxic stress. *Biol Reprod* 80:860-873.
- Friedel, A.M., B.L. Pike, and S.M. Gasser. 2009. ATR/Mec1: coordinating fork stability and repair. *Curr Opin Cell Biol* 21:237-244.
- Fukuchi, K., T. Katsuya, K. Sugimoto, M. Kuremura, H.D. Kim, L. Li, and T. Ogiwara. 2004. LMNA mutation in a 45 year old Japanese subject with Hutchinson-Gilford progeria syndrome. *J Med Genet* 41:e67.
- Fukui, K. 2010. DNA mismatch repair in eukaryotes and bacteria. *J Nucleic Acids* 2010:
- Gao, Y., Y. Sun, K.M. Frank, P. Dikkes, Y. Fujiwara, K.J. Seidl, J.M. Sekiguchi, G.A. Rathbun, W. Swat, J. Wang, R.T. Bronson, B.A. Malynn, M. Bryans, C. Zhu, J. Chaudhuri, L. Davidson, R. Ferrini, T. Stamato, S.H. Orkin, M.E. Greenberg, and F.W. Alt. 1998. A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. *Cell* 95:891-902.
- Giovannetti, A., F. Mazzetta, E. Caprini, A. Aiuti, M. Marzali, M. Pierdominici, A. Cossarizza, L. Chessa, E. Scala, I. Quinti, G. Russo, and M. Fiorilli. 2002. Skewed T-cell receptor repertoire, decreased thymic output, and predominance of terminally differentiated T cells in ataxia telangiectasia. *Blood* 100:4082-4089.
- Goodarzi, A.A., Y. Yu, E. Riballo, P. Douglas, S.A. Walker, R. Ye, C. Harer, C. Marchetti, N. Morrice, P.A. Jeggo, and S.P. Lees-Miller. 2006. DNA-PK autophosphorylation facilitates Artemis endonuclease activity. *EMBO J* 25:3880-3889.
- Gorgoulis, V.G., and T.D. Halazonetis. 2010. Oncogene-induced senescence: the bright and dark side of the response. *Current opinion in cell biology* 22:816-827.
- Goss, K.H., M.A. Risinger, J.J. Kordich, M.M. Sanz, J.E. Straughen, L.E. Slovek, A.J. Capobianco, J. German, G.P. Boivin, and J. Groden. 2002. Enhanced tumor formation in mice heterozygous for Blm mutation. *Science* 297:2051-2053.
- Gray, M.D., J.C. Shen, A.S. Kamath-Loeb, A. Blank, B.L. Sopher, G.M. Martin, J. Oshima, and L.A. Loeb. 1997. The Werner syndrome protein is a DNA helicase. *Nat Genet* 17:100-103.

- Grey, C., F. Baudat, and B. de Massy. 2009. Genome-wide control of the distribution of meiotic recombination. *PLoS Biol* 7:e35.
- Gu, Y., S. Jin, Y. Gao, D.T. Weaver, and F.W. Alt. 1997. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. *Proc Natl Acad Sci U S A* 94:8076-8081.
- Guardavaccaro, D., and M. Pagano. 2006. Stabilizers and destabilizers controlling cell cycle oscillators. *Mol Cell* 22:1-4.
- Guo, C., T.S. Tang, and E.C. Friedberg. 2010. SnapShot: nucleotide excision repair. *Cell* 140:754-754 e751.
- Guo, G., W. Wang, and A. Bradley. 2004. Mismatch repair genes identified using genetic screens in Blm-deficient embryonic stem cells. *Nature* 429:891-895.
- Hanawalt, P.C., and G. Spivak. 2008. Transcription-coupled DNA repair: two decades of progress and surprises. *Nat Rev Mol Cell Biol* 9:958-970.
- Harper, J.W., and S.J. Elledge. 2007. The DNA damage response: ten years after. *Mol Cell* 28:739-745.
- Hartlerode, A.J., and R. Scully. 2009. Mechanisms of double-strand break repair in somatic mammalian cells. *The Biochemical journal* 423:157-168.
- Hasty, P. 2005. The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice. *Mech Ageing Dev* 126:71-77.
- Hayashi, K., K. Yoshida, and Y. Matsui. 2005. A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* 438:374-378.
- Hennekam, R.C. 2006. Hutchinson-Gilford progeria syndrome: review of the phenotype. *Am J Med Genet A* 140:2603-2624.
- Hickson, I.D. 2003. RecQ helicases: caretakers of the genome. *Nat Rev Cancer* 3:169-178.
- Hoeijmakers, J.H. 2009. DNA damage, aging, and cancer. *The New England journal of medicine* 361:1475-1485.
- Huang, S., B. Li, M.D. Gray, J. Oshima, I.S. Mian, and J. Campisi. 1998. The premature ageing syndrome protein, WRN, is a 3'→5' exonuclease. *Nat Genet* 20:114-116.
- Huen, M.S., and J. Chen. 2010. Assembly of checkpoint and repair machineries at DNA damage sites. *Trends Biochem Sci* 35:101-108.
- Huen, M.S., S.M. Sy, and J. Chen. 2010. BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol* 11:138-148.
- Jackson, S.P., and J. Bartek. 2009. The DNA-damage response in human biology and disease. *Nature* 461:1071-1078.
- Johnson, K., D.L. Pflugh, D. Yu, D.G. Hesslein, K.I. Lin, A.L. Bothwell, A. Thomas-Tikhonenko, D.G. Schatz, and K. Calame. 2004. B cell-specific loss of histone 3 lysine 9 methylation in the V(H) locus depends on Pax5. *Nat Immunol* 5:853-861.
- Kamath-Loeb, A.S., L.A. Loeb, E. Johansson, P.M. Burgers, and M. Fry. 2001. Interactions between the Werner syndrome helicase and DNA polymerase delta specifically facilitate copying of tetraplex and hairpin structures of the d(CGG)_n trinucleotide repeat sequence. *J Biol Chem* 276:16439-16446.
- Katyal, S., and P.J. McKinnon. 2008. DNA strand breaks, neurodegeneration and aging in the brain. *Mech Ageing Dev* 129:483-491.

- Keeney, S., C.N. Giroux, and N. Kleckner. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88:375-384.
- Kitao, H., and M. Takata. 2011. Fanconi anemia: a disorder defective in the DNA damage response. *Int J Hematol* 93:417-424.
- Kneitz, B., P.E. Cohen, E. Avdievich, L. Zhu, M.F. Kane, H. Hou, Jr., R.D. Kolodner, R. Kucherlapati, J.W. Pollard, and W. Edelmann. 2000. MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice. *Genes Dev* 14:1085-1097.
- Kolodner, R.D., and G.T. Marsischky. 1999. Eukaryotic DNA mismatch repair. *Curr Opin Genet Dev* 9:89-96.
- Kracker, S., Y. Bergmann, I. Demuth, P.O. Frappart, G. Hildebrand, R. Christine, Z.Q. Wang, K. Sperling, M. Digweed, and A. Radbruch. 2005. Nibrin functions in Ig class-switch recombination. *Proc Natl Acad Sci U S A* 102:1584-1589.
- Kramer, B., W. Kramer, M.S. Williamson, and S. Fogel. 1989. Heteroduplex DNA correction in *Saccharomyces cerevisiae* is mismatch specific and requires functional PMS genes. *Mol Cell Biol* 9:4432-4440.
- Kunkel, T.A., and D.A. Erie. 2005. DNA mismatch repair. *Annu Rev Biochem* 74:681-710.
- Lahdesmaki, A., A.M. Taylor, K.H. Chrzanowska, and Q. Pan-Hammarstrom. 2004. Delineation of the role of the Mre11 complex in class switch recombination. *J Biol Chem* 279:16479-16487.
- Larrea, A.A., S.A. Lujan, and T.A. Kunkel. 2010. SnapShot: DNA mismatch repair. *Cell* 141:730 e731.
- Leach, F.S., N.C. Nicolaides, N. Papadopoulos, B. Liu, J. Jen, R. Parsons, P. Peltomaki, P. Sistonen, L.A. Aaltonen, M. Nystrom-Lahti, and et al. 1993. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* 75:1215-1225.
- Lebel, M., E.A. Spillare, C.C. Harris, and P. Leder. 1999. The Werner syndrome gene product co-purifies with the DNA replication complex and interacts with PCNA and topoisomerase I. *J Biol Chem* 274:37795-37799.
- Lee, J.H., and T.T. Paull. 2004. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 304:93-96.
- Li, B., and L. Comai. 2000. Functional interaction between Ku and the werner syndrome protein in DNA end processing. *J Biol Chem* 275:28349-28352.
- Li, L., E. Salido, Y. Zhou, S. Bhattacharyya, S.M. Yannone, E. Dunn, J. Meneses, A.J. Feeney, and M.J. Cowan. 2005. Targeted disruption of the Artemis murine counterpart results in SCID and defective V(D)J recombination that is partially corrected with bone marrow transplantation. *J Immunol* 174:2420-2428.
- Libby, B.J., L.G. Reinholdt, and J.C. Schimenti. 2003. Positional cloning and characterization of Mei1, a vertebrate-specific gene required for normal meiotic chromosome synapsis in mice. *Proc Natl Acad Sci U S A* 100:15706-15711.
- Lieber, M.R. 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 79:181-211.
- Lieber, M.R., and T.E. Wilson. 2010. SnapShot: Nonhomologous DNA end joining (NHEJ). *Cell* 142:496-496 e491.
- Lindahl, T. 1993. Instability and decay of the primary structure of DNA. *Nature* 362:709-715.

- Lipkin, S.M., P.B. Moens, V. Wang, M. Lenzi, D. Shanmugarajah, A. Gilgeous, J. Thomas, J. Cheng, J.W. Touchman, E.D. Green, P. Schwartzberg, F.S. Collins, and P.E. Cohen. 2002. Meiotic arrest and aneuploidy in MLH3-deficient mice. *Nat Genet* 31:385-390.
- Liu, B., J. Wang, K.M. Chan, W.M. Tjia, W. Deng, X. Guan, J.D. Huang, K.M. Li, P.Y. Chau, D.J. Chen, D. Pei, A.M. Pendas, J. Cadinanos, C. Lopez-Otin, H.F. Tse, C. Hutchison, J. Chen, Y. Cao, K.S. Cheah, K. Tryggvason, and Z. Zhou. 2005. Genomic instability in laminopathy-based premature aging. *Nat Med* 11:780-785.
- Liu, Y., R. Subrahmanyam, T. Chakraborty, R. Sen, and S. Desiderio. 2007. A plant homeodomain in RAG-2 that binds Hypermethylated lysine 4 of histone H3 is necessary for efficient antigen-receptor-gene rearrangement. *Immunity* 27:561-571.
- Lopez-Contreras, A.J., and O. Fernandez-Capetillo. 2010. The ATR barrier to replication-born DNA damage. *DNA Repair (Amst)* 9:1249-1255.
- Lukas, J., C. Lukas, and J. Bartek. 2004. Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. *DNA Repair (Amst)* 3:997-1007.
- Luo, G., M.S. Yao, C.F. Bender, M. Mills, A.R. Bladl, A. Bradley, and J.H. Petrini. 1999. Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A* 96:7376-7381.
- Machwe, A., L. Xiao, and D.K. Orren. 2004. TRF2 recruits the Werner syndrome (WRN) exonuclease for processing of telomeric DNA. *Oncogene* 23:149-156.
- Mahaney, B.L., K. Meek, and S.P. Lees-Miller. 2009. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochem J* 417:639-650.
- Mailand, N., S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, and J. Lukas. 2007. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell* 131:887-900.
- Mandon-Pepin, B., C. Derbois, F. Matsuda, C. Cotinot, D.J. Wolgemuth, K. Smith, K. McElreavey, A. Nicolas, and M. Fellous. 2002. [Human infertility: meiotic genes as potential candidates]. *Gynecol Obstet Fertil* 30:817-821.
- Manis, J.P., Y. Gu, R. Lansford, E. Sonoda, R. Ferrini, L. Davidson, K. Rajewsky, and F.W. Alt. 1998. Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J Exp Med* 187:2081-2089.
- Manis, J.P., J.C. Morales, Z. Xia, J.L. Kutok, F.W. Alt, and P.B. Carpenter. 2004. 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. *Nat Immunol* 5:481-487.
- Maslov, A.Y., and J. Vijg. 2009. Genome instability, cancer and aging. *Biochimica et biophysica acta* 1790:963-969.
- Matsuoka, S., B.A. Ballif, A. Smogorzewska, E.R. McDonald, 3rd, K.E. Hurov, J. Luo, C.E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S.P. Gygi, and S.J. Elledge. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316:1160-1166.
- Maynard, S., S.H. Schurman, C. Harboe, N.C. de Souza-Pinto, and V.A. Bohr. 2009. Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis* 30:2-10.
- McKinnon, P.J. 2009. DNA repair deficiency and neurological disease. *Nat Rev Neurosci* 10:100-112.

- Melcher, R., R. von Golitschek, C. Steinlein, D. Schindler, H. Neitzel, K. Kainer, M. Schmid, and H. Hoehn. 2000. Spectral karyotyping of Werner syndrome fibroblast cultures. *Cytogenet Cell Genet* 91:180-185.
- Michaloglou, C., L.C. Vredevelde, M.S. Soengas, C. Denoyelle, T. Kuilman, C.M. van der Horst, D.M. Majoor, J.W. Shay, W.J. Mooi, and D.S. Peeper. 2005. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436:720-724.
- Mihola, O., Z. Trachtulec, C. Vlcek, J.C. Schimenti, and J. Forejt. 2009. A mouse speciation gene encodes a meiotic histone H3 methyltransferase. *Science* 323:373-375.
- Milyavsky, M., O.I. Gan, M. Trottier, M. Komosa, O. Tabach, F. Notta, E. Lechman, K.G. Hermans, K. Eppert, Z. Konovalova, O. Ornatsky, E. Domany, M.S. Meyn, and J.E. Dick. 2010. A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* 7:186-197.
- Miyamoto, T., E. Koh, N. Sakugawa, H. Sato, H. Hayashi, M. Namiki, and K. Sengoku. 2008. Two single nucleotide polymorphisms in PRDM9 (MEISETZ) gene may be a genetic risk factor for Japanese patients with azoospermia by meiotic arrest. *J Assist Reprod Genet* 25:553-557.
- Mohrin, M., E. Bourke, D. Alexander, M.R. Warr, K. Barry-Holson, M.M. Le Beau, C.G. Morrison, and E. Passegue. 2010. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. *Cell Stem Cell* 7:174-185.
- Moldovan, G.L., and A.D. D'Andrea. 2009. FANCD2 hurdles the DNA interstrand crosslink. *Cell* 139:1222-1224.
- Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.
- Moshous, D., I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, A. Fischer, and J.P. de Villartay. 2001. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell* 105:177-186.
- Moynaham, M.E., and M. Jasin. 2010. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nat Rev Mol Cell Biol* 11:196-207.
- Multani, A.S., and S. Chang. 2007. WRN at telomeres: implications for aging and cancer. *J Cell Sci* 120:713-721.
- Munroe, R.J., R.A. Bergstrom, Q.Y. Zheng, B. Libby, R. Smith, S.W. John, K.J. Schimenti, V.L. Browning, and J.C. Schimenti. 2000. Mouse mutants from chemically mutagenized embryonic stem cells. *Nat Genet* 24:318-321.
- Negrini, S., V.G. Gorgoulis, and T.D. Halazonetis. 2010. Genomic instability--an evolving hallmark of cancer. *Nature reviews. Molecular cell biology* 11:220-228.
- Nick McElhinny, S.A., C.M. Snowden, J. McCarville, and D.A. Ramsden. 2000. Ku recruits the XRCC4-ligase IV complex to DNA ends. *Mol Cell Biol* 20:2996-3003.
- Niedernhofer, L.J., G.A. Garinis, A. Raams, A.S. Lalai, A.R. Robinson, E. Appeldoorn, H. Odijk, R. Oostendorp, A. Ahmad, W. van Leeuwen, A.F. Theil, W. Vermeulen, G.T. van der Horst, P. Meinecke, W.J. Kleijer, J. Vijg, N.G. Jaspers, and J.H. Hoeijmakers. 2006. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. *Nature* 444:1038-1043.
- Nospikel, T. 2009. DNA repair in mammalian cells : Nucleotide excision repair: variations on versatility. *Cell Mol Life Sci* 66:994-1009.

- O'Connell, B.C., and J.W. Harper. 2007. Ubiquitin proteasome system (UPS): what can chromatin do for you? *Curr Opin Cell Biol* 19:206-214.
- O'Driscoll, M., K.M. Cerosaletti, P.M. Girard, Y. Dai, M. Stumm, B. Kysela, B. Hirsch, A. Gennery, S.E. Palmer, J. Seidel, R.A. Gatti, R. Varon, M.A. Oettinger, H. Neitzel, P.A. Jeggo, and P. Concannon. 2001. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell* 8:1175-1185.
- O'Driscoll, M.F., P.A. Smith, and C.M. Magnusson. 2009. Evaluation of a part-time adult diploma nursing programme - 'Tailor-made' provision? *Nurse Educ Today* 29:208-216.
- Osipovich, O., R. Milley, A. Meade, M. Tachibana, Y. Shinkai, M.S. Krangel, and E.M. Oltz. 2004. Targeted inhibition of V(D)J recombination by a histone methyltransferase. *Nat Immunol* 5:309-316.
- Partridge, L., and M. Mangel. 1999. Messages from mortality: the evolution of death rates in the old. *Trends in Ecology and Evolution* 14:438-442.
- Parvanov, E.D., S.H. Ng, P.M. Petkov, and K. Paigen. 2009. Trans-regulation of mouse meiotic recombination hotspots by Rcr1. *PLoS Biol* 7:e36.
- Pei, H., L. Zhang, K. Luo, Y. Qin, M. Chesi, F. Fei, P.L. Bergsagel, L. Wang, Z. You, and Z. Lou. 2011. MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature* 470:124-128.
- Peng, A., and J.L. Maller. 2010. Serine/threonine phosphatases in the DNA damage response and cancer. *Oncogene* 29:5977-5988.
- Pollex, R.L., and R.A. Hegele. 2004. Hutchinson-Gilford progeria syndrome. *Clin Genet* 66:375-381.
- Qiu, W., E.B. Carson-Walter, H. Liu, M. Epperly, J.S. Greenberger, G.P. Zambetti, L. Zhang, and J. Yu. 2008. PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. *Cell Stem Cell* 2:576-583.
- Rastogi, R.P., Richa, A. Kumar, M.B. Tyagi, and R.P. Sinha. 2010. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *J Nucleic Acids* 2010:592980.
- Reddel, C.J., and A.S. Weiss. 2004. Lamin A expression levels are unperturbed at the normal and mutant alleles but display partial splice site selection in Hutchinson-Gilford progeria syndrome. *J Med Genet* 41:715-717.
- Reina-San-Martin, B., H.T. Chen, A. Nussenzweig, and M.C. Nussenzweig. 2004. ATM is required for efficient recombination between immunoglobulin switch regions. *J Exp Med* 200:1103-1110.
- Riballo, E., S.E. Critchlow, S.H. Teo, A.J. Doherty, A. Priestley, B. Broughton, B. Kysela, H. Beamish, N. Plowman, C.F. Arlett, A.R. Lehmann, S.P. Jackson, and P.A. Jeggo. 1999. Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. *Curr Biol* 9:699-702.
- Richard, D.J., E. Bolderson, L. Cubeddu, R.I. Wadsworth, K. Savage, G.G. Sharma, M.L. Nicolette, S. Tsvetanov, M.J. McIlwraith, R.K. Pandita, S. Takeda, R.T. Hay, J. Gautier, S.C. West, T.T. Paull, T.K. Pandita, M.F. White, and K.K. Khanna. 2008. Single-stranded DNA-binding protein hSSB1 is critical for genomic stability. *Nature* 453:677-681.
- Richardson, C., N. Horikoshi, and T.K. Pandita. 2004. The role of the DNA double-strand break response network in meiosis. *DNA Repair (Amst)* 3:1149-1164.

- Rogakou, E.P., D.R. Pilch, A.H. Orr, V.S. Ivanova, and W.M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 273:5858-5868.
- Romanienko, P.J., and R.D. Camerini-Otero. 2000. The mouse Spo11 gene is required for meiotic chromosome synapsis. *Mol Cell* 6:975-987.
- Salk, D., K. Au, H. Hoehn, and G.M. Martin. 1981. Cytogenetics of Werner's syndrome cultured skin fibroblasts: variegated translocation mosaicism. *Cytogenet Cell Genet* 30:92-107.
- San Filippo, J., P. Sung, and H. Klein. 2008. Mechanism of eukaryotic homologous recombination. *Annual review of biochemistry* 77:229-257.
- Sancar, A., L.A. Lindsey-Boltz, T.H. Kang, J.T. Reardon, J.H. Lee, and N. Ozturk. 2010. Circadian clock control of the cellular response to DNA damage. *FEBS letters* 584:2618-2625.
- Sato, H., T. Miyamoto, L. Yogev, M. Namiki, E. Koh, H. Hayashi, Y. Sasaki, M. Ishikawa, D.J. Lamb, N. Matsumoto, O.S. Birk, N. Niikawa, and K. Sengoku. 2006. Polymorphic alleles of the human MEI1 gene are associated with human azoospermia by meiotic arrest. *J Hum Genet* 51:533-540.
- Schrader, C.E., J.E. Guikema, E.K. Linehan, E. Selsing, and J. Stavnezer. 2007. Activation-induced cytidine deaminase-dependent DNA breaks in class switch recombination occur during G1 phase of the cell cycle and depend upon mismatch repair. *J Immunol* 179:6064-6071.
- Schreiber, V., F. Dantzer, J.C. Ame, and G. de Murcia. 2006. Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol* 7:517-528.
- Schwarz, K., G.H. Gauss, L. Ludwig, U. Pannicke, Z. Li, D. Lindner, W. Friedrich, R.A. Seger, T.E. Hansen-Hagge, S. Desiderio, M.R. Lieber, and C.R. Bartram. 1996. RAG mutations in human B cell-negative SCID. *Science* 274:97-99.
- Scully, R., and D.M. Livingston. 2000. In search of the tumour-suppressor functions of BRCA1 and BRCA2. *Nature* 408:429-432.
- Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 3:155-168.
- Shinkai, Y., G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855-867.
- Simsek, D., and M. Jasin. 2010. Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. *Nature structural & molecular biology* 17:410-416.
- So, S., A.J. Davis, and D.J. Chen. 2009. Autophosphorylation at serine 1981 stabilizes ATM at DNA damage sites. *J Cell Biol* 187:977-990.
- Soulas-Sprauel, P., G. Le Guyader, P. Rivera-Munoz, V. Abramowski, C. Olivier-Martin, C. Goujet-Zalc, P. Charneau, and J.P. de Villartay. 2007. Role for DNA repair factor XRCC4 in immunoglobulin class switch recombination. *J Exp Med* 204:1717-1727.
- Soutoglou, E., and T. Misteli. 2008. Activation of the cellular DNA damage response in the absence of DNA lesions. *Science* 320:1507-1510.
- Starcevic, D., S. Dalal, and J.B. Sweasy. 2004. Is there a link between DNA polymerase beta and cancer? *Cell Cycle* 3:998-1001.
- Stavnezer, J., J.E. Guikema, and C.E. Schrader. 2008. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* 26:261-292.

- Stefanini, M., E. Botta, M. Lanzafame, and D. Orioli. 2010. Trichothiodystrophy: from basic mechanisms to clinical implications. *DNA repair* 9:2-10.
- Stewart, G.S., S. Panier, K. Townsend, A.K. Al-Hakim, N.K. Kolas, E.S. Miller, S. Nakada, J. Ylanko, S. Olivarius, M. Mendez, C. Oldreive, J. Wildenhain, A. Tagliaferro, L. Pelletier, N. Taubenheim, A. Durandy, P.J. Byrd, T. Stankovic, A.M. Taylor, and D. Durocher. 2009. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell* 136:420-434.
- Stracker, T.H., and J.H. Petrini. 2011. The MRE11 complex: starting from the ends. *Nat Rev Mol Cell Biol* 12:90-103.
- Su, I.H., A. Basavaraj, A.N. Krutchinsky, O. Hobert, A. Ullrich, B.T. Chait, and A. Tarakhovskiy. 2003. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. *Nat Immunol* 4:124-131.
- Sugasawa, K., Y. Okuda, M. Saijo, R. Nishi, N. Matsuda, G. Chu, T. Mori, S. Iwai, K. Tanaka, and F. Hanaoka. 2005. UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* 121:387-400.
- Suh, E.K., A. Yang, A. Kettenbach, C. Bamberger, A.H. Michaelis, Z. Zhu, J.A. Elvin, R.T. Bronson, C.P. Crum, and F. McKeon. 2006. p63 protects the female germ line during meiotic arrest. *Nature* 444:624-628.
- Sundin, G.W., and M.R. Weigand. 2007. The microbiology of mutability. *FEMS Microbiol Lett* 277:11-20.
- Szilard, L. 1959. On the Nature of the Aging Process. *Proc Natl Acad Sci U S A* 45:30-45.
- Taccioli, G.E., G. Rathbun, E. Oltz, T. Stamato, P.A. Jeggo, and F.W. Alt. 1993. Impairment of V(D)J recombination in double-strand break repair mutants. *Science* 260:207-210.
- Tang, J.B., and R.A. Greenberg. 2010. Connecting the Dots: Interplay Between Ubiquitylation and SUMOylation at DNA Double Strand Breaks. *Genes Cancer* 1:787-796.
- Thonneau, P., S. Marchand, A. Tallec, M.L. Ferial, B. Ducot, J. Lansac, P. Lopes, J.M. Tabaste, and A. Spira. 1991. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989). *Hum Reprod* 6:811-816.
- van der Burg, M., H. Ijspeert, N.S. Verkaik, T. Turul, W.W. Wiegant, K. Morotomi-Yano, P.O. Mari, I. Tezcan, D.J. Chen, M.Z. Zdzienicka, J.J. van Dongen, and D.C. van Gent. 2009. A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining. *J Clin Invest* 119:91-98.
- van der Burg, M., L.R. van Veelen, N.S. Verkaik, W.W. Wiegant, N.G. Hartwig, B.H. Barendregt, L. Brugmans, A. Raams, N.G. Jaspers, M.Z. Zdzienicka, J.J. van Dongen, and D.C. van Gent. 2006. A new type of radiosensitive T-B-NK+ severe combined immunodeficiency caused by a LIG4 mutation. *J Clin Invest* 116:137-145.
- van Engelen, B.G., J.A. Hiel, F.J. Gabreels, L.P. van den Heuvel, D.C. van Gent, and C.M. Weemaes. 2001. Decreased immunoglobulin class switching in Nijmegen Breakage syndrome due to the DNA repair defect. *Hum Immunol* 62:1324-1327.
- Venkatesan, R.N., J.H. Bielas, and L.A. Loeb. 2006. Generation of mutator mutants during carcinogenesis. *DNA Repair (Amst)* 5:294-302.
- Viale, A., F. De Franco, A. Orleth, V. Cambiaghi, V. Giuliani, D. Bossi, C. Ronchini, S. Ronzoni, I. Muradore, S. Monestiroli, A. Gobbi, M. Alcalay, S. Minucci, and P.G. Pelicci. 2009. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. *Nature* 457:51-56.

- Villa, A., S. Santagata, F. Bozzi, S. Giliani, A. Frattini, L. Imberti, L.B. Gatta, H.D. Ochs, K. Schwarz, L.D. Notarangelo, P. Vezzoni, and E. Spanopoulou. 1998. Partial V(D)J recombination activity leads to Omenn syndrome. *Cell* 93:885-896.
- Villa, A., C. Sobacchi, L.D. Notarangelo, F. Bozzi, M. Abinun, T.G. Abrahamsen, P.D. Arkwright, M. Baniyash, E.G. Brooks, M.E. Conley, P. Cortes, M. Duse, A. Fasth, A.M. Filipovich, A.J. Infante, A. Jones, E. Mazzolari, S.M. Muller, S. Pasic, G. Rechavi, M.G. Sacco, S. Santagata, M.L. Schroeder, R. Seger, D. Strina, A. Ugazio, J. Valiaho, M. Vihinen, L.B. Vogler, H. Ochs, P. Vezzoni, W. Friedrich, and K. Schwarz. 2001. V(D)J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. *Blood* 97:81-88.
- Wei, K., A.B. Clark, E. Wong, M.F. Kane, D.J. Mazur, T. Parris, N.K. Kolas, R. Russell, H. Hou, Jr., B. Kneitz, G. Yang, T.A. Kunkel, R.D. Kolodner, P.E. Cohen, and W. Edelmann. 2003. Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. *Genes Dev* 17:603-614.
- Weidenheim, K.M., D.W. Dickson, and I. Rapin. 2009. Neuropathology of Cockayne syndrome: Evidence for impaired development, premature aging, and neurodegeneration. *Mech Ageing Dev* 130:619-636.
- Wilson, D.M., 3rd, and V.A. Bohr. 2007. The mechanics of base excision repair, and its relationship to aging and disease. *DNA Repair (Amst)* 6:544-559.
- Woodward, W.A., M.S. Chen, F. Behbod, M.P. Alfaro, T.A. Buchholz, and J.M. Rosen. 2007. WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America* 104:618-623.
- Wu, L., S.L. Davies, P.S. North, H. Goulaouic, J.F. Riou, H. Turley, K.C. Gatter, and I.D. Hickson. 2000. The Bloom's syndrome gene product interacts with topoisomerase III. *J Biol Chem* 275:9636-9644.
- Yan, C.T., C. Boboila, E.K. Souza, S. Franco, T.R. Hickernell, M. Murphy, S. Gumaste, M. Geyer, A.A. Zarrin, J.P. Manis, K. Rajewsky, and F.W. Alt. 2007. IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature* 449:478-482.
- Yoshida, K., G. Kondoh, Y. Matsuda, T. Habu, Y. Nishimune, and T. Morita. 1998. The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Mol Cell* 1:707-718.
- Yu, C.E., J. Oshima, Y.H. Fu, E.M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G.M. Martin, J. Mulligan, and G.D. Schellenberg. 1996. Positional cloning of the Werner's syndrome gene. *Science* 272:258-262.
- Zhang, M., F. Behbod, R.L. Atkinson, M.D. Landis, F. Kittrell, D. Edwards, D. Medina, A. Tsimelzon, S. Hilsenbeck, J.E. Green, A.M. Michalowska, and J.M. Rosen. 2008. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer research* 68:4674-4682.
- Zhang, Y., and M. Jasin. 2011. An essential role for CtIP in chromosomal translocation formation through an alternative end-joining pathway. *Nature structural & molecular biology* 18:80-84.
- Zhu, J., S. Petersen, L. Tessarollo, and A. Nussenzweig. 2001. Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Curr Biol* 11:105-109.

Double Strand Break Signaling in Health and Diseases

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1. Introduction

Living organisms are constantly subjected to DNA damage whether it originates from intrinsic physiological processes or extrinsic stressors. Of the various types of DNA damage, double strand breaks (DSBs) are the most dangerous since if left unrepaired they can lead to either cell death or genomic instability. For this reason cells have evolved an arsenal of signaling and repair proteins involved in DNA double strand break sensing and repair as well as downstream physiological responses such as apoptosis or cell cycle arrest. DSBs can be generated by reactive oxygen species which are produced during normal metabolism. DSB formation and repair also occurs during the tightly regulated physiological processes of gametogenesis, V(D)J recombination of T- and B-cell receptors and antibody diversification during class switch recombination (CSR). In addition, DSBs can be caused by external agents such as ionizing radiation or chemotherapeutic agents such as etoposide. The importance of DSB signaling and repair is underscored by the many human diseases and syndromes caused by the mutation of genes coding for DNA damage response (DDR) proteins. The study of knockout mouse models for DDR genes has also furthered our understanding of the role of these proteins in DSB repair and in normal physiology. In this chapter, DSB signaling and repair are reviewed. In addition, an overview of the human diseases associated with mutations of DSB signaling and repair proteins is given. Finally, the impact of mouse models on our understanding of DSB signaling and repair and its physiological roles is discussed.

2. Signaling at DNA double strand breaks

2.1 Sensing the DNA double strand breaks

When DSBs are generated they are initially recognized by either the Ku70/Ku80 heterodimer, the Mre11-Rad50-NBS1 (MRN) complex or members of the PARP (PARP1/2) family of proteins (Ciccia & Elledge, 2010). The role of these protein sensors is to bind to and tether the DNA ends, thereby preventing further breakage as well as to recruit additional proteins that are required for DSB signaling and repair. The first group of proteins to be recruited to DSBs after initial sensing of the breaks belongs to the phosphatidylinositol-3-kinase-like protein kinases family. These include ATM (ataxia-telangiectasia mutated), ATR (ATR and Rad3-related) and the catalytic subunit of DNA-PK known as DNA-PKcs. While ATM and DNA-PK respond only to DSBs, ATR also responds to single strand DNA breaks.

Ku70/80 recruits DNA-PKcs to DSBs where it promotes DNA repair by non-homologous end joining whereas both PARP1/2 and the MRN complex lead to the recruitment of ATM which promotes homologous recombination. The MRN complex is recruited to DSBs in both a PARP1/2 dependent and independent manner (Ciccia & Elledge, 2010). ATM is a central component of the cellular response to DSBs and is predicted to have several hundred downstream targets many of which play a role in the DNA damage response (Matsuoka et al, 2007). Under normal conditions ATM is in a homodimeric form. Following DNA damage, it becomes autophosphorylated at Ser1981 and dissociates into its monomeric form and binds the damaged DNA (Bakkenist & Kastan, 2003). There, it leads to the phosphorylation of many downstream targets involved in the DSB response. The phosphorylation of ATM at Ser1981 and its initial binding to DSBs depend upon the MRN complex. MRN consists of three different proteins Mre11, NBS1 and Rad50. Mre11 is a DNA nuclease that interacts with both Rad50 and NBS1 as well as with other Mre11 molecules to form dimers. When paired with the other components of the MRN complex, Mre11 can have both double strand DNA exonuclease activity and single strand DNA endonuclease activity (D'Amours & Jackson, 2002). In addition, Mre11 has two DNA binding sites and intrinsic DNA binding activity. Rad50 is a protein that bears homology to the structural maintenance of chromosome (SMC) family. It is an ATPase and is needed for tethering of DNA ends together during the process of DNA repair. NBS1 has a fork-head-associated (FHA) domain and two BRCT (BRCA1-tandem repeats) domains at its N-terminus which are used to recognize phospho-threonine and phospho-serine residues respectively in Ser-X-Thr motifs. These domains allow RAD50 to interact with several DNA damage signaling proteins following DSB formation. NBS1 also contains a nuclear localization signal (NLS) that allows the translocation of the MRN complex into the nucleus following DNA damage (Lamarche et al, 2010). NBS1 interacts with ATM thereby leading to its recruitment to DSBs. There, ATM is involved in one of the very early response to the formation of DSBs, mainly the phosphorylation of histone variant H2AX on Ser139 to form γ -H2AX (Figure 1). This phosphorylation can extend over a megabase of DNA from the site of DSBs (Modesti & Kanaar, 2001). The formation of γ -H2AX at the sites of DSBs is key for the recruitment of many effector proteins to the break sites including the regulators of cell cycle checkpoint and DNA repair 53BP1, BRCA1 and Rad51 (Bohgaki et al, 2010). The accumulation of γ -H2AX and other DNA damage signaling and repair proteins at the sites of ionizing-radiation induced breaks leads to the formation of microscopically distinct foci known as IR-induced nuclear foci (IRIFs) which can be used experimentally to study IR-induced DNA damage signaling and repair. The ability of H2AX to recruit DNA damage proteins under normal physiological conditions is hampered by its constitutive phosphorylation at Tyr142 by William's syndrome transcription factor (WSTF). This phosphorylation suppresses the ability of H2AX to recruit downstream signaling and effectors of the DNA damage response to the breaks. However, following DNA damage Tyr142 residue is dephosphorylated by the EYA protein phosphatases (Cook et al, 2009). γ -H2AX recruits MDC1, a mediator of the DNA damage response that functions as an adaptor to recruit downstream effector proteins to the break sites. MDC1 has two BRCT domains at its C-terminus and one FHA domain at its N-terminus that allow it to recognize and interact with other DNA damage response proteins (Stewart et al, 2003). The BRCT domains of MDC1 can recognize phosphorylation sites and were shown to mediate MDC1 binding to γ -H2AX.

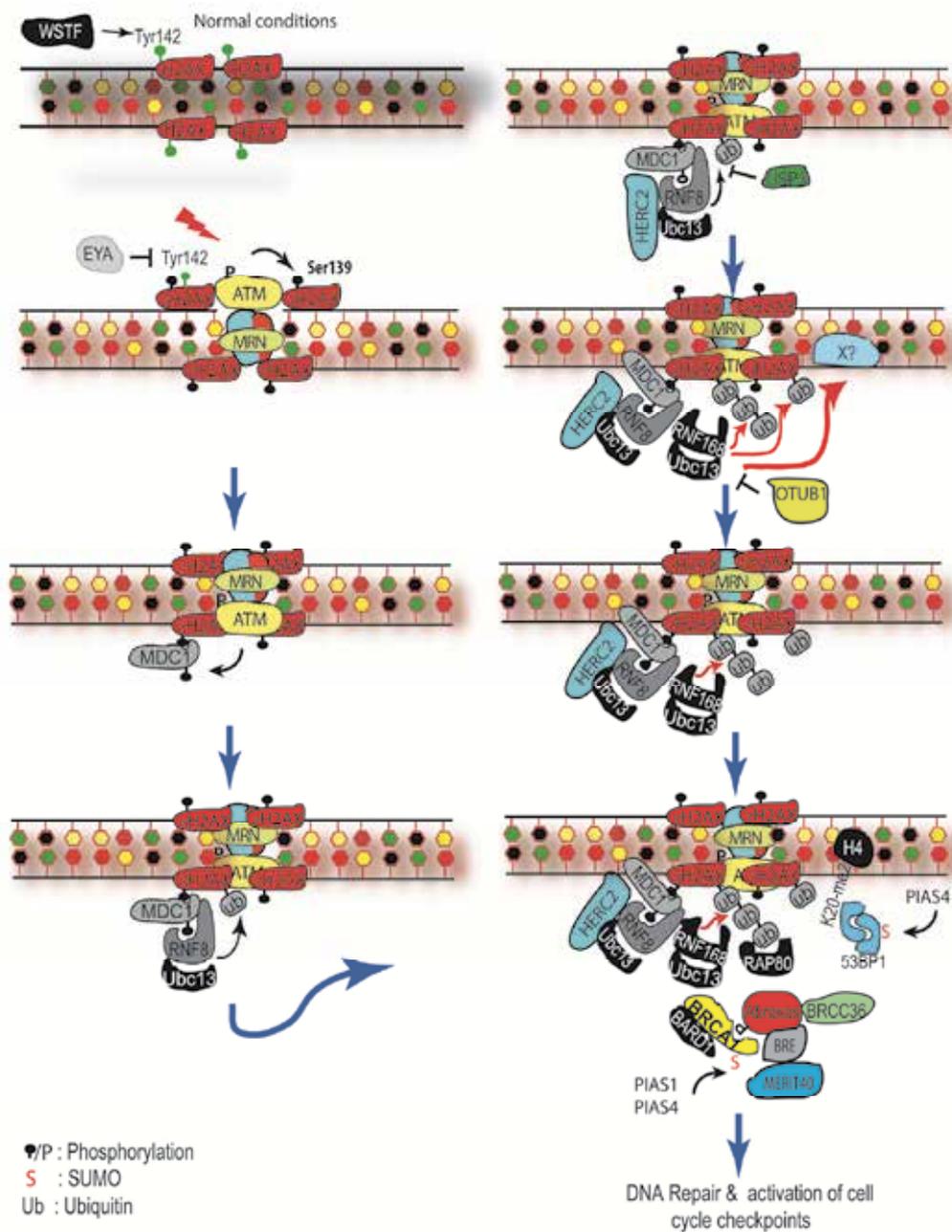


Fig. 1. DNA double strand break signaling

This interaction occurs specifically between the BRCT domain of MDC1 and the Ser139 phosphorylation site of γ -H2AX located at the C-terminus of this protein. MDC1 also interacts with ATM and is phosphorylated by it at its FHA domain (Goldberg et al, 2003; Stewart et al, 2003). Four potential consensus TQFX sites for ATM phosphorylation have been found and two sites, T719 and T752, were confirmed to be phosphorylated by ATM

(Kolas et al, 2007; Matsuoka et al, 2007). MDC1 is thought to be dispensable for the initial binding of ATM to the DSBs and early H2AX phosphorylation but important for ATM and γ -H2AX retention at the damaged DNA sites. In fact, MDC1 acts in a positive feedback loop with γ -H2AX and ATM to amplify the signal at the breaks (Lou et al, 2006). MDC1 was also found to be important for the recruitment of other DNA damage response proteins such as 53BP1 and NBS1 to the break sites. Recent studies have provided us with greater insight about the mechanisms by which MDC1 leads to the recruitment of DNA damage proteins to the DSBs and have highlighted the role of posttranslational modifications such as ubiquitylation and sumoylation in the amplification of the DNA damage response.

2.2 Role of ubiquitylation in the DNA double strand break response

The important role ubiquitylation plays in DNA damage signaling and DNA repair has been recently highlighted (Bohgaki et al, 2010; Panier & Durocher, 2009). Ubiquitin is a small polypeptide of about 8 kDa that can be covalently attached through an isopeptide bond to substrate proteins. This requires the activity of three different enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). The combination of E2 and E3 enzymes used to ubiquitylate target proteins differ from one to the other thereby conferring substrate specificity to the enzyme. Ubiquitin chain attachment can occur through several lysine residues on the ubiquitin polypeptide. The best studied ones are lysine 48 (K48) and lysine 63 (K63). While K48-linked ubiquitin most often leads to proteasomal degradation, K63-linked ubiquitylation is involved in cellular signaling including DSB signaling and DNA repair. Using an siRNA screen for proteins whose absence lead to an impairment of 53BP1 foci formation it was found that knock-down of MDC1 and the E3 ubiquitin ligase RNF8 (Ring Finger protein 8) strongly inhibited 53BP1 recruitment to DSBs (Kolas et al, 2007). RNF8 has a RING domain which is required for its E3 ligase activity and is located at its C-terminus and an FHA domain located at its N-terminus. The FHA domain of RNF8 can recognize phosphorylated MDC1 thereby allowing binding of RNF8 to MDC1. Therefore, MDC1 phosphorylation is required for formation of RNF8 IRIFs. Furthermore, it was found that RNF8 partners up with the E2 ligase UBC13 to mediate K63-linked ubiquitylation of H2A, H2AX and H2B (Huen et al, 2007; Kolas et al, 2007; Mailand et al, 2007). This initial ubiquitylation of chromatin components leads to the recruitment of another ubiquitin ligase RNF168 (Doil et al, 2009; Stewart et al, 2009). RNF168 contains two motifs interacting with ubiquitin (MIUs) and is thought to interact with ubiquitylated chromatin components through its MIUs. At the DNA breaks, RNF168, in collaboration with the E2 ligase UBC13, adds K63-linked polyubiquitin chains to H2A and H2AX. This ultimately results in the subsequent recruitment of other DDR proteins to the break sites. For example, RNF168 is required for the formation of 53BP1 IRIFs and the recruitment of BRCA1 to DSB site is severely reduced in the absence of this E3 ligase (Bohgaki et al, 2011; Doil et al, 2009; Stewart et al, 2009). HERC2 is a recently identified protein that leads to binding and stabilization of the RNF8-UBC13 complex to mediate K63-linked histone ubiquitylation (Bekker-Jensen et al, 2010). Additionally, HERC2 was also found to stabilize RNF168 and to promote recruitment of DSB signaling proteins including 53BP1 and BRCA1 to the break sites. The mechanisms through which chromatin component ubiquitylation leads to the recruitment of 53BP1 and BRCA1 are only partially understood. BRCA1 interacts with the receptor-associated protein 80 (RAP80). RAP80 recruitment to DSBs is dependent on γ -H2AX, MDC1 and RNF8 (Kim et al, 2007; Kolas et al, 2007; Mailand

et al, 2007). RAP80 contains two ubiquitin interacting motifs (UIMs) which are capable of specifically recognizing K-63 linked polyubiquitin chains (Kim et al, 2007). The interaction of RAP80 and BRCA1 is mediated by another protein named Abraxas (Mailand et al, 2007; Wang et al, 2007). The RAP80-Abraxas-BRCA1 complex is thought to bind DSB sites through the recognition of RNF8- and RNF168-ubiquitylated chromatin by the RAP80 UIM domains (Wang et al, 2007). The way 53BP1 is recruited to the break sites is less well understood. 53BP1 recruitment to DSBs is mediated by the binding of its two TUDOR domains to di-methylated histone H4 at lysine (K) 20. Histone methylation is not increased from its constitutive level following ionizing radiation suggesting that changes in chromatin structure following DNA damage might be responsible for the uncovering of the methylated histones and the binding of 53BP1. It is therefore plausible that ubiquitylation of H2A and H2B by RNF8 and RNF168 triggers conformational changes in the chromatin that lead to a better accessibility of 53BP1 to the methylated histones (Mailand et al, 2007). However, this hypothesis still remains to be tested. The ubiquitylation of chromatin components leads to the rapid amplification of the DNA damage response. However, once the DNA is repaired, a rapid deubiquitination of histone H2A, H2B and H2AX should occur to return cells to their steady-state levels. This is accomplished by ubiquitin isopeptidases known as deubiquitinating enzymes or DUBs. Several DUBs have been described to negatively regulate DSB signaling. Ubiquitin-specific protease 3 (USP3) was shown to deubiquitylate H2A and H2B (Nicassio et al, 2007) and to negatively regulate the activity of RNF8 (Doil et al, 2009). BRCC36 binds to RAP80 and is in the BRCA1 complex that also includes Abraxas. BRCC36 along with RAP80 can antagonize the activity of RNF8-UBC13 by acting as a deubiquitinating enzyme for γ -H2AX. It also leads to decreased ubiquitylation signal at DSB sites (Shao et al, 2009). Recently, OTUB1 (OTU domain, ubiquitin aldehyde binding 1) was identified as an RNF168 DUB. OTUB1 does not use its catalytic activity to deubiquitylate RNF168 but rather acts by binding to and inhibiting UBC13, the RNF168 E2 conjugating enzyme (Nakada et al, 2010).

2.3 Role of SUMOylation in DNA double strand break signaling

SUMOylation is a posttranslational modification that involves the covalent linkage of a small ubiquitin-like modifier (SUMO) polypeptide to a target protein (Al-Hakim et al, 2010; Ciccia & Elledge, 2010). The process of SUMOylation is similar to that of ubiquitylation in that it also requires a SUMO-specific E1 (SAE1/SAE2), an E2 conjugating enzyme (Ubc9) and substrate-specific E3 ligases. Three types of SUMO molecules have been identified, SUMO1, SUMO2 and SUMO3. SUMO2 and SUMO3 have strong homology and share the same function and are therefore usually referred to as SUMO2/3. Both SUMO1 and SUMO2/3 are recruited to DSBs and this recruitment is dependent on the presence of RNF8 and RNF168. The E3 ligases PIAS1 and PIAS4 are needed for the accumulation of SUMO molecules to the break sites. PIAS1 is involved in the accumulation of SUMO2/3 but not SUMO1 at DSBs whereas PIAS4 is involved in the accumulation of all three SUMO moieties. PIAS1 and PIAS4 were both found to be important for accumulation of RNF168 and ubiquitylated H2A at DSB sites. Furthermore, PIAS1 and PIAS4 SUMOylate BRCA1 which is thought to increase its ubiquitin E3 ligase activity and the ubiquitylation of H2A by BRCA1 which would further amplify the DNA damage signal (Morris et al, 2009). SUMOylation of 53BP1 by PIAS1 and PIAS4 was also demonstrated and shown to be required for the efficient recruitment of 53BP1 to DSBs (Galanty et al, 2009). In summary,

complex posttranslational modifications such as ubiquitylation, SUMOylation, phosphorylation and methylation, at the sites of DSBs lead to recruitment of proteins that are involved in DNA repair and the regulation of cell cycle checkpoints following DNA damage as will be discussed in the next section.

2.4 Cellular responses following DNA double strand breaks

Following DNA damage, cells are faced with different options. Either to undergo cell cycle arrest and repair the damaged DNA, to undergo senescence or to die by apoptosis. There are three checkpoints put in place to arrest the cell cycle following DNA damage. The G1/S checkpoint, the intra-S checkpoint and the G2/M checkpoint (Warmerdam & Kanaar, 2010). These checkpoints are carefully regulated by ATM and its downstream effectors. ATM phosphorylates and activates the protein kinase Chk2 and the tumor suppressor p53 which then act to enforce cell cycle arrest or apoptosis. Chk2 activation requires phosphorylation by ATM at Thr68 and autophosphorylation at multiple other residues. Chk2 phosphorylation of the cdc25 phosphatases which are needed for cell cycle progression lead to either their degradation (in the case of cdc25A) or their export from the nucleus (for cdc25B and cdc25C) thereby preventing interaction with their respective cdk/cyclin substrates (Donzelli & Draetta, 2003). Chk2 also phosphorylates p53 at Ser20 whereas ATM phosphorylates it at Ser15. This results in the accumulation of p53 and its activation as a transcription factor which transactivates many genes whose products are involved in cell cycle arrest such as p21, GADD45 and 14-3-3 σ . p53 also transactivates several proapoptotic genes such as Noxa, PUMA and Bax, thus triggering apoptotic cell death of the damaged cells. The mechanisms that lead to p53-dependent cell cycle arrest versus p53-dependent apoptosis remain poorly understood but are likely to depend on the multiple posttranslational modifications of p53 (Vousden, 2006).

3. DNA repair

Cells have evolved different pathways to repair DSBs. Currently, homologous recombination (HR) and non-homologous end joining (NHEJ) are recognized as the two major pathways for DSB repair (Kass & Jasin, 2010). The presence of undamaged sister chromatid is required for the error-free HR-mediated DSB repair, whereas NHEJ repair can occur in the absence of a homologous template sequence and is therefore considered to be more error-prone. Alternative NHEJ is another form of DNA repair that does not necessitate the presence of classical NHEJ DNA repair proteins and is characterized by sequence deletion and the introduction of microhomologies within the repaired DNA (Kotnis et al, 2009).

3.1 Non-homologous end joining

NHEJ is the most commonly used pathway for the repair of DSBs. Since it does not require the presence of a homologous sequence on a sister chromatid, it can occur throughout the cell cycle but particularly during G0, G1 and early S-phase (Kass & Jasin, 2010).

The first step of NHEJ is the recognition of the DSBs by the Ku70/Ku80 heterodimer which consists of the Ku70 and Ku80 subunit (Figure 2). The Ku70/Ku80 complex then slides inwards, away from the edge of the DSBs to allow binding of two molecules of the catalytic subunit of DNA-PK, DNA-PKcs. When DNA-PKcs binds to DNA and Ku70/Ku80 it is known as DNA-PK. The two DNA-PKcs molecules bind to each other, thereby bringing

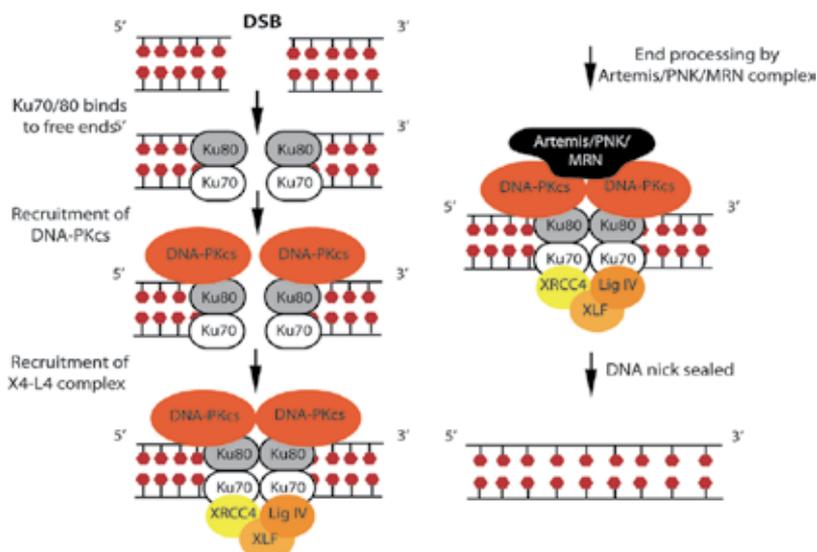


Fig. 2. Repair of DNA double strand breaks by non-homologous end joining

together the DNA ends in a process called synapsis. Binding of Ku70/Ku80 and DNA-PKcs to the DNA ends in this manner protects them against nuclease degradation in the cell. DNA-PKcs is a serine-threonine kinase whose activity is stimulated by binding to double stranded DNA and the Ku heterodimer. Phosphorylation of DNA-PKcs is needed to bring the proteins involved in DNA end processing such as Artemis, the DNA polymerase family members polymerase μ and polymerase λ , and the Polynucleotide kinase (PNK) to the break sites. End processing of the DNA at DSBs allows the removal of DNA lesions that interfere in the ligation process (Mahaney et al, 2009). Artemis is thought to be recruited to DSBs by binding to autophosphorylated DNA-PKcs. While Artemis has inherent 5'→3' exonuclease activity, in the presence of DNA-PK and ATP it also acquires endonuclease activity. DNA-PK promotes the endonucleolytic activity of Artemis versus its exonucleolytic activity and decreases the speed of nucleotide removal. This is important to limit the amount of trimming done at the DNA ends to the minimum. DNA polymerases μ and λ are brought to the DSB sites via their interactions with either Ku70/Ku80 or the XRCC4/DNA ligase complex. Their role at the DSBs involves filling in gaps to allow ligation. DNA polymerase μ is less dependent than DNA polymerase λ on the presence of a template. PNK was also shown to play a role in NHEJ. It associates with XRCC4 and is dependent on its presence for its activity. It can phosphorylate 5'-OH terminal groups and dephosphorylate 3'-ends to restore normal DNA ends that can be ligated by the XRCC4/DNA ligase complex (Chappell et al, 2002). Aprataxin and PNK-like factor (APLF) is an endo-exonuclease that was shown to be required for full NHEJ activity. It can interact with other NHEJ factors such as Ku and XRCC4 and is phosphorylated by ATM in a DNA damage-dependent manner (Macrae et al, 2008). XRCC4 is thought to act as a scaffolding protein that brings many factors involved in NHEJ to the break sites. XRCC4 and DNA ligase IV, along with XLF (also known as Cernunnos) form a complex known as X4-L4. DNA ligase activity is thought to be stimulated by its binding to XRCC4 and to XLF. XRCC4 and XLF can bind to Ku proteins and to DNA. The X4-L4 complex is capable of ligating one strand of DNA at a time which would allow for concomitant processing and ligation of the ends (Hartlerode & Scully, 2009).

3.2 Alternative non-homologous end joining

When one or more classical NHEJ factors (Ku, DNA-PKcs, XRCC4, DNA ligase IV) are missing, DSBs can be repaired through the alternative NHEJ (Alt-NHEJ) pathway. This type of DNA repair relies on the presence of microhomologies at the terminal ends of the DNA breaks. It was found that Alt-NHEJ can occur in the absence of DNA ligase IV suggesting that one of the two remaining ligases (LigI or LigIII in eukaryotes) can function in DSB end ligation. End joining in the absence of LigIV requires 2-3 nucleotides of homology to stabilize the DNA at broken ends whereas no microhomology is needed in normal cells for NHEJ to occur. Furthermore it was found that a 4 nucleotide long microhomology at the break ends greatly decreases the requirement for Ku70, probably because of the increased stabilization of the DNA ends. Alt-NHEJ is stimulated by the presence of CtIP (CtBP-interacting protein) and suppressed by the classical NHEJ factors Ku and XRCC4-LigIV (Bennardo et al, 2008; Simsek & Jasin, 2010). It was found that Alt-NHEJ promotes chromosomal translocations which might explain the increase of hematological cancer incidence in the absence of one or more classical NHEJ factors (Simsek & Jasin, 2010).

3.3 Homologous recombination

Homologous recombination requires the presence of homologous sequences for the accurate repair of DSBs. It usually occurs in the late S phase or G2 phase when a sister chromatid is available to be used as a template for repair (Ciccia & Elledge, 2010; Kass & Jasin, 2010). The first step of HR is the generation of 3'-ssDNA (single stranded DNA) overhangs with 3'-hydroxyl ends which subsequently invade a homologous duplex DNA sequence (Figure 3). Initial processing of the DNA ends requires the MRN complex along with CtIP. Further processing is done by Exo1 in association with the helicase Bloom syndrome protein BLM (Nimonkar et al, 2008). The 3'-ssDNA overhangs generated by this process are then bound by RPA (replication protein A) which is needed to melt the secondary structures of the DNA and protect the DNA ends before the binding of other HR proteins can occur. RPA is required for the recruitment of HR factors such as the DNA-dependent ATPase Rad51 to the break sites (Sleeth et al, 2007).

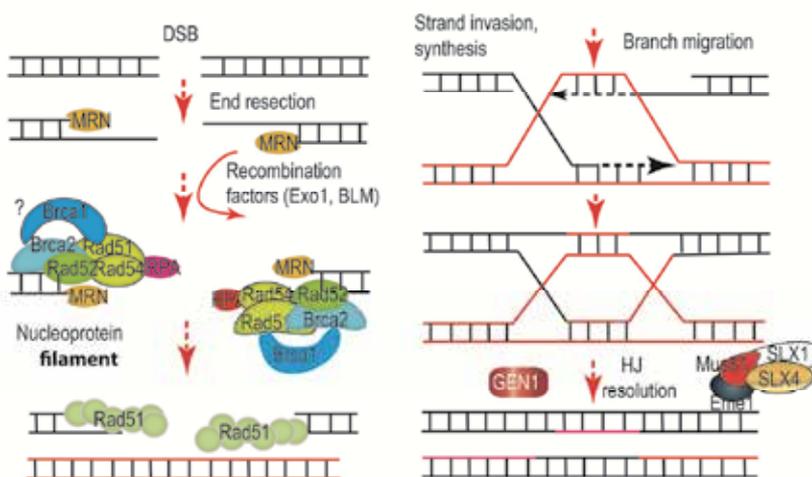


Fig. 3. Double strand break repair by homologous recombination

Rad51, a key HR player, belongs to the Rad52 epistasis group in yeast which is involved in recombinational DNA repair. Rad51 competes with RPA for binding to the DNA. Since it has lower affinity to DNA than RPA, other factors are needed to displace RPA and lead to the formation of Rad51-DNA nucleofilaments. This step requires the presence of the breast cancer susceptibility protein 2 (BRCA2). BRCA2 binds to Rad51 through its BRC repeats and its carboxy-terminus. BRCA2 facilitates the binding of Rad51 to the DNA by promoting RPA displacement and decreasing its ability to bind double stranded DNA. In addition, it inhibits the ATPase activity of Rad51, thereby stabilizing the Rad51-ssDNA complexes (Jensen et al, 2010). Once it binds to the ssDNA, Rad51 catalyzes invasion of a homologous duplex DNA sequence, thereby forming a displacement loop (D-loop). This process is known as synapsis and requires the presence of the Rad54 motor protein. Rad54, a member of the Rad52 epistasis group, binds to the Rad51-DNA nucleofilament, stabilizes it and therefore enhances D-loop formation. However, following synapsis, Rad54 promotes dissociation of Rad51 from double stranded DNA (dsDNA) which allows synthesis of the DNA strand. Rad54-mediated dissociation of Rad51 from dsDNA also allows for rapid Rad51 turnover (Heyer et al, 2010). After the D-loop is formed, DNA repair can occur in three distinct pathways: break-induced replication (BIR), synthesis-dependent strand annealing (SDSA) and double Holliday junctions (dHJ) (Heyer et al, 2010). BIR occurs when only one DNA strand is available to invade duplex DNA. This could occur during replication fork collapse or the uncapping of telomeres. This leads to the formation of a *bona fide* replication fork at this site. DNA synthesis at this site occurs using the regular DNA polymerases (Llorente et al, 2008). During SDSA the invading strand is elongated by DNA synthesis. The D-loop is then reversed, allowing the newly synthesized end to anneal to the resected opposite end of the break. The remaining gaps in the DNA are then filled by DNA synthesis and ligation. SDSA results in non-crossover recombination and is the main pathway used in somatic cells (Sung & Klein, 2006). Finally dHJ mediated recombination involves invasion of the second DNA break into the D loop, DNA synthesis and ligation to join the two invading DNA ends and then dHJ resolution in either a crossover or a non-crossover manner (Sung & Klein, 2006). BLM helicase in conjunction with topoisomerase IIIa leads to the dissolution of HJs to form non-crossover products. The Mus81-Eme1 endonuclease complex, SLX1-SLX4 complex or the human 5'-flap endonuclease GEN1 are capable of resolving dHJ to form both crossover and non-crossover products (Ciccia & Elledge, 2010; Hartlerode & Scully, 2009; Rass et al, 2010).

3.4 Choosing between homologous recombination and non-homologous end joining

The choice of the DNA repair pathway following DSB formation is dependent on many factors. One of the important determinants that allow the cells to choose whether the damaged DNA should be repaired by HR or NHEJ is the cell cycle phase in which it is in. NHEJ can occur throughout the whole cell cycle and is the pathway of choice during the G1 phase whereas DSBs in cells in late S and G2 phases are most likely to be repaired by HR. DNA resection to form 3' ssDNA overhangs is a tightly regulated step because if it occurs it actually commits the cells to repair their DNA using HR versus NHEJ. Binding of CtIP to DSBs promotes resection of the break ends. CtIP levels are regulated in a cell cycle-dependent manner and are elevated during the S, G2 and M phases of the cell cycle but are low during the G1 phase. In addition, CtIP activity is regulated through posttranslational modifications. CtIP is phosphorylated by CDK2 (cyclin dependent kinase 2) during the S

and G2 phases of the cell cycle at two different sites S327 and T847. Phosphorylation of CtIP at S327 allows it to interact with the BRCT domain of BRCA1 and with the MRN complex. This then results in the ubiquitylation of CtIP by BRCA1 (Yu et al, 2006). Posttranslational modification of CtIP by phosphorylation and ubiquitination as well as its interaction with BRCA1 were found to be necessary for it binding to DNA and its role in DNA resection. CtIP is also predicted to be a target of ATM phosphorylation and ATM is required for end resection.

There are many lines of evidence showing competition between the NHEJ and HR pathways for DSB repair. In the absence of the NHEJ factors Ku and XRCC4-LigIV there is an increase in end resection and HR. Conversely, mutations in resection factors such as CtIP result in increased NHEJ (Kass & Jasin, 2010). Recently, 53BP1, a protein involved in cell cycle checkpoint and DNA repair has been implicated in the regulation of the switch between NHEJ and HR repair pathways. Some studies have shown that 53BP1 is important for NHEJ. In addition, 53BP1 is thought to bind to the DSB sites and inhibit DNA resection. Loss of 53BP1 in Brca1-null cells results in increased HR activity in these cells suggesting a model whereby 53BP1 inhibition of end resection is overcome by BRCA1, thereby leading to HR-mediated DNA repair (Bouwman et al, 2010; Bunting et al, 2010). The mechanisms by which BRCA1 can counteract 53BP1 function and promote HR are still unknown but promise to be the focus of intense research in the future.

4. Double strand break repair in normal physiological processes

4.1 Meiotic recombination

Meiosis is a specialized type of cell division occurring during gametogenesis. It allows the production of haploid cells, containing one copy of the genetic material, from diploid cells containing two copies of the genetic material (Kumar et al, 2010). Meiotic recombination is a crucial process during gametogenesis. It occurs during the prophase of the first meiotic division (prophase I) and allows the formation of physical links called chiasmata between two homologous chromosomes. This ensures proper alignment and segregation of the homologous chromosomes during the later phases of meiosis I. DNA recombination during meiosis also allows the exchange of genetic material between homologous chromosomes. This is required to ensure genetic diversity of the organisms and for introduction of mutations needed for the evolution of species. Meiotic recombination requires a highly regulated generation of DSBs followed by their repair through HR. The first step of meiotic recombination is the generation of DSBs during the leptotene stage of prophase I. This stage occurs directly following S phase when DNA replication has occurred. Spo11, a highly conserved type II-like topoisomerase, generates the DSBs needed to initiate meiotic recombination through a transesterification reaction (Keeney et al, 1997). Spo11 binds to both strands of the DNA as a homodimer. Once the DSB is generated, the DNA on which Spo11 is attached is cleaved, thereby releasing a Spo11-oligonucleotide complex. In addition to Spo11, several other proteins were identified as been involved in DSB formation although their mechanism of action is still poorly understood. One of them, Mei4 (MEIosis-specific 4), is needed for DSB formation in both yeast and mammals (Kumar et al., 2010). Mei4 binds to another protein, Rec114 (RECombination 114) which is also essential for DSB formation in yeast. Although the role of Rec114 in mammals is still unknown, its interaction with Mei4 makes it plausible that they function together in DSB generation in mammals (Kumar et al, 2010).

DSBs do not occur evenly throughout the genome but arise more frequently in specific areas called "hotspots". In yeast and mouse it was found that these hotspots are marked by the trimethylation of histone 3 at Lys4 (H3K4Me3) (Borde et al, 2009). Recently, a histone methyltransferase, PRDM9 (PR domain containing 9), was identified as a protein that can bind to and activate hotspots in mammalian genomes (Baudat et al, 2010). After end processing to form 3'-ssDNA, the resected DNA is bound by Rad51 and Dmc1 (Disrupted meiotic cDNA1), a meiosis-specific recombinase. In the yeast *Saccharomyces cerevisiae*, recruitment of Dmc1 to resected DNA ends is dependent upon the two proteins Mei5 and Sae3. Mei5 and Sae3 (Sporulation in the Absence of Spo Eleven 3) form a complex that allows Dmc1 binding to the single stranded DNA and enhances its recombinase activity (Ferrari et al, 2009). The resected ends can then invade double stranded DNA and form a D-loop. Two proteins, Hop2 and Mnd1 which exist in cells as a heterodimeric complex help in the stabilization of the Rad51 and Dmc1 nucleofilaments and increase their ability to invade homologous duplex DNA. Hop2 and Mnd1 function both in yeast and higher eukaryotes, including mammals (Petukhova et al, 2005; Tsubouchi & Roeder, 2002). In contrast to HR occurring in somatic cells which use sister chromatids as a template, the use of a non-sister homologous chromatid is favored during meiotic recombination (Shinohara et al, 1992). The Holliday junctions that form following strand invasion are then resolved to form crossover and non-crossover products. In contrast to what happens during HR in somatic cells, meiotic recombination generates a much higher proportion of crossover products as compared to non-crossover products (Andersen & Sekelsky, 2010). In both *S. cerevisiae* and higher eukaryotes the Msh4-Msh5 (MutS homolog 4-5) complex plays an important role in the resolution of HJs and in crossover formation. It was also suggested that GEN1 could play a role in HJ resolution during human meiotic recombination (Lorenz et al, 2009), however further investigation is needed to prove this hypothesis.

4.2 V(D)J recombination

V(D)J recombination is a crucial process in lymphocyte development through which diverse B and T cell receptors can be generated (Soulas-Sprauel et al, 2007). It involves the assembly of a variable (V), diversity (D) and joining (J) exons to form a B cell or T cell antigen receptor. The multiple combinations that can be obtained by joining together different V, D and J segments are the underlying mechanism for antigen receptor diversity and the ability of the immune system to respond to different types of pathogens. RAG1 and RAG2 (recombination activating genes 1 and 2) are two lymphocyte-specific recombinases that introduce DSBs at specific recombination signal (RS) sequences that border every V, D and J gene segment (Dudley et al, 2005). RS sequences consist of a conserved heptamer and a conserved nonamer separated by a nonconserved 12 bp or 23 bp spacer sequence. In the heavy chain immunoglobulin gene (IgH) for example, RS sequences that surround D sequences have a 12 bp spacer whereas those surrounding the V and J segments have 23 bp. Since recombination can occur only between RS sequences containing a 12 bp spacer and one containing a 23 bp spacer this ensures productive joining of V, D and J sequences (Dudley et al, 2005). RAG mediated cutting of the DNA generates hairpin loops at the joining DNA ends. These hairpin loops are cleaved by the endonuclease Artemis which is recruited and activated by the DNA-PKcs and Ku70/80 complex. The XRCC4/DNAligase IV/XLF complex then religates the joining ends (Soulas-Sprauel et al, 2007).

4.3 Class switch recombination

Class switch recombination (CSR) is a specialized mechanism occurring in B cells that allows the cells to switch from expressing immunoglobulin (Ig) M (IgM) to IgG, IgA or IgE (Stavnezer et al, 2008). This involves replacement of the IgH constant region C μ encoded in IgM with more downstream constant regions such as C γ , C ϵ and C α which code for IgG, IgE and IgA heavy chain constant regions respectively. This replacement of IgH constant regions is mediated through deletion of parts of the chromosome and subsequent recombination. CSR occurs within or nearby specific sequences called the switch regions that precede every constant region. CSR is initiated upon antigenic stimulation of mature B cells and requires at least two rounds of cell division and the presence of a B cell-specific enzyme activation induced cytidine deaminase (AID) that converts deoxycytosine (dC) to deoxyuracil (dU) in the donor and receiver S regions (Muramatsu et al, 2000). The dU is then removed through the base excision repair pathway. The enzyme that cleaves dU in S regions is the uracil DNA glycosylase UNG. The phosphate backbone of the resulting abasic site is then cleaved by the apurinic/apyrimidinic endonucleases APE1 and APE2, two proteins which were found to be essential for CSR, to form a single strand DNA break (Guikema et al, 2007). In order for CSR to occur, DSBs must be created in the donor and acceptor S regions and the single stranded breaks generated by APE1 and 2 are not enough. When single stranded breaks are generated close enough to each other they might lead to the formation of DSBs. Otherwise, DSBs must be generated by mismatch repair (MMR) proteins. It is hypothesized that the MMR heterodimer Msh2-Msh6 (MutS homolog 2-6) recognizes the mismatched U:G pair and binds to it. Mlh1-Pms2 (MutL homolog 1-PostMeiotic Segregation 2) then binds to Msh2-Msh6 to form a heterotetramer capable of recruiting the exonuclease Exo1. Exo1 cuts the DNA between two single stranded breaks thereby resulting in a DSB (Stavnezer et al, 2008).

DSBs in the S regions are religated using the NHEJ repair machinery. However NHEJ factors are not the only proteins involved in CSR. Studies of mouse knockout models have implicated Atm, Mdc-1, γ -H2ax and 53bp1 in the CSR process (Kotnis et al, 2009). More recently, the E3 ligases Rnf8 and Rnf168 have also been implicated in the process of CSR (Bohgaki et al, 2011; Li et al, 2010; Santos et al, 2010; Stewart et al, 2007). Interestingly, in B cells of mice that are deficient in classical NHEJ repair proteins, sequencing of switch junctions have shown a pattern of increased microhomology suggesting that CSR could be occurring through alternative NHEJ pathways in these cells (Kotnis et al, 2009). Of all of the DSB repair proteins, loss of 53bp1 seems to have the most severe effect on CSR with a 90% decrease in CSR in 53bp1-null B cells (Kotnis et al, 2009). There have been several roles proposed for 53BP1 in CSR. One of them is that that 53BP1 inhibits intraswitch religation of DSBs and promotes synapsis between DSBs occurring in distal switch sequences. In addition, 53BP1 inhibits DSB resection which is needed for alternative NHEJ, thereby leading to increased DSB repair through the classical NHEJ pathway and to increased CSR (Bothmer et al, 2010). Despite many advances in the field of CSR and in the function of factors required for this process, further investigation is still required to determine the precise role of each DSB repair protein in CSR.

5. DSB signaling and repair defects: Human diseases and mouse models

The study of rare hereditary diseases and knockout mouse models in which DSB signaling and repair genes are mutated has greatly increased our understanding of the DNA damage

signaling and repair pathways and the underlying mechanisms regulating them (Bohgaki et al, 2010; Hakem, 2008). In this section, a summary of the human syndromes and mouse phenotypes associated with the loss of the major DSB signaling and repair proteins is provided.

5.1 ATM

Mutations in the *ATM* gene result in the devastating disease ataxia-telangiectasia (A-T). A-T is an autosomal recessive disorder. Clinical symptoms of A-T include cerebellar ataxia, oculocutaneous telangiectasias, immunodeficiency, growth retardation, lack of gonadal development, insulin resistance and increased susceptibility to lymphoid cancers (Lavin, 2008). The ataxia manifests itself at an early age when the child starts to walk and worsens with time as the patients usually becomes wheel-chair bound by the end of the first decade of their life. Ataxia in A-T patients is caused by degeneration of Purkinje and granular cells in the cerebellum. A-T patients are immunodeficient, have reduced thymus size and lower serum levels of IgG, IgA and IgE. One of the striking features of A-T is a severe sensitivity to ionizing radiation. This has been observed in both A-T patients who were undergoing radiotherapy and in A-T cells grown in culture. Some patients with A-T develop insulin resistance and diabetes. Although this particular symptom was initially hard to explain through the DNA damage signaling functions of ATM, it has recently become clear that ATM is also a key player in insulin signaling and that it can protect against metabolic disorders (Halaby et al, 2008; Matsuoka et al, 2007). About one third of A-T patients develop lymphoid malignancies.

The phenotype of *Atm*-deficient mice closely resembles what has been observed in A-T patients. The mice are growth-deficient, sterile, immunodeficient and display increased radiosensitivity. In addition, most mice succumb to thymic lymphoma by 6 months of age (Barlow et al, 1996). Furthermore, it has been reported that *Atm*^{-/-} mice have CSR defects (Kotnis et al, 2009). Interestingly, *Atm*^{-/-} mice do not recapitulate entirely the severe neurodegeneration observed in A-T patients. Mild ataxia has been reported in *Atm*^{-/-} mice, however there were no signs of degeneration in the cerebellum such as those observed in A-T patients (Barlow et al, 1996). This suggests that although ATM functions are mostly conserved from mice to humans, it might not be the case in neuronal cells of these organisms.

5.2 The MRN complex

5.2.1 MRE11

Hypomorphic mutations in the human *MRE11* gene lead to A-T like disorder (ATLD). ATLD is a very rare disorder that shares many similarities with A-T disease (Stewart et al, 1999). ATLD patients display progressive cerebellar ataxia and their cells are radiosensitive. However, in contrast with A-T patients, ATLD patients display normal Ig levels, lack of telangiectasia occurrence and they do not have increased susceptibility for cancer development (Taylor et al, 2004).

Straight knockout mice for *Mre11* are embryonic lethal, suggesting that *Mre11* is necessary for embryonic development (Xiao & Weaver, 1997). An ATLD mouse model was developed in which a hypomorphic *Mre11* is expressed (*Mre11*^{ATLD1/ATLD1} mice). These mice appear to have normal growth; however mouse embryonic fibroblasts derived from these mice have increased radiosensitivity, blunted intra-S and G2/M checkpoints and higher levels of genomic instability (Theunissen et al, 2003). However, *Mre11*^{ATLD1/ATLD1} mice did not develop

lymphomas. Interestingly, female *Mre11^{ATLD1/ATLD1}* mice have a severely reduced fertility due to an inability of developing embryos to proliferate properly.

5.2.2 NBS1

NBS1 hypomorphic mutations are the underlying cause for the Nijmegen Breakage syndrome (NBS) (Carney et al, 1998). NBS patients display stunted growth, microencephaly, immunodeficiency, increased radiosensitivity and increased cancer incidence (Digweed & Sperling, 2004). NBS patients have decreased IgA and IgG level which shows that NBS1 plays an important in CSR (van Engelen et al, 2001). Some female patients also showed ovarian failure and amenorrhea. Lymphomas are the most common malignancies observed in NBS patients although other cancers such as medulloblastomas were also diagnosed in these patients (Digweed & Sperling, 2004).

As with *Mre11*, straight knockout mice of *Nbs1* are not viable (Zhu et al, 2001). Mice with conditional deletion of *Nbs1* in B cells had increased genomic instability in B cells and CSR defects (Reina-San-Martin et al, 2005). However and interestingly so, mice with hypomorphic mutation of *Nbs1* that mimics a mutation observed in NBS1 patients display a generally milder phenotype than what is observed in NBS patients (Williams et al, 2002). Mouse embryonic fibroblasts derived from these mice show increased sensitivity to DNA damaging compounds, defects in cell cycle checkpoints and increased chromosomal instability. However, these mice with homozygous *Nbs1* hypomorph mutation do not show immunodeficiency, increased susceptibility to tumor development or female sterility.

5.2.3 RAD50

Recently, a human disorder caused by mutations in the *RAD50* gene was characterized (Waltes et al, 2009). This disorder was described as being NBS-like since the only known patient with this disorder shares similar features with NBS patients. Clinical features of the *RAD50* (NBS-like) disorder patient include microencephaly, growth retardation and slight ataxia. The patient has a normal immune system and did not develop any tumors by the age of 23. Cells derived from the patient displayed radiosensitivity, G1/S and G2/M checkpoint defects, radioresistant DNA synthesis and increased genomic instability.

Rad50-null mutant mice are not viable and die early during embryonic development. Viable *Rad50* hypomorphic mice were generated (Bender et al, 2002). These mice have strong growth defects and most die from anemia caused by hematopoietic stem cell failure. Mutant mice that survive develop lymphomas and leukemia and males exhibit degeneration in the testes. Mouse embryonic fibroblasts with the *Rad50* hypomorphic mutation are not sensitive to radiation or DNA damaging agents and do not perform radioresistant DNA synthesis.

5.3 RNF168

RNF168 was recently identified as the gene mutated in the RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties) syndrome (Stewart et al, 2009; Stewart et al, 2007). The RIDDLE syndrome was identified in only one patient to date and is characterized by immunodeficiency with decreased IgG levels but slightly increased IgA and IgM levels. The RIDDLE patient also displayed a mild decrease in motor and learning abilities, shorter stature and facial dysmorphism. Fibroblasts derived from the RIDDLE patient showed increased radiosensitivity. Interestingly, another patient with

RNF168 gene mutation was recently identified (Devgan et al, 2011). This patient also displayed short stature, cellular radiosensitivity and low serum IgA. In addition, this patient was reported to also display A-T like symptoms including ataxia, ocular telangiectasias, microcephaly, and immunodeficiency with very low IgA levels. Both patients with *RNF168* deficiency did not develop tumors.

Rnf168 null mice were recently generated (Bohgaki et al, 2011). These mice display normal growth and development and do not develop malignancies. They do however exhibit immunodeficiency mainly characterized by defects in CSR. Cells deficient for *Rnf168* display increased radiosensitivity and genomic instability and elevated cancer risk was observed in mice lacking both *Rnf168* and *p53*.

5.4 RNF8

Human syndromes caused by mutations of the *RNF8* gene have yet to be identified. However, *Rnf8* mouse knockout models have been generated and have produced interesting phenotypes that are worth discussing here. *Rnf8*^{-/-} mice are viable and are born in normal mendelian ratio (Li et al, 2010; Santos et al, 2010). They have growth defects, male sterility, increased radiosensitivity and immunodeficiency. *Rnf8*^{-/-} mice display reduced CSR and increased genomic instability. A broad spectrum of tumors including lymphomas, sarcomas and breast tumors developed in *Rnf8*^{-/-} mice (Li et al, 2010). It would be interesting to determine whether mutations of *RNF8* gene lead to human genetic disorders or if *RNF8* loss correlates with cancer development in humans.

5.5 BRCA1 and BRCA2

BRCA1 and *BRCA2* are two breast and ovarian cancer susceptibility genes (O'Donovan & Livingston, 2010). Germline mutation of one *BRCA1* allele results in an up to 80% cumulative risk of breast cancer and a 30-40% risk of ovarian cancer by 70 years of age. Carriers of a *BRCA2* mutation have a 50% cumulative risk of breast cancer and a 10-15% risk of developing ovarian cancer by age 70. In addition, germline *BRCA2* mutations have been implicated in familial prostate cancer (McKinnon & Caldecott, 2007). Tumors in *BRCA1* or *BRCA2* mutation carriers most often lose the second *BRCA1* or *BRCA2* allele through loss of heterozygosity. Recently, it has been discovered that *BRCA1* and *BRCA2*-negative tumors are very sensitive to a class of compounds known as PARP inhibitors. PARP inhibitors leave unrepaired single strand breaks in the DNA. If these unrepaired breaks meet a replication fork either fork collapse or DSBs. In the absence of *BRCA1* and *BRCA2* DSBs are left unrepaired, thereby leading to cell death. In this way PARP inhibitors are able to specifically target and kill *BRCA1* and *BRCA2*-null cells, making these inhibitors powerful therapeutic candidates for *BRCA1* and *BRCA2* negative breast and ovarian tumors. This treatment strategy might also work for tumors with somatic inactivation of *BRCA1* and *BRCA2* (Carden et al, 2010).

Brc1 and *Brc2* knockout mice are embryonic lethal, reflecting the essential requirement for these two proteins (Hakem et al, 1996; Suzuki et al, 1997). *Brc1*-deficient cells displayed radiosensitivity and increased chromosomal abnormalities (Mak et al, 2000; McPherson et al, 2004). Females carrying *Brc1* targeted mutations in mammary tissue exhibit a long latency time before development of mammary tumors, however this latency time is reduced considerably in the absence of tumor suppressor proteins such as *p53* and *Chk2* (McPherson et al, 2004; Xu et al, 1999). Interestingly, it was recently shown that loss of *53bp1* in *Brc1*-null mammary epithelium prevented mammary tumor development (Bunting et al, 2010), suggesting that *53bp1* could eventually be targeted to treated *Brc1*-deficient breast cancer.

Similar to what was observed with *Brca1* conditional mutant mice, loss of *Brca2* expression in mouse mammary epithelium leads to increased incidence of mammary tumors, the latency of which is shortened in the absence of a *p53* allele (Jonkers et al, 2001). Recently, study of a conditional knockout of *Brca2* in mouse prostate epithelium showed that loss of *Brca2* leads to increased prostate cancer incidence which is accelerated in the absence of *p53* (Francis et al, 2010).

5.6 DNA ligase IV

Hypomorphic mutations in the *DNA ligase IV* gene in humans give rise to the ligase IV (Lig4) syndrome. Lig4 syndrome is an autosomal recessive disorder characterized by microencephaly, growth retardation, mental retardation, decreased red and white blood cell count, immunodeficiency and increased cancer susceptibility (O'Driscoll et al, 2001). Cells from Lig4 patients display increased radiosensitivity and are defective in NHEJ DSB repair, but they have normal cell cycle checkpoints (O'Driscoll et al, 2001).

Knocking out *DNA ligase IV* in mice results in late embryonic lethality with massive neuronal apoptosis and lymphocyte development arrest due to lack of V(D)J recombination (Frank et al, 1998). Mice with hypomorphic mutation of *DNA ligase IV* were obtained through a mutagenesis screen. These mice have growth defects, are immunodeficient and have hematopoietic stem cell exhaustion with age (Nijnik et al, 2007).

5.7 Artemis

Artemis is the gene mutated in radiosensitive-severe combined immunodeficiency (RS-SCID) (Moshous et al, 2001). RS-SCID is characterized by normal development but increased radiosensitivity and a complete absence of mature B and T cells that can be attributable to defects in V(D)J recombination. While null mutations of *Artemis* give rise to RD-SCID, hypomorphic mutations lead to a plethora of less severe immunodeficiency syndromes that are characterized by increased incidence of Epstein-Barr virus-induced lymphomas (Moshous et al, 2003).

Artemis knockout mice are viable and display normal growth. B cell development in these mice is arrested at early progenitor stages. In contrast to what is observed in humans some T cells are able to undergo V(D)J recombination and mature normally leading to a "leaky" SCID phenotype (Rooney et al, 2002). Artemis-deficient cells display increased radiosensitivity and genomic instability. Recently, Artemis-null mice were generated in a different genetic background (Xiao et al, 2009). These mice exhibit complete arrest of B and T lymphocyte development and do not present a leaky phenotype and thus recapitulate more closely RS-SCID symptoms.

5.8 DNA-PKcs

The first *DNA-PKcs* human gene mutation was recently identified in a patient presenting classical symptoms of RS-SCID (van der Burg et al, 2009). This patient did not have mature B or T cells but had normal natural killer cell numbers. Cells from the patient were unable to properly repair DNA double strand breaks and were deficient in NHEJ.

DNA-PKcs was initially identified as the gene inactivated in the classical scid mice. Later on, knockout mice for *DNA-PKcs* recapitulated the phenotypes of the scid mice (Gao et al, 1998). *DNA-PKcs*^{-/-} mice exhibited no growth defects but they displayed arrested B and T cell development at early progenitor stages, impaired V(D)J recombination and increased cellular radiosensitivity.

5.9 NHEJ1

NHEJ1 (*Cernunnos-XLF*) gene mutations were identified in patients with immunodeficiency and microcephaly (Buck et al, 2006). These patients are characterized by bird-like features, microcephaly, progressive loss of B and T cells and growth retardation. They have low levels of circulating IgA and IgG. Cells derived from these patients are radiosensitive but display normal cell cycle checkpoint.

A mouse model in which *Nhej1* is deleted has been generated (Li et al, 2008). These mice display a much milder phenotype than what is seen in humans. *Nhej1*-null mice are viable, born at the mendelian ratio and have normal growth and development. Although B and T cell numbers are reduced in *Nhej1*-deficient mice, a normal development of immune cells is observed and V(D)J recombination was not impaired in *Nhej1*-null lymphocytes. On the other hand, B cells from *Nhej1*-deficient mice had defective CSR. Concomitant loss of *Nhej1* and p53 resulted in the rapid occurrence of thymic lymphomas and medulloblastomas.

5.10 Ku70 and Ku80

Mutations in *Ku70* or *Ku80* genes have not yet been described in humans. However knockout mice have been generated for these two proteins. Similar to what has been observed for knockout mice of other NHEJ factors described above, *Ku70* and *Ku80* deficient mice display immunodeficiency with arrested B and T cell development and defective V(D)J recombination (Gu et al, 1997; Nussenzweig et al, 1996). Interestingly though, *Ku70*^{-/-} and *Ku80*^{-/-} mice displayed significant growth defects and reduced size compared to wild-type mice.

6. Conclusion

DNA double strand breaks are constantly generated in our cells either through external stressors such as radiation or through internal programmed events that are needed for normal physiological processes such as gametogenesis, V(D)J recombination and class switch recombination. The importance of quickly detecting and repairing these breaks is underscored by the plethora of human syndromes caused by mutation of genes coding for DSB signaling and repair proteins. These syndromes share many similarities which include neurological defects, growth defects, immunodeficiency, radiosensitivity, sterility and increased cancer incidence. Although many of these symptoms have been recapitulated in knockout mouse models of DSB response proteins, some discrepancies between human syndromes and mouse models are sometimes observed which highlight differential role or redundancy between DSB response proteins in humans and mice. Nevertheless, study of these models have provided great insight into the physiological functions of DSB response proteins and have led to rapid discoveries in this field. Finally, these studies resulted in a better understanding of the etiology of certain diseases such as cancer and provided potential new ways of treating these diseases.

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8. References

- Al-Hakim A., Escribano-Diaz, C., Landry M.C, O' Donnel, L., Panier S., Szilard R.K. & Durocher D. (2010) The ubiquitous role of ubiquitin in the DNA damage response. *DNA repair*, Vol. 9, No. 12, pp. 1229-1240, ISSN 1568-7856
- Andersen, S.L. & Sekelsky, J. (2010) Meiotic Versus Mitotic Recombination: Two Different Routes for Double-Strand Break Repair: The Different Functions of Meiotic Versus Mitotic Dsb Repair Are Reflected in Different Pathway Usage and Different Outcomes. *Bioessays*, Vol.32, No.12, pp.1058-1066, ISSN 1521-1878
- Bakkenist, C.J. & Kastan, M.B. (2003) DNA Damage Activates Atm through Intermolecular Autophosphorylation and Dimer Dissociation. *Nature*, Vol.421, No.6922, pp.499-506, ISSN 0028-0836
- Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D. & Wynshaw-Boris, A. (1996) Atm-Deficient Mice: A Paradigm of Ataxia Telangiectasia. *Cell*, Vol.86, No.1, pp.159-171, ISSN 0092-8674
- Baudat, F., Buard, J., Grey, C., Fledel-Alon, A., Ober, C., Przeworski, M., Coop, G. & de Massy, B. (2010) Prdm9 Is a Major Determinant of Meiotic Recombination Hotspots in Humans and Mice. *Science*, Vol.327, No.5967, pp.836-840, ISSN 1095-9203
- Bekker-Jensen, S., Rendtlew Danielsen, J., Fugger, K., Gromova, I., Nerstedt, A., Lukas, C., Bartek, J., Lukas, J. & Mailand, N. (2010) Herc2 Coordinates Ubiquitin-Dependent Assembly of DNA Repair Factors on Damaged Chromosomes. *Nat Cell Biol*, Vol.12, No.1, pp.80-86; sup pp 81-12, ISSN 1476-4679
- Bender, C.F., Sikes, M.L., Sullivan, R., Huye, L.E., Le Beau, M.M., Roth, D.B., Mirzoeva, O.K., Oltz, E.M. & Petrini, J.H. (2002) Cancer Predisposition and Hematopoietic Failure in Rad50(S/S) Mice. *Genes Dev*, Vol.16, No.17, pp.2237-2251, ISSN 0890-9369
- Bennardo, N., Cheng, A., Huang, N. & Stark, J.M. (2008) Alternative-Nhej Is a Mechanistically Distinct Pathway of Mammalian Chromosome Break Repair. *PLoS Genet*, Vol.4, No.6, pp.e1000110, ISSN 1553-7404
- Bohgaki, T., Bohgaki, M., Cardoso, R., Panier, S., Stewart, G.S., Sanchez, O., Durocher, D., Hakem, A. & Hakem, R. (2011) Genomic Instability, Defective Spermatogenesis, Immunodeficiency and Cancer in a Mouse Model of the Riddle Syndrome. *Plos Genetics*, In press.
- Bohgaki, T., Bohgaki, M. & Hakem, R. (2010) DNA Double-Strand Break Signaling and Human Disorders. *Genome Integr*, Vol.1, No.1, pp.15, ISSN 2041-9414
- Borde, V., Robine, N., Lin, W., Bonfils, S., Geli, V. & Nicolas, A. (2009) Histone H3 Lysine 4 Trimethylation Marks Meiotic Recombination Initiation Sites. *EMBO J*, Vol.28, No.2, pp.99-111, ISSN 1460-2075
- Bothmer, A., Robbiani, D.F., Feldhahn, N., Gazumyan, A., Nussenzweig, A. & Nussenzweig, M.C. (2010) 53bp1 Regulates DNA Resection and the Choice between Classical and Alternative End Joining During Class Switch Recombination. *J Exp Med*, Vol.207, No.4, pp.855-865, ISSN 1540-9538
- Bouwman, P., Aly, A., Escandell, J.M., Pieterse, M., Bartkova, J., van der Gulden, H., Hiddingh, S., Thanasoula, M., Kulkarni, A., Yang, Q., Haffty, B.G., Tommiska, J., Blomqvist, C., Drapkin, R., Adams, D.J., Nevanlinna, H., Bartek, J., Tarsounas, M., Ganesan, S. & Jonkers, J. (2010) 53bp1 Loss Rescues Brca1 Deficiency and Is

- Associated with Triple-Negative and Brca-Mutated Breast Cancers. *Nat Struct Mol Biol*, Vol.17, No.6, pp.688-695, ISSN 1545-9985
- Buck, D., Malivert, L., de Chasseval, R., Barraud, A., Fondaneche, M.C., Sanal, O., Plebani, A., Stephan, J.L., Hufnagel, M., le Deist, F., Fischer, A., Durandy, A., de Villartay, J.P. & Revy, P. (2006) Cernunnos, a Novel Nonhomologous End-Joining Factor, Is Mutated in Human Immunodeficiency with Microcephaly. *Cell*, Vol.124, No.2, pp.287-299, ISSN 0092-8674
- Bunting, S.F., Callen, E., Wong, N., Chen, H.T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L., Xu, X., Deng, C.X., Finkel, T., Nussenzweig, M., Stark, J.M. & Nussenzweig, A. (2010) 53bp1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. *Cell*, Vol.141, No.2, pp.243-254, ISSN 1097-4172
- Carden, C.P., Yap, T.A. & Kaye, S.B. (2010) Parp Inhibition: Targeting the Achilles' Heel of DNA Repair to Treat Germline and Sporadic Ovarian Cancers. *Curr Opin Oncol*, Vol.22, No.5, pp.473-480, ISSN 1531-703X
- Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates, J.R., 3rd, Hays, L., Morgan, W.F. & Petrini, J.H. (1998) The Hmre11/Hrad50 Protein Complex and Nijmegen Breakage Syndrome: Linkage of Double-Strand Break Repair to the Cellular DNA Damage Response. *Cell*, Vol.93, No.3, pp.477-486, ISSN 0092-8674
- Chappell, C., Hanakahi, L.A., Karimi-Busheri, F., Weinfeld, M. & West, S.C. (2002) Involvement of Human Polynucleotide Kinase in Double-Strand Break Repair by Non-Homologous End Joining. *EMBO J*, Vol.21, No.11, pp.2827-2832, ISSN 0261-4189
- Ciccia, A. & Elledge, S.J. (2010) The DNA Damage Response: Making It Safe to Play with Knives. *Molecular Cell* Vol.40, No.2, pp.179-204
- Cook, P.J., Ju, B.G., Telese, F., Wang, X., Glass, C.K. & Rosenfeld, M.G. (2009) Tyrosine Dephosphorylation of H2ax Modulates Apoptosis and Survival Decisions. *Nature*, Vol.458, No.7238, pp.591-596, ISSN 1476-4687
- D'Amours, D. & Jackson, S.P. (2002) The Mre11 Complex: At the Crossroads of Dna Repair and Checkpoint Signalling. *Nat Rev Mol Cell Biol*, Vol.3, No.5, pp.317-327, ISSN 1471-0072
- Devgan, S.S., Sanal, O., Doil, C., Nakamura, K., Nahas, S.A., Pettijohn, K., Bartek, J., Lukas, C., Lukas, J. & Gatti, R.A. (2011) Homozygous Deficiency of Ubiquitin-Ligase Ring-Finger Protein Rnf168 Mimics the Radiosensitivity Syndrome of Ataxia-Telangiectasia. *Cell Death Differ*, ISSN 1476-5403
- Digweed, M. & Sperling, K. (2004) Nijmegen Breakage Syndrome: Clinical Manifestation of Defective Response to DNA Double-Strand Breaks. *DNA Repair (Amst)*, Vol.3, No.8-9, pp.1207-1217, ISSN 1568-7864
- Doil, C., Mailand, N., Bekker-Jensen, S., Menard, P., Larsen, D.H., Pepperkok, R., Ellenberg, J., Panier, S., Durocher, D., Bartek, J., Lukas, J. & Lukas, C. (2009) Rnf168 Binds and Amplifies Ubiquitin Conjugates on Damaged Chromosomes to Allow Accumulation of Repair Proteins. *Cell*, Vol.136, No.3, pp.435-446, ISSN 1097-4172
- Donzelli, M. & Draetta, G.F. (2003) Regulating Mammalian Checkpoints through Cdc25 Inactivation. *EMBO Rep*, Vol.4, No.7, pp.671-677, ISSN 1469-221X

- Dudley, D.D., Chaudhuri, J., Bassing, C.H. & Alt, F.W. (2005) Mechanism and Control of V(D)J Recombination Versus Class Switch Recombination: Similarities and Differences. *Adv Immunol*, Vol.86, pp.43-112, ISSN 0065-2776
- Ferrari, S.R., Grubb, J. & Bishop, D.K. (2009) The Mei5-Sae3 Protein Complex Mediates Dmc1 Activity in *Saccharomyces Cerevisiae*. *J Biol Chem*, Vol.284, No.18, pp.11766-11770, ISSN 0021-9258
- Francis, J.C., McCarthy, A., Thomsen, M.K., Ashworth, A. & Swain, A. (2010) Brca2 and Trp53 Deficiency Cooperate in the Progression of Mouse Prostate Tumourigenesis. *PLoS Genet*, Vol.6, No.6, pp.e1000995, ISSN 1553-7404
- Frank, K.M., Sekiguchi, J.M., Seidl, K.J., Swat, W., Rathbun, G.A., Cheng, H.L., Davidson, L., Kangaloo, L. & Alt, F.W. (1998) Late Embryonic Lethality and Impaired V(D)J Recombination in Mice Lacking DNA Ligase Iv. *Nature*, Vol.396, No.6707, pp.173-177, ISSN 0028-0836
- Galanty, Y., Belotserkovskaya, R., Coates, J., Polo, S., Miller, K.M. & Jackson, S.P. (2009) Mammalian Sumo E3-Ligases Pias1 and Pias4 Promote Responses to DNA Double-Strand Breaks. *Nature*, Vol.462, No.7275, pp.935-939, ISSN 1476-4687
- Gao, Y., Chaudhuri, J., Zhu, C., Davidson, L., Weaver, D.T. & Alt, F.W. (1998) A Targeted DNA-Pkcs-Null Mutation Reveals DNA-Pk-Independent Functions for Ku in V(D)J Recombination. *Immunity*, Vol.9, No.3, pp.367-376, ISSN 1074-7613
- Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J. & Jackson, S.P. (2003) Mdc1 Is Required for the Intra-S-Phase DNA Damage Checkpoint. *Nature*, Vol.421, No.6926, pp.952-956, ISSN 0028-0836
- Gu, Y., Seidl, K.J., Rathbun, G.A., Zhu, C., Manis, J.P., van der Stoep, N., Davidson, L., Cheng, H.L., Sekiguchi, J.M., Frank, K., Stanhope-Baker, P., Schlissel, M.S., Roth, D.B. & Alt, F.W. (1997) Growth Retardation and Leaky Scid Phenotype of Ku70-Deficient Mice. *Immunity*, Vol.7, No.5, pp.653-665, ISSN 1074-7613
- Guikema, J.E., Linehan, E.K., Tsuchimoto, D., Nakabeppu, Y., Strauss, P.R., Stavnezer, J. & Schrader, C.E. (2007) Ape1- and Ape2-Dependent DNA Breaks in Immunoglobulin Class Switch Recombination. *J Exp Med*, Vol.204, No.12, pp.3017-3026, ISSN 1540-9538
- Hakem, R. (2008) DNA-Damage Repair; the Good, the Bad, and the Ugly. *EMBO J*, Vol.27, No.4, pp.589-605, ISSN 1460-2075
- Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., Firpo, E., Hui, C.C., Roberts, J., Rossant, J. & Mak, T.W. (1996) The Tumor Suppressor Gene Brca1 Is Required for Embryonic Cellular Proliferation in the Mouse. *Cell*, Vol.85, No.7, pp.1009-1023, ISSN 0092-8674
- Halaby, M.J., Hibma, J.C., He, J. & Yang, D.Q. (2008) Atm Protein Kinase Mediates Full Activation of Akt and Regulates Glucose Transporter 4 Translocation by Insulin in Muscle Cells. *Cell Signal*, Vol.20, No.8, pp.1555-1563, ISSN 0898-6568
- Hartlerode, A.J. & Scully, R. (2009) Mechanisms of Double-Strand Break Repair in Somatic Mammalian Cells. *Biochem J*, Vol.423, No.2, pp.157-168, ISSN 1470-8728
- Heyer, W.D., Ehmsen, K.T. & Liu, J. (2010) Regulation of Homologous Recombination in Eukaryotes. *Annu Rev Genet*, Vol.44, pp.113-139, ISSN 1545-2948
- Huen, M.S., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M.B. & Chen, J. (2007) Rnf8 Transduces the DNA-Damage Signal Via Histone Ubiquitylation and Checkpoint Protein Assembly. *Cell*, Vol.131, No.5, pp.901-914, 0092-8674

- Jensen, R.B., Carreira, A. & Kowalczykowski, S.C. (2010) Purified Human Brca2 Stimulates Rad51-Mediated Recombination. *Nature*, Vol.467, No.7316, pp.678-683, ISSN 1476-4687
- Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M. & Berns, A. (2001) Synergistic Tumor Suppressor Activity of Brca2 and P53 in a Conditional Mouse Model for Breast Cancer. *Nat Genet*, Vol.29, No.4, pp.418-425, ISSN 1061-4036
- Kass, E.M. & Jasin, M. (2010) Collaboration and Competition between DNA Double-Strand Break Repair Pathways. *FEBS Lett*, Vol.584, No.17, pp.3703-3708, ISSN 1873-3468
- Keeney, S., Giroux, C.N. & Kleckner, N. (1997) Meiosis-Specific DNA Double-Strand Breaks Are Catalyzed by Spo11, a Member of a Widely Conserved Protein Family. *Cell*, Vol.88, No.3, pp.375-384, ISSN 0092-8674
- Kim, H., Chen, J. & Yu, X. (2007) Ubiquitin-Binding Protein Rap80 Mediates Brca1-Dependent DNA Damage Response. *Science*, Vol.316, No.5828, pp.1202-1205, ISSN 1095-9203
- Kolas, N.K., Chapman, J.R., Nakada, S., Ylanko, J., Chahwan, R., Sweeney, F.D., Panier, S., Mendez, M., Wildenhain, J., Thomson, T.M., Pelletier, L., Jackson, S.P. & Durocher, D. (2007) Orchestration of the DNA-Damage Response by the Rnf8 Ubiquitin Ligase. *Science*, Vol.318, No.5856, pp.1637-1640, ISSN 1095-9203
- Kotnis, A., Du, L., Liu, C., Popov, S.W. & Pan-Hammarstrom, Q. (2009) Non-Homologous End Joining in Class Switch Recombination: The Beginning of the End. *Philos Trans R Soc Lond B Biol Sci*, Vol.364, No.1517, pp.653-665, ISSN 1471-2970
- Kumar, R., Bourbon, H.M. & de Massy, B. (2010) Functional Conservation of Mei4 for Meiotic DNA Double-Strand Break Formation from Yeasts to Mice. *Genes Dev*, Vol.24, No.12, pp.1266-1280, ISSN 1549-5477
- Lamarche, B.J., Orazio, N.I. & Weitzman, M.D. (2010) The Mrn Complex in Double-Strand Break Repair and Telomere Maintenance. *FEBS Lett*, Vol.584, No.17, pp.3682-3695, ISSN 1873-3468
- Lavin, M.F. (2008) Ataxia-Telangiectasia: From a Rare Disorder to a Paradigm for Cell Signalling and Cancer. *Nat Rev Mol Cell Biol*, Vol.9, No.10, pp.759-769, ISSN 1471-0080
- Li, G., Alt, F.W., Cheng, H.L., Brush, J.W., Goff, P.H., Murphy, M.M., Franco, S., Zhang, Y. & Zha, S. (2008) Lymphocyte-Specific Compensation for Xlf/Cernunnos End-Joining Functions in V(D)J Recombination. *Mol Cell*, Vol.31, No.5, pp.631-640, ISSN 1097-4164
- Li, L., Halaby, M.J., Hakem, A., Cardoso, R., El Ghamrasni, S., Harding, S., Chan, N., Bristow, R., Sanchez, O., Durocher, D. & Hakem, R. (2010) Rnf8 Deficiency Impairs Class Switch Recombination, Spermatogenesis, and Genomic Integrity and Predisposes for Cancer. *J Exp Med*, Vol.207, No.5, pp.983-997, ISSN 1540-9538
- Llorente, B., Smith, C.E. & Symington, L.S. (2008) Break-Induced Replication: What Is It and What Is It For? *Cell Cycle*, Vol.7, No.7, pp.859-864, ISSN 1551-4005
- Lorenz, A., West, S.C. & Whitby, M.C. (2009) The Human Holliday Junction Resolvase Gen1 Rescues the Meiotic Phenotype of a Schizosaccharomyces Pombe Mus81 Mutant. *Nucleic Acids Res*, Vol.38, No.6, pp.1866-1873, ISSN 1362-4962
- Lou, Z., Minter-Dykhous, K., Franco, S., Gostissa, M., Rivera, M.A., Celeste, A., Manis, J.P., van Deursen, J., Nussenzweig, A., Paull, T.T., Alt, F.W. & Chen, J. (2006) Mdc1

- Maintains Genomic Stability by Participating in the Amplification of Atm-Dependent DNA Damage Signals. *Mol Cell*, Vol.21, No.2, pp.187-200, ISSN 1097-2765
- Macrae, C.J., McCulloch, R.D., Ylanko, J., Durocher, D. & Koch, C.A. (2008) Aplf (C2orf13) Facilitates Nonhomologous End-Joining and Undergoes Atm-Dependent Hyperphosphorylation Following Ionizing Radiation. *DNA Repair (Amst)*, Vol.7, No.2, pp.292-302, ISSN 1568-7864
- Mahaney, B.L., Meek, K. & Lees-Miller, S.P. (2009) Repair of Ionizing Radiation-Induced DNA Double-Strand Breaks by Non-Homologous End-Joining. *Biochem J*, Vol.417, No.3, pp.639-650, ISSN 1470-8728
- Mailand, N., Bekker-Jensen, S., Fastrup, H., Melander, F., Bartek, J., Lukas, C. & Lukas, J. (2007) Rnf8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. *Cell*, Vol.131, No.5, pp.887-900, ISSN 0092-8674
- Mak, T.W., Hakem, A., McPherson, J.P., Shehabeldin, A., Zabolcki, E., Migon, E., Duncan, G.S., Bouchard, D., Wakeham, A., Cheung, A., Karaskova, J., Sarosi, I., Squire, J., Marth, J. & Hakem, R. (2000) Brca1 Is Required for T Cell Lineage Development but Not Tcr Loci Rearrangement. *Nat Immunol*, Vol.1, No.1, pp.77-82, ISSN 1529-2908
- Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S.P. & Elledge, S.J. (2007) Atm and Atr Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. *Science*, Vol.316, No.5828, pp.1160-1166, ISSN 1095-9203
- McKinnon, P.J. & Caldecott, K.W. (2007) DNA Strand Break Repair and Human Genetic Disease. *Annu Rev Genomics Hum Genet*, Vol.8, pp.37-55, ISSN 1527-8204
- McPherson, J.P., Lemmers, B., Hirao, A., Hakem, A., Abraham, J., Migon, E., Matysiak-Zabolcki, E., Tamblyn, L., Sanchez-Sweatman, O., Khokha, R., Squire, J., Hande, M.P., Mak, T.W. & Hakem, R. (2004) Collaboration of Brca1 and Chk2 in Tumorigenesis. *Genes Dev*, Vol.18, No.10, pp.1144-1153, ISSN 0890-9369
- Modesti, M. & Kanaar, R. (2001) DNA Repair: Spot(Light)S on Chromatin. *Curr Biol*, Vol.11, No.6, pp.R229-232, ISSN 0960-9822
- Morris, J.R., Boutell, C., Keppler, M., Densham, R., Weekes, D., Alamshah, A., Butler, L., Galanty, Y., Pangon, L., Kiuchi, T., Ng, T. & Solomon, E. (2009) The Sumo Modification Pathway Is Involved in the Brca1 Response to Genotoxic Stress. *Nature*, Vol.462, No.7275, pp.886-890, ISSN 1476-4687
- Moshous, D., Pannetier, C., Chasseval Rd, R., Deist Fl, F., Cavazzana-Calvo, M., Romana, S., Macintyre, E., Canioni, D., Brousse, N., Fischer, A., Casanova, J.L. & Villartay, J.P. (2003) Partial T and B Lymphocyte Immunodeficiency and Predisposition to Lymphoma in Patients with Hypomorphic Mutations in Artemis. *J Clin Invest*, Vol.111, No.3, pp.381-387, ISSN 0021-9738
- Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. & Honjo, T. (2000) Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (Aid), a Potential Rna Editing Enzyme. *Cell*, Vol.102, No.5, pp.553-563, ISSN 0092-8674
- Nakada, S., Tai, I., Panier, S., Al-Hakim, A., Iemura, S., Juang, Y.C., O'Donnell, L., Kumakubo, A., Munro, M., Sicheri, F., Gingras, A.C., Natsume, T., Suda, T. &

- Durocher, D. (2010) Non-Canonical Inhibition of DNA Damage-Dependent Ubiquitination by Otub1. *Nature*, Vol.466, No.7309, pp.941-946, ISSN 1476-4687
- Nicassio, F., Corrado, N., Vissers, J.H., Areces, L.B., Bergink, S., Marteijn, J.A., Geverts, B., Houtsmuller, A.B., Vermeulen, W., Di Fiore, P.P. & Citterio, E. (2007) Human Usp3 Is a Chromatin Modifier Required for S Phase Progression and Genome Stability. *Curr Biol*, Vol.17, No.22, pp.1972-1977, ISSN 0960-9822
- Nijnik, A., Woodbine, L., Marchetti, C., Dawson, S., Lambe, T., Liu, C., Rodrigues, N.P., Crockford, T.L., Cabuy, E., Vindigni, A., Enver, T., Bell, J.I., Slijepcevic, P., Goodnow, C.C., Jeggo, P.A. & Cornall, R.J. (2007) DNA Repair Is Limiting for Haematopoietic Stem Cells During Ageing. *Nature*, Vol.447, No.7145, pp.686-690, ISSN 1476-4687
- Nimonkar, A.V., Ozsoy, A.Z., Genschel, J., Modrich, P. & Kowalczykowski, S.C. (2008) Human Exonuclease 1 and Blm Helicase Interact to Resect DNA and Initiate DNA Repair. *Proc Natl Acad Sci U S A*, Vol.105, No.44, pp.16906-16911, ISSN 1091-6490
- Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzweig, M.C. & Li, G.C. (1996) Requirement for Ku80 in Growth and Immunoglobulin V(D)J Recombination. *Nature*, Vol.382, No.6591, pp.551-555, ISSN 0028-0836
- O'Donovan, P.J. & Livingston, D.M. (2010) Brca1 and Brca2: Breast/Ovarian Cancer Susceptibility Gene Products and Participants in DNA Double-Strand Break Repair. *Carcinogenesis*, Vol.31, No.6, pp.961-967, ISSN 1460-2180
- O'Driscoll, M., Cerosaletti, K.M., Girard, P.M., Dai, Y., Stumm, M., Kysela, B., Hirsch, B., Gennery, A., Palmer, S.E., Seidel, J., Gatti, R.A., Varon, R., Oettinger, M.A., Neitzel, H., Jeggo, P.A. & Concannon, P. (2001) DNA Ligase Iv Mutations Identified in Patients Exhibiting Developmental Delay and Immunodeficiency. *Mol Cell*, Vol.8, No.6, pp.1175-1185, ISSN 1097-2765
- Panier, S. & Durocher, D. (2009) Regulatory Ubiquitylation in Response to DNA Double-Strand Breaks. *DNA Repair (Amst)*, Vol.8, No.4, pp.436-443, ISSN 1568-7864
- Petukhova, G.V., Pezza, R.J., Vanevski, F., Ploquin, M., Masson, J.Y. & Camerini-Otero, R.D. (2005) The Hop2 and Mnd1 Proteins Act in Concert with Rad51 and Dmc1 in Meiotic Recombination. *Nat Struct Mol Biol*, Vol.12, No.5, pp.449-453, ISSN 1545-9993
- Rass, U., Compton, s.A., Matos, J., Singleton, M.R., Ip, S.C.Y., Blanco, M.G., Griffith, J.D. & West, S.C. (2010) Mechanism of Holliday Junction Resolution by the Human Gen1 Protein. *Genes and Development*, Vol.24, pp.1559-1569
- Reina-San-Martin, B., Nussenzweig, M.C., Nussenzweig, A. & Difilippantonio, S. (2005) Genomic Instability, Endoreduplication, and Diminished Ig Class-Switch Recombination in B Cells Lacking Nbs1. *Proc Natl Acad Sci U S A*, Vol.102, No.5, pp.1590-1595, ISSN 0027-8424
- Rooney, S., Sekiguchi, J., Zhu, C., Cheng, H.L., Manis, J., Whitlow, S., DeVido, J., Foy, D., Chaudhuri, J., Lombard, D. & Alt, F.W. (2002) Leaky Scid Phenotype Associated with Defective V(D)J Coding End Processing in Artemis-Deficient Mice. *Mol Cell*, Vol.10, No.6, pp.1379-1390, ISSN 1097-2765
- Santos, M.A., Huen, M.S., Jankovic, M., Chen, H.T., Lopez-Contreras, A.J., Klein, I.A., Wong, N., Barbancho, J.L., Fernandez-Capetillo, O., Nussenzweig, M.C., Chen, J. & Nussenzweig, A. (2010) Class Switching and Meiotic Defects in Mice Lacking the E3 Ubiquitin Ligase Rnf8. *J Exp Med*, Vol.207, No.5, pp.973-981, ISSN 1540-9538

- Shao, G., Lilli, D.R., Patterson-Fortin, J., Coleman, K.A., Morrissey, D.E. & Greenberg, R.A. (2009) The Rap80-Brc36 De-Ubiquitinating Enzyme Complex Antagonizes Rnf8-Ubc13-Dependent Ubiquitination Events at DNA Double Strand Breaks. *Proc Natl Acad Sci U S A*, Vol.106, No.9, pp.3166-3171, ISSN 1091-6490
- Shinohara, A., Ogawa, H. & Ogawa, T. (1992) Rad51 Protein Involved in Repair and Recombination in *S. Cerevisiae* Is a RecA-Like Protein. *Cell*, Vol.69, No.3, pp.457-470, ISSN 0092-8674
- Simsek, D. & Jasin, M. (2010) Alternative End-Joining Is Suppressed by the Canonical Nhej Component Xrcc4-Ligase Iv During Chromosomal Translocation Formation. *Nat Struct Mol Biol*, Vol.17, No.4, pp.410-416, ISSN 1545-9985
- Sleeth, K.M., Sorensen, C.S., Issaeva, N., Dziegielewska, J., Bartek, J. & Helleday, T. (2007) Rpa Mediates Recombination Repair During Replication Stress and Is Displaced from DNA by Checkpoint Signalling in Human Cells. *J Mol Biol*, Vol.373, No.1, pp.38-47, ISSN 0022-2836
- Soulas-Sprauel, P., Rivera-Munoz, P., Malivert, L., Le Guyader, G., Abramowski, V., Revy, P. & de Villartay, J.P. (2007) V(D)J and Immunoglobulin Class Switch Recombinations: A Paradigm to Study the Regulation of DNA End-Joining. *Oncogene*, Vol.26, No.56, pp.7780-7791, ISSN 1476-5594
- Stavnezer, J., Guikema, J.E. & Schrader, C.E. (2008) Mechanism and Regulation of Class Switch Recombination. *Annu Rev Immunol*, Vol.26, pp.261-292, ISSN 0732-0582
- Stewart, G.S., Maser, R.S., Stankovic, T., Bressan, D.A., Kaplan, M.I., Jaspers, N.G., Raams, A., Byrd, P.J., Petrini, J.H. & Taylor, A.M. (1999) The DNA Double-Strand Break Repair Gene Hmre11 Is Mutated in Individuals with an Ataxia-Telangiectasia-Like Disorder. *Cell*, Vol.99, No.6, pp.577-587, ISSN 0092-8674
- Stewart, G.S., Panier, S., Townsend, K., Al-Hakim, A.K., Kolas, N.K., Miller, E.S., Nakada, S., Ylanko, J., Olivarius, S., Mendez, M., Oldreive, C., Wildenhain, J., Tagliaferro, A., Pelletier, L., Taubenheim, N., Durandy, A., Byrd, P.J., Stankovic, T., Taylor, A.M. & Durocher, D. (2009) The Riddle Syndrome Protein Mediates a Ubiquitin-Dependent Signaling Cascade at Sites of DNA Damage. *Cell*, Vol.136, No.3, pp.420-434, ISSN 1097-4172
- Stewart, G.S., Stankovic, T., Byrd, P.J., Wechsler, T., Miller, E.S., Huissoon, A., Drayson, M.T., West, S.C., Elledge, S.J. & Taylor, A.M. (2007) Riddle Immunodeficiency Syndrome Is Linked to Defects in 53bp1-Mediated DNA Damage Signaling. *Proc Natl Acad Sci U S A*, Vol.104, No.43, pp.16910-16915, ISSN 0027-8424
- Stewart, G.S., Wang, B., Bignell, C.R., Taylor, A.M. & Elledge, S.J. (2003) Mdc1 Is a Mediator of the Mammalian DNA Damage Checkpoint. *Nature*, Vol.421, No.6926, pp.961-966, ISSN 0028-0836
- Sung, P. & Klein, H. (2006) Mechanism of Homologous Recombination: Mediators and Helicases Take on Regulatory Functions. *Nat Rev Mol Cell Biol*, Vol.7, No.10, pp.739-750, ISSN 1471-0072
- Suzuki, A., de la Pompa, J.L., Hakem, R., Elia, A., Yoshida, R., Mo, R., Nishina, H., Chuang, T., Wakeham, A., Itie, A., Koo, W., Billia, P., Ho, A., Fukumoto, M., Hui, C.C. & Mak, T.W. (1997) Brca2 Is Required for Embryonic Cellular Proliferation in the Mouse. *Genes Dev*, Vol.11, No.10, pp.1242-1252, ISSN 0890-9369

- Taylor, A.M., Groom, A. & Byrd, P.J. (2004) Ataxia-Telangiectasia-Like Disorder (Atld)-Its Clinical Presentation and Molecular Basis. *DNA Repair (Amst)*, Vol.3, No.8-9, pp.1219-1225, ISSN 1568-7864
- Theunissen, J.W., Kaplan, M.I., Hunt, P.A., Williams, B.R., Ferguson, D.O., Alt, F.W. & Petrini, J.H. (2003) Checkpoint Failure and Chromosomal Instability without Lymphomagenesis in Mre11(Atld1/Atld1) Mice. *Molecular Cell*, Vol.12, No.6, pp.1511-1523
- Tsubouchi, H. & Roeder, G.S. (2002) The Mnd1 Protein Forms a Complex with Hop2 to Promote Homologous Chromosome Pairing and Meiotic Double-Strand Break Repair. *Mol Cell Biol*, Vol.22, No.9, pp.3078-3088, ISSN 0270-7306
- van der Burg, M., Ijspeert, H., Verkaik, N.S., Turul, T., Wiegant, W.W., Morotomi-Yano, K., Mari, P.O., Tezcan, I., Chen, D.J., Zdzienicka, M.Z., van Dongen, J.J. & van Gent, D.C. (2009) A DNA-Pkcs Mutation in a Radiosensitive T-B- Scid Patient Inhibits Artemis Activation and Nonhomologous End-Joining. *J Clin Invest*, Vol.119, No.1, pp.91-98, ISSN 0021-9738
- van Engelen, B.G., Hiel, J.A., Gabreels, F.J., van den Heuvel, L.P., van Gent, D.C. & Weemaes, C.M. (2001) Decreased Immunoglobulin Class Switching in Nijmegen Breakage Syndrome Due to the DNA Repair Defect. *Hum Immunol*, Vol.62, No.12, pp.1324-1327, ISSN 0198-8859
- Vousden, K.H. (2006) Outcomes of P53 Activation--Spoilt for Choice. *J Cell Sci*, Vol.119, No.Pt 24, pp.5015-5020, ISSN 0021-9533
- Waltes, R., Kalb, R., Gatei, M., Kijas, A.W., Stumm, M., Sobock, A., Wieland, B., Varon, R., Lerenthal, Y., Lavin, M.F., Schindler, D. & Dork, T. (2009) Human Rad50 Deficiency in a Nijmegen Breakage Syndrome-Like Disorder. *Am J Hum Genet*, Vol.84, No.5, pp.605-616, ISSN 1537-6605
- Wang, B., Matsuoka, S., Ballif, B.A., Zhang, D., Smogorzewska, A., Gygi, S.P. & Elledge, S.J. (2007) Abraxas and Rap80 Form a Brca1 Protein Complex Required for the DNA Damage Response. *Science*, Vol.316, No.5828, pp.1194-1198, ISSN 1095-9203
- Warmerdam, D.O. & Kanaar, R. (2010) Dealing with DNA Damage: Relationships between Checkpoint and Repair Pathways. *Mutat Res*, Vol.704, No.1-3, pp.2-11, ISSN 0027-5107
- Williams, B.R., Mirzoeva, O.K., Morgan, W.F., Lin, J., Dunnick, W. & Petrini, J.H. (2002) A Murine Model of Nijmegen Breakage Syndrome. *Curr Biol*, Vol.12, No.8, pp.648-653, ISSN 0960-9822
- Xiao, Y. & Weaver, D.T. (1997) Conditional Gene Targeted Deletion by Cre Recombinase Demonstrates the Requirement for the Double-Strand Break Repair Mre11 Protein in Murine Embryonic Stem Cells. *Nucleic Acids Res*, Vol.25, No.15, pp.2985-2991, ISSN 0305-1048
- Xiao, Z., Yannone, S.M., Dunn, E. & Cowan, M.J. (2009) A Novel Missense Rag-1 Mutation Results in T-B-Nk+ Scid in Athabaskan-Speaking Dine Indians from the Canadian Northwest Territories. *Eur J Hum Genet*, Vol.17, No.2, pp.205-212, ISSN 1476-5438
- Xu, X., Wagner, K.U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A. & Deng, C.X. (1999) Conditional Mutation of Brca1 in Mammary Epithelial Cells Results in Blunted Ductal Morphogenesis and Tumour Formation. *Nat Genet*, Vol.22, No.1, pp.37-43, ISSN 1061-4036

- Yu, X., Fu, S., Lai, M., Baer, R. & Chen, J. (2006) Brca1 Ubiquitinates Its Phosphorylation-Dependent Binding Partner CtIP. *Genes and Development*, Vol.20, pp.1721-1726
- Zhu, J., Petersen, S., Tessarollo, L. & Nussenzweig, A. (2001) Targeted Disruption of the Nijmegen Breakage Syndrome Gene Nbs1 Leads to Early Embryonic Lethality in Mice. *Curr Biol*, Vol.11, No.2, pp.105-109, ISSN 0960-9822

DNA Damage Repair and Cancer: The Role of RAD51 Protein and Its Genetic Variants

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1. Introduction

Genomes are continually attacked by both endogenous and exogenous agents that damage DNA. DNA damage in the form of DNA breaks can lead to chromosome translocations, cell cycle arrest, and apoptosis. Homologous recombination is an essential biological process that ensures the accurate repair of DNA breaks and thereby contributes to genomic integrity (Kuzminov 1999; Paques and Haber 1999).

DNA double-strand breaks (DSBs) are considered the principal lethal DNA damage resulting from ionizing radiation and cross-linking drugs. In addition, DSB can arise from endogenous processes, such as replication fork stalling during attempted replication over a single strand-break and topoisomerase poisons that are common therapies in the treatment of human cancers (Arnaudeau, Lundin et al. 2001).

It is of great importance that cells recognise DSBs and act upon them rapidly and efficiently, because cell death or impaired cell function can occur if these are left unrepaired or are repaired inaccurately. In addition to DNA repair mechanisms, cell cycle checkpoint activation processes are initiated in response to DNA damage. More specifically, DNA damage signals to arrest cell cycle progression, giving the cell more time to repair what might otherwise be a fatal lesion (Henning and Sturzbecher 2003).

2. DNA damage – repair pathways

Faithful genome transmission requires the co-ordination of a network of pathways such as cell cycle checkpoint, DNA replication, DNA repair/recombination and programmed cell death. In response to DNA damage, cells arrest their cell cycle progression, thus providing time for repair, or activate programmed cell death - both responses preventing transmission of genetic instability (Khanna and Jackson 2001). Thus, maintenance of the genomic integrity by DNA repair genes is an essential step in normal cellular growth and differentiation (Hoeijmakers 2001).

Failure to repair DNA lesions such as DSBs can lead to mutations, genomic instability, and cell death. Due to the severe consequences of DSBs, cells have developed two major repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Helleday, Lo et al. 2007).

HR takes advantage of large sequence homologies to repair DSBs. These homologous sequences can be found on homologous chromosome or in DNA repeats. HR refers to several processes, the two most documented in mammalian cells being single-strand annealing (SSA) and gene conversion associated or not with crossing over (Lin, Sperle et al. 1984). SSA process occurs between direct repeat sequences (Lin, Sperle et al. 1984) and is initiated by homologous pairing, except that—unlike HR—the homology is between short stretches of single-stranded DNA at staggered DSBs, and pairing precedes re-ligation, not strand exchange. It is a non-conservative process and error-prone because sequence information can be lost or rearranged when ends overlapping by as little as 30 bp are unsuitably joined (Henning and Sturzbecher 2003). Homologous recombination requires a homologous intact sequence and results in gene conversion associated or not with crossing over (Szostak, Orr-Weaver et al. 1983). Gene conversion is a conservative, generally error-free process, although it can also generate genetic variability. Gene conversion is involved in meiosis and molecular evolution (Daboussi, Dumay et al. 2002).

The mitotic cell cycle is an important determinant in the choice of the right repair pathway for a given physiological situation. Repair by HR predominates during S/G phases of the cell cycle, when sister chromatids, the preferred substrate for error-free exchange, are present (Henning and Sturzbecher 2003).

Non-homologous end joining (NHEJ) ligates the two broken DNA ends and does not require extensive sequence homologies between the two recombining DNA molecules. During the process, limited degradation of the DNA ends or DNA capture can lead to deletion or insertion of nucleotides or DNA fragments. It is thus a potentially error-prone process (Smith and Jackson 1999).

3. Role of mammalian protein RAD51

Mammalian RAD51 protein is a structural, biochemical and genetic homologue of the bacterial RecA and of the yeast RAD51 recombination proteins. Interestingly, overexpression of RAD51 alone is sufficient to stimulate gene conversion in mammalian cells (Vispe, Cazaux et al. 1998; Arnaudeau, Helleday et al. 1999; Lambert and Lopez 2000). In contrast, expression of dominant negative forms of RAD51 is enough to abolish almost totally gene conversion between tandem repeat sequences (Lambert and Lopez 2000). These data suggest that RAD51 plays a pivotal role in gene conversion regulation.

In vitro, RAD51 protein promotes DNA homologous pairing and strand exchange, in association with other proteins of the gene conversion complex (Benson, Baumann et al. 1998). In cultured mammalian cells, RAD51 is involved in spontaneous gene conversion as well as in HR induced by γ -rays (Lambert and Lopez 2000), alkylating agents, UV-C and replication elongation inhibitors (Saintigny, Delacote et al. 2001). More precisely, RAD51 controls DSB repair via gene conversion leading to gene conversion associated or not with crossing over (Lambert and Lopez 2000).

Finally, RAD51 partly participates in induced sister chromatid exchange in mammalian cells (Lambert and Lopez 2001). These roles of mammalian RAD51 in gene conversion are very similar to the roles of yeast RAD51. However, there are important differences between yeast and vertebrate RAD51 (Daboussi, Dumay et al. 2002).

The *RAD51* gene consists of 10 exons and spans at least 30 kb. All exon-intron boundaries follow the GT-AC rule. Further sequencing of the region 5' of the first exon revealed that noncoding exon 1 contained a CpG island that was approximately 900 bp in size. This

putative promoter region contains several recognition sites for Sp1 transcription factors but lacks a TATA box (Schmutte, Tomblin et al. 1999). The presence of several putative Sp1 promoter binding sites is consistent with the observed cell cycle-dependent expression of *RAD51* (Johnson 1992). The translation start codon is located in exon 2, and the average size of the coding exons is 112 bp (Schmutte, Tomblin et al. 1999).

RAD51 gene encodes a highly conserved well-characterized DNA repair protein (Liu, Lamerdin et al. 1998) (Table 1). *RAD51* gene is located at chromosome position 15q15.1 (Takahashi, Matsuda et al. 1994), a region that exhibits loss of heterozygosity in a large of cancers, including those of the lung, the colorectum and the breast (Wick, Petersen et al. 1996).

<i>RAD51</i>	
Gene symbol	<i>hRAD51</i>
Molecular Weight (Da)	36966
Gene type	Protein coding (<i>RAD51</i>)
Function	Involved in the homologous recombination and repair of DNA
Gene Map Locus	15q15.1
Localization primary	Nucleus
Protein interactions	BRCA1, <i>RAD51C</i> , ABL, P53, BRCA2, <i>RAD54</i> -like protein, <i>RAD54B</i> , <i>RAD52</i> , ERCC2, Cell cycle checkpoint kinase (<i>CHEK1/CHK1</i>), ATM, <i>RAD51C</i> , XRCC3, XRCC2, <i>RAD51B</i>
Described polymorphisms	5' UTR G135C and 5' UTR G172T

Table 1. Characteristics of the gene *RAD51*.

When cells are exposed to genotoxic agents or irradiation, such as mitomycin C, UV, and ionizing radiation, *RAD51* protein is recruited to sites of DNA damage where it mediates the search for a homologous sequence during homologous recombination (Buchhop, Gibson et al. 1997; Vispe, Cazaux et al. 1998). It has been revealed that the *RAD51* nuclear foci are the sites of repair of DNA damage (Tashiro, Kotomura et al. 1996).

This protein is therefore required for meiotic and mitotic recombination and plays a central role in homology-dependent recombinational repair of DSBs (Levy-Lahad, Lahad et al. 2001). Upon its regulated recruitment to sites of DNA breaks, *RAD51* forms a nucleoprotein filament by polymerizing onto single-stranded DNA at the processed break. This filament catalyses DNA strand exchange with an undamaged sister chromatid or homologous chromosome, which serve as templates for the restoration of missing genetic information (Li and Heyer 2008; San Filippo, Sung et al. 2008).

So when HR is used for repair, in eukaryotes it is promoted by the recombinase *RAD51*, which binds to 3'-tailed single strands at the end of DSBs in a helical fashion and promotes pairing with homologous DNA sequences as a prelude to strand invasion and repair of the DSBs (Sung and Klein 2006) (Figure 1).

In response to DNA damage, *RAD51* is translocated from the cytosol to the nucleus (Haaf, Golub et al. 1995). In the nucleus, *RAD51* sequesters into foci together with other proteins involved in homologous recombination, e. g., *RAD52* (Liu and Maizels 2000).

RAD51 is required for the resistance to ionising radiation (Ohnishi, Taki et al. 1998), and high levels of *RAD51* have been correlated with resistance to chemotherapeutics agents

(Maacke, Jost et al. 2000; Slupianek, Hoser et al. 2002). Importantly, RAD51 is also essential for embryonic survival in the absence of exogenous DNA damaging agents and has a role in the repair of spontaneously occurring chromosome breaks in proliferating cells of higher eukaryotes (Sonoda, Sasaki et al. 1998). This protein has been shown to be involved in the repair of different kinds of DNA lesions during replication (Lundin, Schultz et al. 2003). Thus, RAD51 is likely to promote genomic stability in eukaryotic cells (Orre, Falt et al. 2006). However, despite its role in maintaining genomic integrity, it has been proposed that the aberrant increase in RAD51 expression found in tumour cells may contribute to genomic instability by stimulating aberrant recombination between short repetitive elements and homologous sequences (Xia, Shammass et al. 1997; Flygare, Falt et al. 2001).

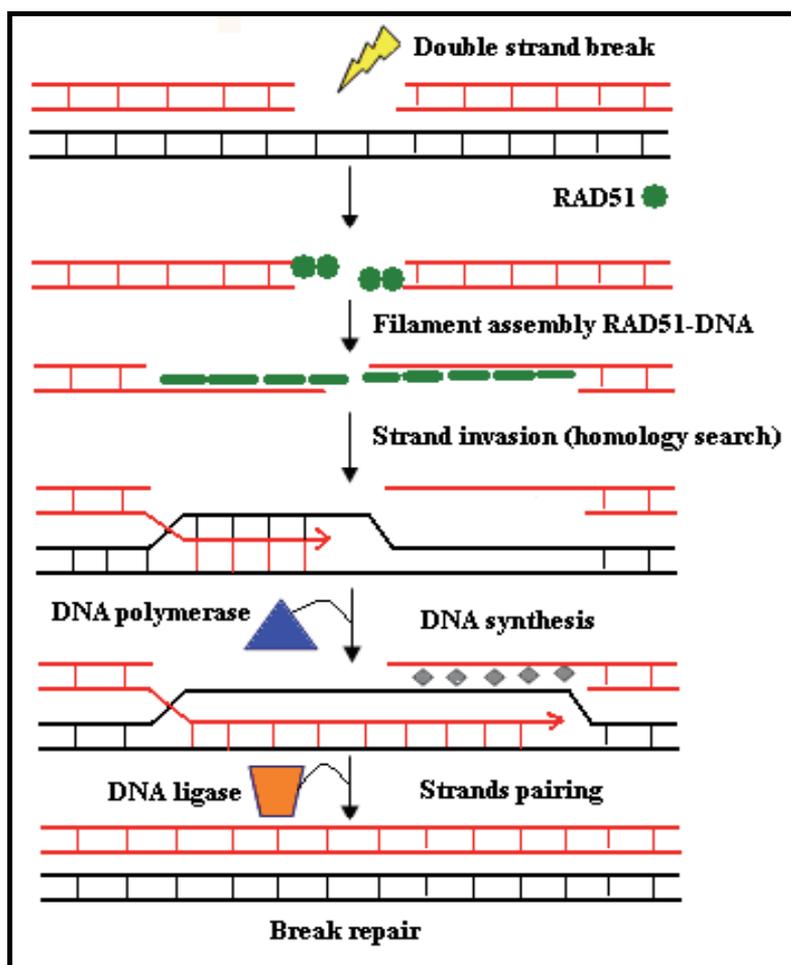


Fig. 1. Mechanism of DNA repair by RAD51 (homologous recombination).

The regulation of RAD51 appears to occur through at least two ways: (a) transcriptionally, by genes that confer a proliferative potential, as well as by checkpoint signaling pathways that regulate DNA damage responses; and (b) at the protein level, where interactions with other molecules leads to distinct cellular localization in RAD51 nuclear foci. It is possible

that RAD51 regulation may occur at the transcriptional level in a cell type-, cell cycle-, or damage response-coordinated manner.

RAD51 gene expression is controlled by a variety of transcriptional activators and repressors (Hasselbach, Haase et al. 2005; Arias-Lopez, Lazaro-Trueba et al. 2006), but is not affected by DNA damage (Henson, Tsai et al. 2006).

The accurate functioning of the DNA-repair proteins is a crucial step in maintaining genomic homeostasis and preventing carcinogenesis.

4. Levels of RAD51 protein expression

RAD51 expression is cell cycle-regulated, being lowest in resting cells. In proliferating cells, RAD51 expression peaks in the S/G2 phases of the cell cycle (Flygare, Benson et al. 1996; Yamamoto, Taki et al. 1996), indicating a role of the protein in intrachromosomal recombinational repair (Flygare, Falt et al. 2001).

Several studies have shown RAD51 protein expression levels to be elevated in immortalized cells and a wide variety of human cancer cell lines (Xia, Shamma et al. 1997; Raderschall, Stout et al. 2002). Given this high RAD51 expression, it is possible that RAD51 overexpression followed by hyperrecombination may contribute to genomic instability and malignant transformation (Vispe, Cazaux et al. 1998; Yanez and Porter 1999). Moreover, a growing body of literature suggests that RAD51 overexpression can increase cellular resistance to radiation and some chemotherapeutic drugs (Maacke, Jost et al. 2000; Henning and Sturzbecher 2003; Qiao, Wu et al. 2005). This could be of clinical importance for the treatment of cancer patients with radio- and/or chemotherapy (Flygare, Falt et al. 2001).

Aberrant overexpression of RAD51 protein could confer several advantages to tumor cells. First, the DNA repair function of RAD51 may protect cells from DNA damage and apoptosis. Secondly, overstimulation of homologous recombination and chromatid exchange mechanisms by RAD51 protein (Xia, Shamma et al. 1997; Arnaudeau, Helleday et al. 1999) may contribute to genomic instability and genetic diversity of tumour cells (Raderschall, Stout et al. 2002).

Hasselbach and co-workers (Hasselbach, Haase et al. 2005) identified three separate cis-acting sequence elements within the *RAD51* transcriptional promoter, one ensuring basal levels of expression and two elements limiting expression to relatively low levels. The characterisation of transcription factor binding might help to explain high-level expression of RAD51 in a variety of solid tumours.

The mechanisms underlying the observed radioresistance accompanying RAD51 overexpression are poorly understood. It is possible that an increased DSB repair capacity following RAD51 upregulation is responsible for the increased radioresistance. Alternatively, the overexpression of RAD51 might affect other cellular processes influencing cell survival, e.g., cell cycle progression (Flygare, Falt et al. 2001). The tumour suppressors p53 (Buchhop, Gibson et al. 1997) and BRCA2 (Patel, Yu et al. 1998; Yuan, Lee et al. 1999) associate with RAD51 and play roles in DNA repair and cell cycle checkpoint pathways (Dasika, Lin et al. 1999).

The reasons for RAD51 overexpression in cancer cells are not entirely understood. It is not the result of gene duplication or protein stability, but is thought to occur at the level of transcriptional regulation in the promoter region (Raderschall, Stout et al. 2002).

Since many tumours exhibit resistance to therapeutic drugs that damage DNA, it is important to understand the molecular mechanisms causing this DNA damage resistance. In

several cases it appears that the resistance is from enhanced RAD51 expression, due to oncogene-induced expression of RAD51 and inhibition of pathways that limit RAD51 protein levels. Cells that express the oncogenes BCR/ABL have increased levels of RAD51 protein (Slupianek, Schmutte et al. 2001). This occurs through STAT5-dependent transcription of RAD51 and inhibition of RAD51 protein cleavage by caspase-3. These cells have increased resistance to cisplatin and mitomycin C, drugs whose damage requires RAD51 for repair. Additionally, double strand break-induced homologous recombination is elevated in these cells (Klein 2008).

Therefore, another level of regulation of RAD51 activity occurs through protein modification. RAD51 is a target of the BCR/ABL kinase and is phosphorylated on tyrosine 315. Mutation of this tyrosine residue to phenylalanine resulted in increased sensitivity to cisplatin and mitomycin C, suggesting that RAD51 recombinational repair of DNA crosslink damage is controlled through RAD51 tyrosine 315 (Klein 2008).

Thus, upon DNA damage, RAD51 is phosphorylated by c-Abl. In addition, RAD51 is cleaved by caspase-3 during apoptosis (Slupianek, Schmutte et al. 2001; Klein 2008). Together, these findings suggest that the cellular level of the RAD51 protein is important for the control of homologous recombination and survival after DNA damage (Hansen, Lundin et al. 2003).

A study demonstrated that RAD51 expression levels in human cell lines were modulated by introducing various fusion tyrosine kinase (FTK) proteins. All of the FTK's, except one, elevated RAD51 expression levels (5- to 8-fold) relative to the parental cell line. The RAD51 expression levels correlated with cellular resistance to cisplatin, and this resistance was partially reversed by blocking RAD51 expression with an anti-sense strategy (Slupianek, Hoser et al. 2002).

Earlier studies found that due to its central role in recombination, RAD51 was likely to be a target for regulatory factors that coordinate DNA repair, transcription, replication and cell-cycle progression. The tumour-suppressor protein p53 is one of several factors that could interact directly with human RAD51 (Buchhop, Gibson et al. 1997). The p53 protein has a well-established role in linking progression through the cell cycle with genome integrity. This function is likely to require contact with the DNA-repair machinery, and RAD51 is therefore a potential target. There are some indications that the presence of TP53 affects the activities of RAD51 (Levine 1997). Overexpression of the c-myc, β -catenin or human papilloma virus E7 oncogenes results in induction of RAD51 and increased protein levels (Pauklin, Kristjuhan et al. 2005). RAD51 induction is dependent on the ATM and ATR kinases acting on p53 phosphorylation and downregulation of RAD51 levels. Increased RAD51 levels are correlated with an induction of the DNA damage response when oncogenes are overexpressed, using formation of γ -H2AX foci as a marker of DNA damage. Further studies suggested that the ARF tumor suppressor pathway also regulated RAD51 levels through p53 activation (Klein 2008).

Therefore, the tumor suppressor protein p53, which is frequently mutated in cancer, interacts with the RAD51 core promoter and RAD51 protein to inhibit both its expression and activity (Linke, Sengupta et al. 2003; Arias-Lopez, Lazaro-Trueba et al. 2006), while the transcription factor STAT5 has been shown to stimulate the expression of RAD51 (Slupianek, Schmutte et al. 2001; Slupianek, Hoser et al. 2002).

There are only a few studies that have investigated RAD51 expression levels in human tumors. Since increased levels of RAD51 have been correlated with elevated recombination rates (Xia, Shammam et al. 1997; Vispe, Cazaux et al. 1998), but also

increased genomic instability, the consequences of increased RAD51 expression were studied in human cells. Elevated RAD51 protein levels have been detected in human pancreatic adenocarcinoma cell lines (Maacke, Jost et al. 2000) and in cells derived from a patient with Bloom's syndrome (Magnusson, Sandstrom et al. 2000), a disorder that confers pronounced genomic instability. It has also been shown that elevated levels of RAD51 correlate with increased invasiveness of breast cancer (Maacke, Opitz et al. 2000) and can be used as an independent prognostic marker for mean survival time in patients with non-small cell lung cancer (Qiao, Wu et al. 2005).

It has already been demonstrated that increased RAD51 protein expression leads to a perturbation of the cellular state of equilibrium, reflected in alterations of gene expression patterns detectable at the mRNA level. Up-regulation of p53 and auto-regulated decrease of RAD51 protein indicate that high RAD51 protein levels may have induced stress responses in our system (Orre, Falt et al. 2006). However, the resulting RAD51 protein level remained higher than that observed in cells expressing RAD51 and was similar to the degree of RAD51 up-regulation observed in many cancer cell lines (Raderschall, Stout et al. 2002).

However, additional studies will be required to determine at which point during the multistage process of tumorigenesis RAD51 up-regulation occurs and to understand its clinical significance. Raderschall and co-workers (Raderschall, Stout et al. 2002) showed possible diagnostic and therapeutic applications. Firstly, RAD51 could serve as a diagnostic/prognostic marker to improve tumour classification. More importantly, down-regulation of RAD51 protein by RAD51 antisense oligonucleotides (Ohnishi, Taki et al. 1998) or RAD51-inhibitory drugs could be used to sensitize tumours to radiation or chemotherapy.

Therefore, various studies of DNA damage and repair in cancer are important, because they can give not only deeper insight into molecular mechanisms of carcinogenesis, but may also yield information on risk markers for cancer and help to improve cancer therapy as well as fight its hindrances (Synowiec, Stefanska et al. 2008).

5. Genetic variants and cancer

In the process of generating a draft sequence of the human genome, it has become clear that the extent of genetic variation is much larger than previously estimated (Lander, Linton et al. 2001; Venter, Adams et al. 2001). The most common variations in human genome are single nucleotide polymorphisms (SNPs), which are polymorphisms with only one nucleotide substitution. By definition, SNPs are single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater (Brookes 1999; Risch 2000).

These genetic variants are defined as low penetrance susceptibility alleles, providing an altered risk for cancer development. This risk appears to be influenced by individual SNPs profile in key genes for cancer susceptibility (Brookes 1999).

The association between exposure factor (polymorphism) and the disease is evaluated by relative risk (RR) estimation, indicating the probability of disease development in the group of polymorphic variant carriers. The great majority of molecular epidemiology studies on cancer are of case-control type; therefore RR is evaluated through Odds Ratio (OR). OR represents an association magnitude and supplies helpful information on causality and

definition of attributable risk, which is the proportion of all cases that is attributed to the risk factor (Knudsen, Loft et al. 2001).

SNPs and haplotype analysis in cancer research may contribute to the determination of high risk groups and help cancer prevention and development of new therapeutic orientations.

Several studies have reported that variations in genes involved in DNA repair and in the maintenance of genome integrity may be responsible in the increase of cancer risk (Jara, Acevedo et al. 2007). Thus, there is increasing volume of data supporting the hypothesis that genetic polymorphisms in various DNA repair genes result in reduced DNA repair capacity, in this way, being associated with increased susceptibility to various human solid tumours (Qiao, Spitz et al. 2002; Au, Salama et al. 2003; Hung, Hall et al. 2005).

Presence of polymorphisms in DNA repair genes could change the DNA-repair capacity and subsequently modulate the response to DNA-damaging agents and alter an individual's susceptibility to cancer (Hu, Mohrenweiser et al. 2002).

5.1 *RAD51* gene polymorphisms (G135C and G172T)

Two single-nucleotide polymorphisms (SNPs) polymorphisms have been described in the 5'- untranslated region (5'-UTR) of *RAD51* gene, a G to C substitution at position +135 bp, and a G to T substitution at position +172 bp from the start of the cDNA sequence (Levy-Lahad, Lahad et al. 2001; Wang, Spurdle et al. 2001; Rollinson, Smith et al. 2007). Promoter activity is significantly enhanced by substituting G at the polymorphic positions +135 and +172 for C and T, respectively (Hasselbach, Haase et al. 2005).

The biological effect of these polymorphisms is yet to be elucidated and will be important to investigate (Blasiak, Przybylowska et al. 2003). However, *RAD51* G135C polymorphism could affect mRNA splicing, regulation of transcription, translation efficiency or mRNA stability by association of 5'UTR region with regulatory elements (Gray 1998), leading to altered polypeptide product levels, which could affect the function of the final product - the *RAD51* protein (Poplawski, Arabski et al. 2006). Because a guanine-to-cytosine substitution at position +135 of the *RAD51* is a gain-of-function mutation, it is expected to result in increased activity of *RAD51*. This effect is opposite to those found for most of the other genetic variations in DNA repair genes, which result in the decrease of function (Chistiakov, Voronova et al. 2008).

Human *RAD51*, known to function in DNA repair, interacts with a number of proteins implicated in breast cancer, including *BRCA1* and *BRCA2*. Few studies have investigated the role of *RAD51* gene variations in familial breast cancer (Jara, Acevedo et al. 2007). However, some authors hypothesize that several polymorphisms of DNA repair genes could modify either DNA capacity or fidelity, which may contribute to familial and sporadic breast cancer susceptibility (Costa, Pinto et al. 2007). These genes involved in DNA repair, especially those that interact with the product of the *BRCA1* or *BRCA2* genes, are of particular interest as cancer risk modifiers in *BRCA1/2* mutation carriers. Both *BRCA1* and *BRCA2* participate in DNA double-strand break repair through homologous recombination (Venkitaraman 2002).

Therefore, the problem of genetic variability of the *RAD51* gene in breast cancer is worth studying for at least two reasons: (1) the involvement of *RAD51* in the stability of the genome and (2) its potential to modify the penetrance of *BRCA1/BRCA2* mutations, which can increase susceptibility for breast cancer (Blasiak, Przybylowska et al. 2003).

It was reported that the G135C polymorphism of the *RAD51* gene is a clinically significant modifier of *BRCA2* penetrance, specifically in raising breast cancer risk at younger ages (Levy-Lahad, Lahad et al. 2001).

Previous studies have linked the *RAD51* 135C allele with altered susceptibility to both breast cancer and ovarian cancer. In breast cancer, although a study found no association for the genetic variant (Kuschel, Auranen et al. 2002), Wang *et al.* (Wang, Spurdle et al. 2001) reported an increased risk of breast cancer and a lower risk of ovarian cancer amongst cases also possessing a *BRCA2* mutation, however, no association was seen for individuals known to have a *BRCA1* mutation. Apparently conflicting results have been reported by Jakubowska *et al.* (Jakubowska, Gronwald et al. 2007). These researchers investigated the role of the *RAD51* G135C polymorphism in breast and ovarian cancer in case-control populations of Polish women matched for *BRCA1* mutation and age. The results revealed that women who harboured the C allele had almost two times reduced risk of breast and ovarian cancer risk compared with women who harboured only the G allele. Moreover, it was shown in this study that the site of the *BRCA1* mutation did not influence the effect of the *RAD51* C allele, indicating that this polymorphism contributes to prevention of the disease among *BRCA1* carriers.

These differences in associated risk among for *BRCA1* mutation carriers may be due to chance, but also could be explained by the nature of the *BRCA1* mutations reported in the two studies. The most common mutation seen in the Jakubowska study was the 5382insC, which results in a truncated protein but which retains an intact *RAD51* binding site (Jakubowska, Narod et al. 2003). The primary mutation reported in the Wang study was the 185delAG, which also results in a truncated protein but abolishes the *BRCA1-RAD51* binding site. This suggests that for a protective effect to be seen in *BRCA1* mutation carriers, the *RAD51* interaction site must be present, enabling the *RAD51* 135C allele to enhance the activity of mutant *BRCA1* (Jakubowska, Narod et al. 2003).

Another study showed an elevated breast cancer risk associated with the *RAD51* 135C allele in *BRCA2* mutation carriers, but not in *BRCA1* mutation carriers (Levy-Lahad, Lahad et al. 2001; Wang, Spurdle et al. 2001).

Synowiec *et al.* (Synowiec, Stefanska et al. 2008) showed previously that the G135C polymorphism was not an independent marker in breast cancer, but it could be associated with an increased breast cancer risk in *BRCA2* mutation carriers (Blasiak, Przybylowska et al. 2003; Sliwinski, Krupa et al. 2005), confirming similar results from other studies (Levy-Lahad, Lahad et al. 2001; Antoniou, Sinilnikova et al. 2007). They also observed a protective effect against breast cancer occurrence for the G/C genotype of this polymorphism (OR 0.25; 95% CI 0.10-0.63). The results from a combined analysis of 19 studies revealed an increased risk of breast cancer in the C/C homozygotes with *BRCA2* mutation [41].

Jara *et al.* (Jara, Acevedo et al. 2007) proposed that *RAD51* G135C polymorphism presents an increased risk of familial breast cancer in women with age < 50 years at diagnosis, and this polymorphism may be a breast cancer risk variant. This finding should be confirmed in other populations.

BRCA2 is required for the orderly assembly of *RAD51* on single stranded DNA ends. In the absence of *BRCA2*, initiation of accurate HR is impaired and repair errors will rapidly accumulate (Powell, Willers et al. 2002). The increased risk associated with the *RAD51* 135C allele suggests an increase in repair errors. The biological explanation for this is uncertain but may reflect the use of an alternative pathway such as NHEJ (Moynahan, Pierce et al. 2001), or may be a result of error prone HR (Tutt, Bertwistle et al. 2001).

Costa *et al.* (Costa, Pinto et al. 2007) in a case-control study, showed an association of *RAD51* 135C allele and increased breast cancer risk only among women with family history of breast cancer, suggesting that this polymorphism contributed to the familial breast cancer in

the Portuguese population, in opposition to reported results in a Brazilian population (Dufloth, Costa et al. 2005). Concerning sporadic breast cancer risk, similar results to Costa and co-workers findings were obtained by other studies in Australian women (Webb, Hopper et al. 2005) and in the Anglo-Saxon population (Kuschel, Auranen et al. 2002), where no association was obtained.

In order to confirm these results, Kadouri *et al.* (Kadouri, Easton et al. 2001) evaluated the effect of the *RAD51* G135C polymorphism on breast cancer risk in *BRCA1/2* mutation carriers and in non-carrier breast cancer cases, mainly of Ashkenazi origin. These researchers reported a modifying effect for the *RAD51* G135C polymorphism in *BRCA2* carriers, similar to the effect shown in two previous studies. This is the first modifier gene identified in *BRCA2* carriers. The clinical implication of these findings is still limited; however, it hints at differences in molecular mechanisms involved in tumour development in *BRCA1* and *BRCA2* carriers. The study of polymorphisms in other DNA repair genes could further elucidate the mechanism of tumorigenesis in *BRCA1* and *BRCA2* carriers.

Recent structural studies suggest a mechanism for the regulation of *RAD51* activity by *BRCA2*, and cancer-associated mutations affecting the domain where *RAD51* binds to *BRCA2* or reduced level of the protein itself disrupt this interaction leading to impaired DNA repair via HR (Galkin, Esashi et al. 2005; Martin, Winkelmann et al. 2005). Because mutations in the *BRCA2* gene may be associated with breast and ovarian cancer and results from multi-site cancer phenotype, genetic variation in the *RAD51* gene may contribute to cancer (Martinez, Herzog et al. 2004). Some studies suggest that the G135C polymorphism of the *RAD51* gene may have a phenotypic effect, manifested in the changes in the extent of oxidative DNA damage. Recently, HR has been implicated in the repair of stalled replication forks (Michel, Grompone et al. 2004). This type of cellular events can occur as a consequence of oxidation of DNA. DNA double strand breaks (DSBs), which are the main substrate for HR, can arise directly from reactive oxygen species (ROS) (Galli, Piroddi et al. 2005).

A study in gastric cancer suggested that the G135C polymorphism of the *RAD51* gene may be linked with gastric cancer by the modulation of the cellular response to oxidative stress. In this work, the authors correlated the genetic constitution expressed by genotypes of the G135C polymorphism with susceptibility to DNA damage and efficacy of DNA repair in human lymphocytes of gastric cancer patients (Poplawski, Arabski et al. 2006). The results of this study suggest that the variants of the G135C polymorphism of the *RAD51* gene can be associated with the occurrence of gastric cancer in individuals with a high level of oxidative DNA damage or impaired repair of such damage, which can be a consequence of another genetic variation or/and environmental factor(s). Therefore, this polymorphism can be considered as an additional marker in gastric cancer. However, this study had mainly preliminary character and further research, performed on a larger group, is needed to establish a correlation between gastric cancer and the G/C polymorphism of the *RAD51* gene (Poplawski, Arabski et al. 2006).

It is known that in humans, inherited defects in HR pathways are known to predispose to acute myeloid leukaemia (AML), an example of this, Fanconi anemia (FA) (Bogliolo, Cabre et al. 2002) is characterized by spontaneous and mutagen-induced chromosome instability. Recently *BRCA2*, was identified as an FA protein, linking this pathway to HR through the interaction of *BRCA2* with *RAD51* (Godthelp, Artwert et al. 2002). There is a study that highlights the importance of the link between *RAD51*, *BRCA1*, *BRCA2* and a risk for AML. An increased risk for AML has been noted in patients previously diagnosed with breast cancer (Pagano, Pulsoni et al. 2001). Rollinson *et al.* (Rollinson, Smith et al. 2007) observed a

protective effect for the *RAD51* 135–172 C–G haplotype suggesting that it may be associated with increased *RAD51* expression, modulating HR and protecting the cells against aberrant DNA repair events, thus reducing the risk of AML.

Therapy-related acute myeloid leukemia (t-AML) is a devastating complication of chemotherapy and/or radiotherapy for a primary cancer. The risk of the development of t-AML was found to be associated with the G-to-C polymorphism at –135 of the 5' untranslated region (135G/C-5'UTR) of *RAD51* (Seedhouse and Russell 2007). The promoter activity of the *RAD51* gene is enhanced by the G-to-C substitution (135G/C-5'UTR), resulting in high levels of *RAD51* expression in individuals with the variation. *RAD51*'s role in t-AML was also supported by an indirect finding that *RAD51* was upregulated in mismatch repair-deficient murine embryonic stem cells. This process can be recapitulated by treatment with alkylating agents. Mismatch repair deficiency has been proposed to play an early role in therapy-related carcinogenesis. These data indicate that high levels of *RAD51* not only confer resistance to DNA-damaging agents but also contribute to the development of therapy-related cancers (Miyagawa 2008).

We previously reported a study evaluating the prognostic and predictive role of *RAD51* G135C polymorphism in non-small lung cancer (NSCLC) patients treated with combined platinum taxanes/gemcitabine first line chemotherapy (Nogueira, Catarino et al. 2009). In this study, our results demonstrated that the C allele is associated with a higher survival time, conferring a better prognosis than the GG genotype carrier patients. Thus, individuals carrying the C allele showed a longer overall survival after chemotherapy, compared with individuals carrying the allele G. This study also indicates that the influence of *RAD51* G135C polymorphism in treatment response of NSCLC patients seems to be modulated by smoking history. Our results demonstrate that smoker or ex-smoker patients carriers of *RAD51* 135C allele present a higher mean overall survival time (Nogueira, Catarino et al. 2009). According to the results obtained, we believe that *RAD51* genotypes could be useful molecular markers for predicting the clinical outcome of NSCLC patients.

The following table shows the main characteristics of some association studies between polymorphisms in the *RAD51* gene and risk for cancer (table 2).

6. Conclusion

New factors and pathways with the ability to recognize and repair DSBs are being discovered and studied. DSB production is now recognized as a general occurrence in cells, and these lesions frequently arise through endogenous and exogenous events. As a consequence of evolution from prokaryotes to eukaryotes, cells have developed complex mechanisms which can recognize and repair this type of severe damage rapidly and correctly. Cells have been exposed to many types of environmental stresses, and these stresses can sometimes lead to sub-lethal damage. In order to survive and function under adverse conditions, it is necessary to repair or eliminate DNA damage, and as a consequence, cells have developed a number of complex repair systems to enable their survival and functioning. Knowledge and understanding of these complex systems will make contributions to biology and medicine (Ohnishi, Mori et al. 2009).

A recent series of findings established a connection between apoptosis, HR regulation and tumorigenesis. Regulation of *RAD51* activity appears to be essential in these regulation networks. It is questionable whether other kinases or signalling processes can affect *RAD51* regulation. These data should enhance understanding of the general mechanisms

Authors	RAD51 SNP	Tumoral model	Population (case/control)	Ethnicity	Genotyping methods	Results
Synowiec <i>et al.</i> (2008)	5' UTR G135C	Breast cancer	41/48	European	PCR-RFLP	Polymorphism was not an independent marker in breast cancer, but it could be associated with an increased breast cancer risk in BRCA2 mutation carriers
Costa <i>et al.</i> (2007)	5' UTR G135C	Breast cancer	285/442	European	PCR-RFLP	Association of RAD51 135C allele and increased breast cancer risk only among women with family history of breast cancer. No association for sporadic breast cancer risk
Kadouri <i>et al.</i> (2001)	5' UTR G135C	Breast cancer	309/152	Jewish	PCR-RFLP	Elevated risk for breast cancer in carriers of BRCA2 mutations who also carry a 135C allele. No association for BRCA1 carriers. No association for BRCA1 non-carriers
Jara <i>et al.</i> (2007)	5' UTR G135C	Breast cancer	143/ 247	South American	PCR-RFLP	Increased risk of familial breast cancer in women with age < 50 years at diagnosis
Blasiak <i>et al.</i> (2003)	5' UTR G135C	Breast cancer	46/60	European	PCR-RFLP	No association between the polymorphism and appearance and progression of breast cancer

Jakubowska <i>et al.</i> (2007)	5' UTR G135C	Breast and ovarian cancer	485/485	European	PCR-RFLP	Women who harbour the C allele have almost twice the reduction in breast cancer risk and ovarian cancer risk compared with women who harbour the G allele
Wang <i>et al.</i> (2001)	5' UTR G135C	Breast and ovarian cancer	317/263	Mixed	PCR-RFLP	Increased risk of breast cancer and a lower risk of ovarian cancer for cases with a BRCA2 mutation. No association in cases with a BRCA1 mutation
Nogueira <i>et al.</i> (2009)	5' UTR G135C	Non- small lung cancer	234/-	European	PCR-RFLP	C allele associated with a higher survival time. Smoker or ex-smoker patients carrying RAD51 135C allele presented a higher mean overall survival time, but this association was not observed in non-smoker patients
Pagano <i>et al.</i> (2001)	5' UTR G135C 5' UTR G172T	Acute myeloid leukaemia	3934/-	European	PCR-RFLP	Increased risk for AML in patients previously diagnosed with breast cancer. Protective effect for the RAD51 135-172 C-G haplotype.

Rollinson <i>et al.</i> (2007)	5' UTR G135C 5' UTR G172T	Acute myeloid leukaemia	479/952	European	Real-Time PCR	Protective effect for the RAD51 135-172 C-G haplotype, reducing the risk of AML.
Poplawski <i>et al.</i> (2006)	5' UTR G135C	Gastric cancer	30/30	European	PCR-RFLP	The variants of the G135C polymorphism can be associated with the occurrence of gastric cancer in individuals with a high level of oxidative DNA damage or impaired repair of such damage.

Table 2. Main characteristics of some studies included in this review

controlling genome stability, their connections with cell cycle control, apoptosis regulation and more generally predisposition to tumour development (Daboussi, Dumay *et al.* 2002). Most of case-control studies searching for the contribution of genetic alterations within DNA repair genes to susceptibility to radiation-related cancer have been focused on genes involved in HR. Additional efforts are needed to find novel genetic variants of DNA repair genes involved in HR that confer susceptibility to radiation-induced cancer as well as to confirm already discovered disease-associated variants. To date, significant advances have been achieved in evaluating the role of genetic variations within DNA repair genes in clinical radiosensitivity in cancer. *RAD51* gene polymorphisms have been suggested to be associated with radiosensitivity in cancer (Chistiakov, Voronova *et al.* 2008).

Recently, several national and international clinical research projects have been initiated to find markers of genetic predisposition to radiation-induced cancer and clinical radiosensitivity in tumour tissues. However, over the next few years, a considerable molecular characterization of large-scale cohorts of individuals who show therapeutic radiation sensitivity is likely to be achieved. The construction and use of genetic-risk profiles may provide significant improvements in the efficacy of population-based programs of intervention for cancers. This also should help in predicting radiosensitivity that will eventually allow individual tailoring of treatment and reduce the risk of developing acute reactions in anticancer radiotherapy (Kuhne, Riballo *et al.* 2004; Chistiakov, Voronova *et al.* 2008).

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8. References

- Antoniou, A. C., O. M. Sinilnikova, et al. (2007). "RAD51 135G-->C modifies breast cancer risk among BRCA2 mutation carriers: results from a combined analysis of 19 studies." *Am J Hum Genet* 81(6): 1186-200.
- Arias-Lopez, C., I. Lazaro-Trueba, et al. (2006). "p53 modulates homologous recombination by transcriptional regulation of the RAD51 gene." *EMBO Rep* 7(2): 219-24.
- Arnaudeau, C., T. Helleday, et al. (1999). "The RAD51 protein supports homologous recombination by an exchange mechanism in mammalian cells." *J Mol Biol* 289(5): 1231-8.
- Arnaudeau, C., C. Lundin, et al. (2001). "DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells." *J Mol Biol* 307(5): 1235-45.
- Au, W. W., S. A. Salama, et al. (2003). "Functional characterization of polymorphisms in DNA repair genes using cytogenetic challenge assays." *Environ Health Perspect* 111(15): 1843-50.
- Benson, F. E., P. Baumann, et al. (1998). "Synergistic actions of Rad51 and Rad52 in recombination and DNA repair." *Nature* 391(6665): 401-4.
- Blasiak, J., K. Przybylowska, et al. (2003). "Analysis of the G/C polymorphism in the 5'-untranslated region of the RAD51 gene in breast cancer." *Acta Biochim Pol* 50(1): 249-53.
- Bogliolo, M., O. Cabre, et al. (2002). "The Fanconi anaemia genome stability and tumour suppressor network." *Mutagenesis* 17(6): 529-38.
- Brookes, A. J. (1999). "The essence of SNPs." *Gene* 234(2): 177-86.
- Buchhop, S., M. K. Gibson, et al. (1997). "Interaction of p53 with the human Rad51 protein." *Nucleic Acids Res* 25(19): 3868-74.
- Chistiakov, D. A., N. V. Voronova, et al. (2008). "Genetic variations in DNA repair genes, radiosensitivity to cancer and susceptibility to acute tissue reactions in radiotherapy-treated cancer patients." *Acta Oncol* 47(5): 809-24.
- Costa, S., D. Pinto, et al. (2007). "DNA repair polymorphisms might contribute differentially on familial and sporadic breast cancer susceptibility: a study on a Portuguese population." *Breast Cancer Res Treat* 103(2): 209-17.
- Daboussi, F., A. Dumay, et al. (2002). "DNA double-strand break repair signalling: the case of RAD51 post-translational regulation." *Cell Signal* 14(12): 969-75.
- Dasika, G. K., S. C. Lin, et al. (1999). "DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis." *Oncogene* 18(55): 7883-99.
- Dufloth, R. M., S. Costa, et al. (2005). "DNA repair gene polymorphisms and susceptibility to familial breast cancer in a group of patients from Campinas, Brazil." *Genet Mol Res* 4(4): 771-82.
- Flygare, J., F. Benson, et al. (1996). "Expression of the human RAD51 gene during the cell cycle in primary human peripheral blood lymphocytes." *Biochim Biophys Acta* 1312(3): 231-6.
- Flygare, J., S. Falt, et al. (2001). "Effects of HsRad51 overexpression on cell proliferation, cell cycle progression, and apoptosis." *Exp Cell Res* 268(1): 61-9.
- Galkin, V. E., F. Esashi, et al. (2005). "BRCA2 BRC motifs bind RAD51-DNA filaments." *Proc Natl Acad Sci U S A* 102(24): 8537-42.

- Galli, F., M. Piroddi, et al. (2005). "Oxidative stress and reactive oxygen species." *Contrib Nephrol* 149: 240-60.
- Godthelp, B. C., F. Artwert, et al. (2002). "Impaired DNA damage-induced nuclear Rad51 foci formation uniquely characterizes Fanconi anemia group D1." *Oncogene* 21(32): 5002-5.
- Gray, N. K. (1998). "Translational control by repressor proteins binding to the 5'UTR of mRNAs." *Methods Mol Biol* 77: 379-97.
- Haaf, T., E. I. Golub, et al. (1995). "Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes." *Proc Natl Acad Sci U S A* 92(6): 2298-302.
- Hansen, L. T., C. Lundin, et al. (2003). "The role of RAD51 in etoposide (VP16) resistance in small cell lung cancer." *Int J Cancer* 105(4): 472-9.
- Hasselbach, L., S. Haase, et al. (2005). "Characterisation of the promoter region of the human DNA-repair gene Rad51." *Eur J Gynaecol Oncol* 26(6): 589-98.
- Helleday, T., J. Lo, et al. (2007). "DNA double-strand break repair: from mechanistic understanding to cancer treatment." *DNA Repair (Amst)* 6(7): 923-35.
- Henning, W. and H. W. Sturzbecher (2003). "Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance." *Toxicology* 193(1-2): 91-109.
- Henson, S. E., S. C. Tsai, et al. (2006). "Pir51, a Rad51-interacting protein with high expression in aggressive lymphoma, controls mitomycin C sensitivity and prevents chromosomal breaks." *Mutat Res* 601(1-2): 113-24.
- Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." *Nature* 411(6835): 366-74.
- Hu, J. J., H. W. Mohrenweiser, et al. (2002). "Symposium overview: genetic polymorphisms in DNA repair and cancer risk." *Toxicol Appl Pharmacol* 185(1): 64-73.
- Hung, R. J., J. Hall, et al. (2005). "Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review." *Am J Epidemiol* 162(10): 925-42.
- Jakubowska, A., J. Gronwald, et al. (2007). "The RAD51 135 G>C polymorphism modifies breast cancer and ovarian cancer risk in Polish BRCA1 mutation carriers." *Cancer Epidemiol Biomarkers Prev* 16(2): 270-5.
- Jakubowska, A., S. A. Narod, et al. (2003). "Breast cancer risk reduction associated with the RAD51 polymorphism among carriers of the BRCA1 5382insC mutation in Poland." *Cancer Epidemiol Biomarkers Prev* 12(5): 457-9.
- Jara, L., M. L. Acevedo, et al. (2007). "RAD51 135G>C polymorphism and risk of familial breast cancer in a South American population." *Cancer Genet Cytogenet* 178(1): 65-9.
- Johnson, L. F. (1992). "G1 events and the regulation of genes for S-phase enzymes." *Curr Opin Cell Biol* 4(2): 149-54.
- Kadouri, L., D. F. Easton, et al. (2001). "CAG and GGC repeat polymorphisms in the androgen receptor gene and breast cancer susceptibility in BRCA1/2 carriers and non-carriers." *Br J Cancer* 85(1): 36-40.
- Khanna, K. K. and S. P. Jackson (2001). "DNA double-strand breaks: signaling, repair and the cancer connection." *Nat Genet* 27(3): 247-54.
- Klein, H. L. (2008). "The consequences of Rad51 overexpression for normal and tumor cells." *DNA Repair (Amst)* 7(5): 686-93.

- Knudsen, L. E., S. H. Loft, et al. (2001). "Risk assessment: the importance of genetic polymorphisms in man." *Mutat Res* 482(1-2): 83-8.
- Kuhne, M., E. Riballo, et al. (2004). "A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity." *Cancer Res* 64(2): 500-8.
- Kuschel, B., A. Auranen, et al. (2002). "Variants in DNA double-strand break repair genes and breast cancer susceptibility." *Hum Mol Genet* 11(12): 1399-407.
- Kuzminov, A. (1999). "Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda." *Microbiol Mol Biol Rev* 63(4): 751-813, table of contents.
- Lambert, S. and B. S. Lopez (2000). "Characterization of mammalian RAD51 double strand break repair using non-lethal dominant-negative forms." *Embo J* 19(12): 3090-9.
- Lambert, S. and B. S. Lopez (2001). "Role of RAD51 in sister-chromatid exchanges in mammalian cells." *Oncogene* 20(45): 6627-31.
- Lander, E. S., L. M. Linton, et al. (2001). "Initial sequencing and analysis of the human genome." *Nature* 409(6822): 860-921.
- Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." *Cell* 88(3): 323-31.
- Levy-Lahad, E., A. Lahad, et al. (2001). "A single nucleotide polymorphism in the RAD51 gene modifies cancer risk in BRCA2 but not BRCA1 carriers." *Proc Natl Acad Sci U S A* 98(6): 3232-6.
- Li, X. and W. D. Heyer (2008). "Homologous recombination in DNA repair and DNA damage tolerance." *Cell Res* 18(1): 99-113.
- Lin, F. L., K. Sperle, et al. (1984). "Homologous recombination in mouse L cells." *Cold Spring Harb Symp Quant Biol* 49: 139-49.
- Lin, F. L., K. Sperle, et al. (1984). "Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process." *Mol Cell Biol* 4(6): 1020-34.
- Linke, S. P., S. Sengupta, et al. (2003). "p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination." *Cancer Res* 63(10): 2596-605.
- Liu, N., J. E. Lamerdin, et al. (1998). "XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages." *Mol Cell* 1(6): 783-93.
- Liu, Y. and N. Maizels (2000). "Coordinated response of mammalian Rad51 and Rad52 to DNA damage." *EMBO Rep* 1(1): 85-90.
- Lundin, C., N. Schultz, et al. (2003). "RAD51 is involved in repair of damage associated with DNA replication in mammalian cells." *J Mol Biol* 328(3): 521-35.
- Maacke, H., K. Jost, et al. (2000). "DNA repair and recombination factor Rad51 is over-expressed in human pancreatic adenocarcinoma." *Oncogene* 19(23): 2791-5.
- Maacke, H., S. Opitz, et al. (2000). "Over-expression of wild-type Rad51 correlates with histological grading of invasive ductal breast cancer." *Int J Cancer* 88(6): 907-13.
- Magnusson, K. P., M. Sandstrom, et al. (2000). "p53 splice acceptor site mutation and increased HsRAD51 protein expression in Bloom's syndrome GM1492 fibroblasts." *Gene* 246(1-2): 247-54.
- Martin, J. S., N. Winkelmann, et al. (2005). "RAD-51-dependent and -independent roles of a *Caenorhabditis elegans* BRCA2-related protein during DNA double-strand break repair." *Mol Cell Biol* 25(8): 3127-39.
- Martinez, S. L., J. Herzog, et al. (2004). "Loss of five amino acids in BRCA2 is associated with ovarian cancer." *J Med Genet* 41(2): e18.

- Michel, B., G. Grompone, et al. (2004). "Multiple pathways process stalled replication forks." *Proc Natl Acad Sci U S A* 101(35): 12783-8.
- Miyagawa, K. (2008). "Clinical relevance of the homologous recombination machinery in cancer therapy." *Cancer Sci* 99(2): 187-94.
- Moynahan, M. E., A. J. Pierce, et al. (2001). "BRCA2 is required for homology-directed repair of chromosomal breaks." *Mol Cell* 7(2): 263-72.
- Nogueira, A., R. Catarino, et al. (2009). "Influence of DNA repair RAD51 gene variants in overall survival of non-small cell lung cancer patients treated with first line chemotherapy." *Cancer Chemother Pharmacol*.
- Ohnishi, T., E. Mori, et al. (2009). "DNA double-strand breaks: their production, recognition, and repair in eukaryotes." *Mutat Res* 669(1-2): 8-12.
- Ohnishi, T., T. Taki, et al. (1998). "In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the RAD51 gene." *Biochem Biophys Res Commun* 245(2): 319-24.
- Orre, L. M., S. Falt, et al. (2006). "Rad51-related changes in global gene expression." *Biochem Biophys Res Commun* 341(2): 334-42.
- Pagano, L., A. Pulsoni, et al. (2001). "Acute myeloid leukemia in patients previously diagnosed with breast cancer: experience of the GIMEMA group." *Ann Oncol* 12(2): 203-7.
- Paques, F. and J. E. Haber (1999). "Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*." *Microbiol Mol Biol Rev* 63(2): 349-404.
- Patel, K. J., V. P. Yu, et al. (1998). "Involvement of Brca2 in DNA repair." *Mol Cell* 1(3): 347-57.
- Pauklin, S., A. Kristjuhan, et al. (2005). "ARF and ATM/ATR cooperate in p53-mediated apoptosis upon oncogenic stress." *Biochem Biophys Res Commun* 334(2): 386-94.
- Poplawski, T., M. Arabski, et al. (2006). "DNA damage and repair in gastric cancer--a correlation with the hOGG1 and RAD51 genes polymorphisms." *Mutat Res* 601(1-2): 83-91.
- Powell, S. N., H. Willers, et al. (2002). "BRCA2 keeps Rad51 in line. High-fidelity homologous recombination prevents breast and ovarian cancer?" *Mol Cell* 10(6): 1262-3.
- Qiao, G. B., Y. L. Wu, et al. (2005). "High-level expression of Rad51 is an independent prognostic marker of survival in non-small-cell lung cancer patients." *Br J Cancer* 93(1): 137-43.
- Qiao, Y., M. R. Spitz, et al. (2002). "Modulation of repair of ultraviolet damage in the host-cell reactivation assay by polymorphic XPC and XPD/ERCC2 genotypes." *Carcinogenesis* 23(2): 295-9.
- Raderschall, E., K. Stout, et al. (2002). "Elevated levels of Rad51 recombination protein in tumor cells." *Cancer Res* 62(1): 219-25.
- Risch, N. J. (2000). "Searching for genetic determinants in the new millennium." *Nature* 405(6788): 847-56.
- Rollinson, S., A. G. Smith, et al. (2007). "RAD51 homologous recombination repair gene haplotypes and risk of acute myeloid leukaemia." *Leuk Res* 31(2): 169-74.
- Saintigny, Y., F. Delacote, et al. (2001). "Characterization of homologous recombination induced by replication inhibition in mammalian cells." *Embo J* 20(14): 3861-70.

- San Filippo, J., P. Sung, et al. (2008). "Mechanism of eukaryotic homologous recombination." *Annu Rev Biochem* 77: 229-57.
- Schmutte, C., G. Tomblin, et al. (1999). "Characterization of the human Rad51 genomic locus and examination of tumors with 15q14-15 loss of heterozygosity (LOH)." *Cancer Res* 59(18): 4564-9.
- Seedhouse, C. and N. Russell (2007). "Advances in the understanding of susceptibility to treatment-related acute myeloid leukaemia." *Br J Haematol* 137(6): 513-29.
- Sliwinski, T., R. Krupa, et al. (2005). "Polymorphisms of the BRCA2 and RAD51 genes in breast cancer." *Breast Cancer Res Treat* 94(2): 105-9.
- Slupianek, A., G. Hoser, et al. (2002). "Fusion tyrosine kinases induce drug resistance by stimulation of homology-dependent recombination repair, prolongation of G(2)/M phase, and protection from apoptosis." *Mol Cell Biol* 22(12): 4189-201.
- Slupianek, A., C. Schmutte, et al. (2001). "BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance." *Mol Cell* 8(4): 795-806.
- Smith, G. C. and S. P. Jackson (1999). "The DNA-dependent protein kinase." *Genes Dev* 13(8): 916-34.
- Sonoda, E., M. S. Sasaki, et al. (1998). "Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death." *Embo J* 17(2): 598-608.
- Sung, P. and H. Klein (2006). "Mechanism of homologous recombination: mediators and helicases take on regulatory functions." *Nat Rev Mol Cell Biol* 7(10): 739-50.
- Synowiec, E., J. Stefanska, et al. (2008). "Association between DNA damage, DNA repair genes variability and clinical characteristics in breast cancer patients." *Mutat Res* 648(1-2): 65-72.
- Szostak, J. W., T. L. Orr-Weaver, et al. (1983). "The double-strand-break repair model for recombination." *Cell* 33(1): 25-35.
- Takahashi, E., Y. Matsuda, et al. (1994). "Chromosome mapping of the human (RECA) and mouse (Reca) homologs of the yeast RAD51 and Escherichia coli recA genes to human (15q15.1) and mouse (2F1) chromosomes by direct R-banding fluorescence in situ hybridization." *Genomics* 19(2): 376-8.
- Tashiro, S., N. Kotomura, et al. (1996). "S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes." *Oncogene* 12(10): 2165-70.
- Tutt, A., D. Bertwistle, et al. (2001). "Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences." *Embo J* 20(17): 4704-16.
- Venkitaraman, A. R. (2002). "Cancer susceptibility and the functions of BRCA1 and BRCA2." *Cell* 108(2): 171-82.
- Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." *Science* 291(5507): 1304-51.
- Vispe, S., C. Cazaux, et al. (1998). "Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation." *Nucleic Acids Res* 26(12): 2859-64.
- Wang, W. W., A. B. Spurdle, et al. (2001). "A single nucleotide polymorphism in the 5' untranslated region of RAD51 and risk of cancer among BRCA1/2 mutation carriers." *Cancer Epidemiol Biomarkers Prev* 10(9): 955-60.
- Webb, P. M., J. L. Hopper, et al. (2005). "Double-strand break repair gene polymorphisms and risk of breast or ovarian cancer." *Cancer Epidemiol Biomarkers Prev* 14(2): 319-23.

- Wick, W., I. Petersen, et al. (1996). "Evidence for a novel tumor suppressor gene on chromosome 15 associated with progression to a metastatic stage in breast cancer." *Oncogene* 12(5): 973-8.
- Xia, S. J., M. A. Shamma, et al. (1997). "Elevated recombination in immortal human cells is mediated by HsRAD51 recombinase." *Mol Cell Biol* 17(12): 7151-8.
- Yamamoto, A., T. Taki, et al. (1996). "Cell cycle-dependent expression of the mouse Rad51 gene in proliferating cells." *Mol Gen Genet* 251(1): 1-12.
- Yanez, R. J. and A. C. Porter (1999). "Gene targeting is enhanced in human cells overexpressing hRAD51." *Gene Ther* 6(7): 1282-90.
- Yuan, S. S., S. Y. Lee, et al. (1999). "BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo." *Cancer Res* 59(15): 3547-51.

The Role of Error-Prone Alternative Non-Homologous End-Joining in Genomic Instability in Cancer

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1. Introduction

To maintain the integrity of the genome, cells have evolved a complex set of pathways that function in response to DNA damage. Components of this response include (i) cell cycle checkpoints that prevent damaged DNA from being replicated, (ii) induction of programmed cell death to prevent the transmission of potentially mutagenic genetic changes and (iii) DNA repair pathways that remove various types of DNA lesions such as single base lesions, single strand breaks (SSB)s or double strand breaks (DSB)s.

DSBs are considered the most lethal form of DNA damage because, unlike almost any other types of DNA damage that have an intact undamaged template strand to guide the repair, the integrity of both strands of the duplex is lost (Khanna and Jackson, 2001). DSBs can be induced by environmental factors such as ionizing radiation, ultraviolet light, therapeutic treatment but also occur as a consequence of specific physiological processes such as DNA replication, the V(D)J recombination in B and T-lymphocytes or the immunoglobulin class switch recombination (CSR) within immunoglobulin variable domains in B-lymphocytes occurring during the development and maturation of the immune system (Ferguson and Alt, 2001, Revy et al., 2005). In order to maintain the integrity of the DNA information, cells recruit stringent DSB repair machinery to ensure the efficient repair of various types of DNA damage. Thus, failure to properly repair the DSBs may cause chromosomal abnormalities, which in turn, may lead to genomic instability and predispose the cells to malignant transformation. Moreover, the importance of DNA repair in protecting against DSB-induced genomic instability is suggested by the increased incidence of cancer in autosomal recessive DNA repair deficient human syndromes, such as BRCA1/2 deficient breast cancers (Futreal et al., 1994). Thus, since genomic instability is a common characteristic of both inherited and sporadic forms of cancer cells, it is likely that abnormalities in DNA repair contribute to the development and progression of sporadic cancers (Khanna and Jackson, 2001).

DSBs can be repaired by two major pathways, homology-directed repair (HR) and non-homologous end-joining (NHEJ) (Helleday et al., 2007). HR is active during the late S and G2 phases of the cell cycle and uses the intact sister chromatid as the template for repair

(Khanna and Jackson, 2001, Hartlerode and Scully, 2009). This pathway is a highly efficacious and error-free form of repair and is mainly responsible for the repair of DSBs caused by stalled/or collapsed replication forks induced for example by chemotherapeutic agents that abrogate DNA replication (Keller et al., 2001). HR mechanisms and their implication in genomic stability are reviewed in detail in Khanna and Jackson, 2001, Helleday et al., 2007, Hartlerode and Scully, 2009.

NHEJ repairs DSBs quite differently from HR by joining DNA ends directly. This form of repair is independent of extensive DNA sequence homology, and therefore errors can be introduced during the processing and joining of non-compatible DNA ends (Khanna and Jackson, 2001, Lieber, 2008, Hartlerode and Scully, 2009). NHEJ occurs throughout the cell cycle and is the major DSB repair pathway in G₀, G₁ and early S phase. NHEJ is the preferential pathway for repair of DSBs in mammalian cells (Lieber et al., 2003, Lieber, 2008).

Here, we describe the mechanism(s) and the role(s) of the error-prone NHEJ pathway in the maintenance of genomic instability in cancer and discuss how targeting NHEJ is a promising therapeutic strategy in cancer.

2. Error-prone NHEJ pathway: Mechanisms and properties in normal and cancer cells

Classical or C-NHEJ contributes to the repair of DSBs caused by endogenous and exogenous DNA damaging agents and also plays an important role in the repair of programmed DSBs in normal mammalian cells, made during V(D)J or CSR (Lieber et al., 2006). In addition, evidence now exists for an alternative version of NHEJ (ALT-NHEJ) (Nussenzweig and Nussenzweig, 2007) that exists at low levels in normal cells (Sallmyr et al., 2008b) and is enhanced in the absence of C-NHEJ. Here, we discuss the mechanisms and properties of C-NHEJ and ALT-NHEJ in normal and cancer cells.

2.1 The C-NHEJ pathway

There appears to be two phases of C-NHEJ: a rapid phase and a slower phase (Riballo et al., 2004). The rapid phase will repair most of the simple lesions which do not require any type of processing. In contrast, the slower phase of NHEJ reflects both the repair of (i) DSBs that occur in condensed chromatin and (ii) more complex DSBs that require processing before ligation (Riballo et al., 2004, Goodarzi et al., 2008).

The C-NHEJ pathway is initiated by the Ku70/Ku86 heterodimer also called Ku, a ring shaped complex that binds DSBs (Walker et al., 2001). This leads to the recruitment of the catalytic subunit of DNA dependent protein kinase (DNA PKcs) (Mimori and Hardin, 1986, Falzon et al., 1993, Gottlieb and Jackson, 1993) to form the activated DNA PK (Calsou et al., 1999, Singleton et al., 1999). The kinase activity of DNA PK is critical for C-NHEJ (Lees-Miller et al., 1990). DNA PK also phosphorylates other proteins, such as Artemis, which binds to DNA PKcs (Ma et al., 2002), activating its endonuclease activity at both 3' and 5' overhangs. The physical juxtaposition of DNA ends involves interactions between DNA-bound DNA PKcs molecules (Yaneva et al., 1997, DeFazio et al., 2002). If DNA ends can be directly ligated then the repair only requires ligation by XLF/DNA ligase IV/XRCC4, after interaction with DNA PK (Ahnesorg et al., 2006, Buck et al., 2006). However, a large fraction of DSBs generated by agents such as, ionizing radiation, are not directly ligatable, and require additional processing (Chen et al., 2000, Loblrich and Jeggo, 2005). Many proteins are

involved in processing these DNA ends, including polynucleotide kinase (PNK) which interacts with XRCC4 (Chappell et al., 2002), the nucleases Flap endonuclease-1 (FEN-1) (Wu et al., 1999) and Artemis (Chen et al., 2000), and the Polymerase X family members, Pol μ and λ (Ma et al., 2004). As a consequence of these processing reactions, the joining of DSBs by C-NHEJ often results in the loss or addition of a few nucleotides and the presence of short complementary sequences, microhomologies, at the break site that presumably contribute to the alignment of the DNA ends (Roth et al., 1985, Roth and Wilson, 1986). A schematic of the C-NHEJ is presented in **Figure 1**.

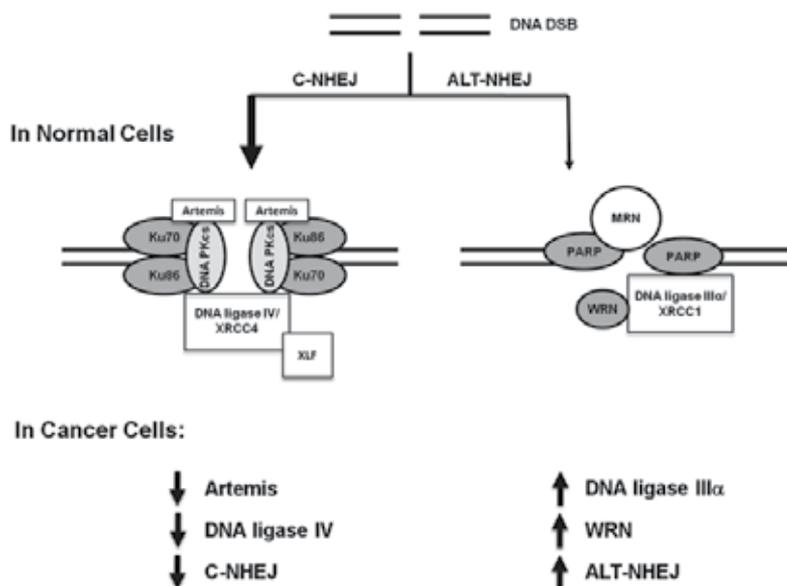


Fig. 1. In normal cells (upper panel), ALT-NHEJ pathway is a minor DSB repair pathway compared with C-NHEJ. In cancer cells (lower panel), the steady state levels of key C-NHEJ proteins are reduced whereas the steady state levels of key ALT-NHEJ are increased. This results in increased activity of the ALT-NHEJ pathway and reduced activity of the C-NHEJ pathway. Figure modified from Rassool and Tomkinson, 2010.

2.2 The ALT-NHEJ pathway

There are several lines of evidence for an alternative or back-up version of NHEJ that is enhanced in the absence of C-NHEJ (Riballo et al., 2004, Wang et al., 2006, Nussenzweig and Nussenzweig, 2007) (**Figure 1**). While these studies have begun to define more precisely the characteristics, mechanisms, regulation and roles of ALT-NHEJ in the development and maintenance of cancer, much of this pathway(s) remains to be elucidated. In the next section, the current state of our knowledge of ALT-NHEJ will be discussed.

2.2.1 Key signatures of ALT-NHEJ

The key features of the ALT-NHEJ pathway are that the repair junctions are characterized by larger deletions, insertions, and longer tracts of microhomology compared with those generated by C-NHEJ, and a much higher frequency of chromosomal translocations (Nussenzweig and Nussenzweig, 2007).

2.2.1.1 Microhomologies

Mechanistically, 3' single stranded overhangs containing longer tracts of microhomology are used to mediate ALT-NHEJ (Corneo et al., 2007, Yan et al., 2007, Bennardo et al., 2008, Deriano et al., 2009, Dinkelmann et al., 2009, Rass et al., 2009, Xie et al., 2009). This generally involves the loss of the intervening DNA sequences between the microhomology containing regions, resulting in larger DNA deletions. The regions of microhomology always reside at the precise site of repair and can be used as a marker to define these repair events. Moreover, while, ALT-NHEJ is associated with the generation of 3' single stranded overhang at the sites of DSBs, the presence of the DNA end-processing factor CtIP, appears to be required for microhomology-mediated joins upon depletion of the C-NHEJ component Ku70 (Lee-Theilen et al., 2011). Notably, microhomology sequences suggestive of ALT-NHEJ have been found at the recombination junctions of radiation-induced genomic rearrangements (Morris and Thacker, 1993, Nohmi et al., 1999) implying that radiation-induced DSBs can be repaired by ALT-NHEJ. Moreover, microhomologies are frequently detected at the breakpoints of chromosomal deletions and translocations in human cancer cells (Canning and Dryja, 1989, Dryja et al., 1989, Smanik et al., 1995, Wiemels and Greaves, 1999).

2.2.1.2 Translocation frequency

Several groups have observed that in the absence of C-NHEJ proteins, chromosomal translocations occur with increased frequency (Boboila et al., 2010b, Simsek and Jasin, 2010). These authors thus suggested that C-NHEJ suppresses chromosomal translocations. An alternative explanation for the increase in translocation frequency when C-NHEJ is absent, is that end-joining may be inefficient due to missing or mutant NHEJ components, and this may lead to the accumulation of multiple unrepaired DSBs. There is evidence that the repair kinetics of ALT-NHEJ is slower than that of C-NHEJ, in that end-joining assays performed in cells lacking DNA ligase IV are about 10 times slower than in cells proficient for C-NHEJ (Yan et al., 2007, Han and Yu, 2008). Thus, slowed NHEJ would be expected to increase the time of overlap during which two breaks would remain unrepaired, thereby increasing the chance of translocation events (Lieber, 2010).

Recent studies have suggested that oncogenes critical in the pathogenesis of leukemias directly or indirectly down regulate steady state levels of key C-NHEJ proteins, and in concert, upregulate key ALT-NHEJ proteins, leading to an increase in the frequency of deletions and translocations, which likely drive genomic instability, disease progression or resistance to treatment (Chen et al., 2008, Sallmyr et al., 2008b, Fan et al., 2010, Li et al., 2011).

2.2.2 Components involved in ALT-NHEJ

The presence or absence of Ku at the DSB dictate whether repair occurs *via* C-NHEJ or ALT-NHEJ, respectively (Fattah et al., 2010, Cheng et al., 2011). Several DNA repair proteins have been implicated in ALT-NHEJ repair. These include, DNA ligase III α /XRCC1, poly(ADP) ribose polymerase-1 (PARP-1), the MRN complex (Mre11/Rad50/Nbs1), WRN and CtIP (Audebert et al., 2004, Wang et al., 2005, Wang et al., 2006, Rass et al., 2009, Robert et al., 2009, Xie et al., 2009, Lee-Theilen et al., 2011, Cheng et al., 2011, Zhang and Jasin, 2011).

Given that ALT-NHEJ is initiated by resected DNA ends, the question arises, which factors can bind resected DSBs to start this repair process? Recent work identified PARP-1 as an

additional potential contributor to ALT-NHEJ (Audebert et al., 2004). PARP-1 recognizes DNA strand interruptions *in vivo* and triggers its own modification as well as that of other proteins by the sequential addition of ADP-ribose to form polymers. PARP-1 intervenes in base excision and single strand annealing (SSA) and now also operates in ALT-NHEJ (Audebert et al., 2004, Wang et al., 2006). While its role in ALT-NHEJ remains to be clearly elucidated, Wang et al. showed that PARP-1 binds to DNA ends in direct competition with Ku (Wang et al., 2006). When essential components of C-NHEJ are absent, PARP-1 is recruited for DSB repair, particularly in the absence of Ku proteins (Wang et al., 2006, Cheng et al., 2011).

The next question that arises is, which factor(s) is involved in the final joining reaction of ALT-NHEJ? Several studies implicate DNA ligase III α in ALT-NHEJ (Audebert et al., 2004, Wang et al., 2005, Haber, 2008). For example, using extract fractionation studies, Wang et al., showed that the majority of DNA end joining activity in extracts of HeLa cells could be attributed to DNA ligase III α (Wang et al., 2005). In addition, immunodepletion of DNA ligase III α from cell extracts caused loss of activity that could be recovered by the addition of the joining activity contributed by the purified enzyme. These experiments also ruled out a significant contribution to the end joining activity by DNA ligase I and DNA ligase IV. Furthermore, Wang et al., also addressed this question using RNA interference to investigate the requirements for DNA ligase III α and DNA ligase IV in the repair of DSBs (Wang et al., 2005). *In vivo* plasmid assays showed that DNA ligase IV-deficient mouse embryonic fibroblasts (MEFs) retained significant DNA end joining activity that could be reduced by up to 80% in cells knocked down for DNA ligase III α using RNAi (Wang et al., 2005). These *in vivo* observations are in line with DNA ligase III α being a candidate component for ALT-NHEJ. Other studies have implicated additional factors in ALT-NHEJ, such as PNK, FEN-1 (Gottlich et al., 1998, Wang et al., 2003, Audebert et al., 2004, Wang et al., 2006), and it is expected that additional factors will also be identified in the future (Figure 1).

2.2.3 Where ALT-NHEJ fits into the hierarchy of DSB repair?

While there is strong evidence that ALT-NHEJ is enhanced in cells that are defective for C-NHEJ, the question of where ALT-NHEJ fits into the hierarchy of DSB repair with respect to the cell cycle, and what would be the consequences of this repair at the genomic level, are still relatively unclear (Figure 2).

2.2.3.1 ALT-NHEJ and cell cycle

While it is well documented that HR is efficiently carried out only in the late S and G2 phases of the cell cycle using the newly synthesized sister chromatid, whereas C-NHEJ is the major DSB repair pathway in G0, G1 and early S phase (Lieber et al., 2003, Lieber et al., 2006), recent studies suggest that ALT-NHEJ may also be cell cycle dependent. During DNA replication, the newly replicated chromatids are held together by cohesin and this sister chromatid cohesion is maintained until mitosis. When a DSB occurs, the intact sister chromatid is preferentially used to repair the DSB by HR. If HR is defective, as it is demonstrated in BRCA 1/2 deficient cells, DSB is likely to be repaired by the following error-prone pathways (Tutt et al., 2001, Venkitaraman, 2001): (i) SSA that generates intrachromosomal deletions between repeated sequences, (ii) C-NHEJ pathway that generates small intrachromosomal deletions and insertions, and (iii) ALT-NHEJ pathway that generates larger deletions and chromosomal translocations. One of the roles of the DNA PK complex assembled on the DNA end is to

protect the DNA end from resection (Huertas, 2010). If C-NHEJ is defective, it is likely that end resection will occur (**Figure 2**). While the above hypothetical scenarios for error-prone repair of DSBs are envisioned, recent studies suggest that ALT-NHEJ may occur more frequently in G2. Mladenov and Iliakis enquired whether ALT-NHEJ was cell cycle dependent. In this study, MEFs with defects in C-NHEJ and/or HR were irradiated, G1 and G2 cells were isolated by cell sorting, and repair was examined by using pulse field gel electrophoresis (Mladenov and Iliakis, 2011). They found that wild-type and HR defective (*Rad54*^{-/-}) MEFs repaired DSBs with similar efficiency in G1 and G2 phases. In contrast, C-NHEJ defective (*DNA ligase IV*^{-/-}, *DNA PKcs*^{-/-}, and *Ku70*^{-/-}) MEFs showed a more pronounced repair defect in G1 phase than in G2 phase. Importantly, *DNA ligase IV*^{-/-}/*Rad54*^{-/-} MEFs repaired DSBs as efficiently as *DNA ligase IV*^{-/-} MEFs in G2 suggesting that the increased repair efficiency in G2 phase relies on the enhanced function of ALT-NHEJ rather than on HR. Furthermore, *in vivo* and *in vitro* plasmid end joining assays confirmed an enhanced function of ALT-NHEJ in G2 phase (Mladenov and Iliakis, 2011). Additional studies along the same lines using mutant Chinese hamster cells with defects in the DNA PKcs, Ku86 or XRCC4 components of C-NHEJ, or in the XRCC2 and XRCC3 components of HR confirmed these observations (Wu et al., 2008). Wild-type cells and mutants of HR repaired DSBs with similar efficiency in G1 and G2 phases. Mutants of C-NHEJ, showed more pronounced repair in G2 phase than in G1. These results in aggregate demonstrate a new and potentially important cell cycle regulation of ALT-NHEJ and generate a framework to investigate the mechanistic basis of HR contribution to DSB repair and its possible interactions with ALT-NHEJ.

Yet another study by Shibata et al., also examined the regulation of repair pathway usage at DSBs in G2 (Shibata et al., 2011). They identified the speed of DSB repair as a major component influencing repair pathway usage showing that DNA damage and chromatin complexity are factors influencing DSB repair rate and pathway choice. They found that loss of C-NHEJ proteins slowed DSB repair allowing increased resection. In contrast, loss of HR does not impair repair by C-NHEJ although CtIP-dependent end-resection precludes C-NHEJ usage. These data suggest that C-NHEJ initially attempts the repair of DSBs and, if rapid rejoining does not ensue, then resection occurs promoting repair by HR using the homologous chromosome as template, but this may result in loss of heterozygosity (LOH). It is likely that if repair does not occur by HR, DNA ends will be repaired by error-prone pathways, such as SSA and ALT-NHEJ, pathways that require end-resected DSBs (Shibata et al., 2011) (**Figure 2**).

2.2.3.2 Factors regulating ALT-NHEJ

Unlike C-NHEJ, the mechanism(s) for regulation of ALT-NHEJ and the factors involved in this repair pathway(s) are not clearly understood. The presence of Ku proteins appear to determine whether DSBs are repaired by C-NHEJ *vs.* ALT-NHEJ (Bennardo et al., 2008, Fattah et al., 2010, Cheng et al., 2011). Fattah et al., utilized an end-joining assay in isogenic human colon carcinoma cell lines and human somatic HCT116 with targeted deletions of the key C-NHEJ factors (Ku, DNA PKcs, XLF, and DNA ligase IV). The end-joining assay was a plasmid based repair assay of a DSB made within reporter plasmid pEGFP-Pem1-Ad2 and reconstitution of green fluorescent protein (GFP). They found that absence of key C-NHEJ factors resulted in cell lines that were profoundly impaired in DSB repair activity. Unexpectedly, Ku86-deleted cells showed wild-type levels of DNA DSB repair activity but the events were mainly repaired by microhomology joining. Using siRNA technology, ALT-

NHEJ repair activity could also be efficiently activated in *DNA ligase IV*^{-/-} and *DNA PKcs*^{-/-} cells by subsequently reducing the level of Ku70. Recently, Cheng et al., demonstrated that Ku is the main factor preventing PARP-1 and MRN mobilization to the site of DSBs (Cheng et al., 2011). These studies demonstrate that Ku proteins are the critical C-NHEJ factors that regulate DSB repair pathway choice. Similarly, studies of Bennardo et al., compared the genetic requirements for ALT-NHEJ, using a series of chromosome integrated reporters to monitor repair of DSBs by the I-SceI endonuclease in mouse embryonic stem (ES) cells and the HEK293 cell line (Bennardo et al., 2008). Each individual reporter was designed such that repair of I-SceI-induced DSBs by a specific pathway restored a GFP expression cassette. Such repair was then scored in individual cells as green fluorescence using flow cytometric analysis. They found that the *Ku70*^{-/-} cells exhibited a 4-fold increase in the restoration of the GFP⁺ gene over wild-type cells, and that this increase was reversed by co-transfection of a Ku70 expression vector (Bennardo et al., 2008). Thus, the ALT-NHEJ repair events appeared not only to be Ku-independent, but also appear to be inhibited by Ku proteins (Bennardo et al., 2008, Cheng et al., 2011). Bennardo and Stark have also highlighted the importance of the presence of ataxia telangiectasia-mutated (ATM) in matching correct DNA ends during end-joining and preventing the joining of multiple chromosome ends that can lead to chromosomal translocation and genomic instability (Bennardo and Stark, 2010). They found that genetic or chemical disruption of ATM caused a substantial increase in incorrect end joining (Distal-EJ), but not correct end joining (Proximal-EJ). Moreover, the increase in Distal-EJ caused by ATM disruption was dependent on the presence of C-NHEJ factors, specifically DNA PKcs, XRCC4, and XLF. Thus, these authors concluded that ATM is important to limit incorrect end utilization during C-NHEJ. In yet another study, Zha et al. showed that ATM and XLF have fundamental roles in processing and joining DNA ends during V(D)J recombination, but that these roles were masked by functional redundancies. They found that combined deficiency of ATM and XLF nearly blocked mouse lymphocyte development due to an inability to process and join chromosomal V(D)J recombination DSB intermediates. Combined XLF and ATM deficiency also severely impaired C-NHEJ, but not ALT-NHEJ, during CSR. Redundant ATM and XLF functions in C-NHEJ appeared to be mediated by ATM kinase activity and are not required for extra-chromosomal V(D)J recombination, indicating a role for chromatin-associated ATM substrates. These authors also found a role for H2AX, protein involved in the recruitment of DNA repair factors to nuclear foci after DSBs (Rogakou et al., 1998). Conditional H2AX inactivation in XLF-deficient pro-B lines leads to V(D)J recombination defects associated with marked degradation of unjoined V(D)J ends, revealing that H2AX also has a role in the repair process (Zha et al., 2011).

Mechanistically, it is believed that during ALT-NHEJ both broken ends are resected to generate 3'-single-stranded overhangs (Huertas, 2010). Given that Ku-deficiency can lead to elevated DSB end-processing, these results raise the possibility that ALT-NHEJ, SSA and HR share end-processing as a common intermediate. Thus, Bennardo et al., determined also whether end-resecting factor CtIP is important for ALT-NHEJ, by performing siRNA knock-down of CtIP in HEK293 cell lines with integrated GFP reporter plasmids and stable expression of the inducible I-SceI protein and examined ALT-NHEJ repair in CtIP-depleted cells *vs.* control cells. They observed that ALT-NHEJ was significantly reduced in CtIP-depleted cells suggesting that CtIP-mediated DSB end-processing promotes ALT-NHEJ but also SSA and HR (Bennardo et al., 2008). Interestingly, disrupting RAD51 and RAD52 expression decrease HR and SSA activity respectively without perturbing ALT-NHEJ repair

(Bennardo et al., 2008). In recent studies, Zhang and Jasin, showed that depletion of CtIP, resulted in a substantial decrease in the chromosomal translocation frequency in mouse cells and a significantly lower usage of microhomology at the translocation breakpoint junctions. This suggests that CtIP-mediated ALT-NHEJ has a primary role in translocation formation (Zhang and Jasin, 2011).

Several studies have also implicated the MRN complex in ALT-NHEJ repair (Rass et al., 2009, Xie et al., 2009). Recent studies examined the role of the nuclease MRE11 in CSR. They showed that loss of the nuclease MRE11 resulted in milder defects, compared with loss of the whole MRN complex. This suggested that the MRN complex performed activities in end-joining, in addition to the nuclease activity of MRE11. Studies employing atomic force microscopy have visualized the MRN complex bridging DNA at distances of 1200 angstroms (Moreno-Herrero et al., 2005). Thus, MRN may perform bridging functions that may be particularly suited for CSR. In addition, since chromosomal translocations are frequently observed in ALT-NHEJ, the MRN complex may also play a role in end-bridging of distant DSBs, resulting in chromosomal rearrangements (**Figure 2**).

A schematic representation of the regulation and the hierarchy of the DSB repair pathways is presented in **Figure 2**.

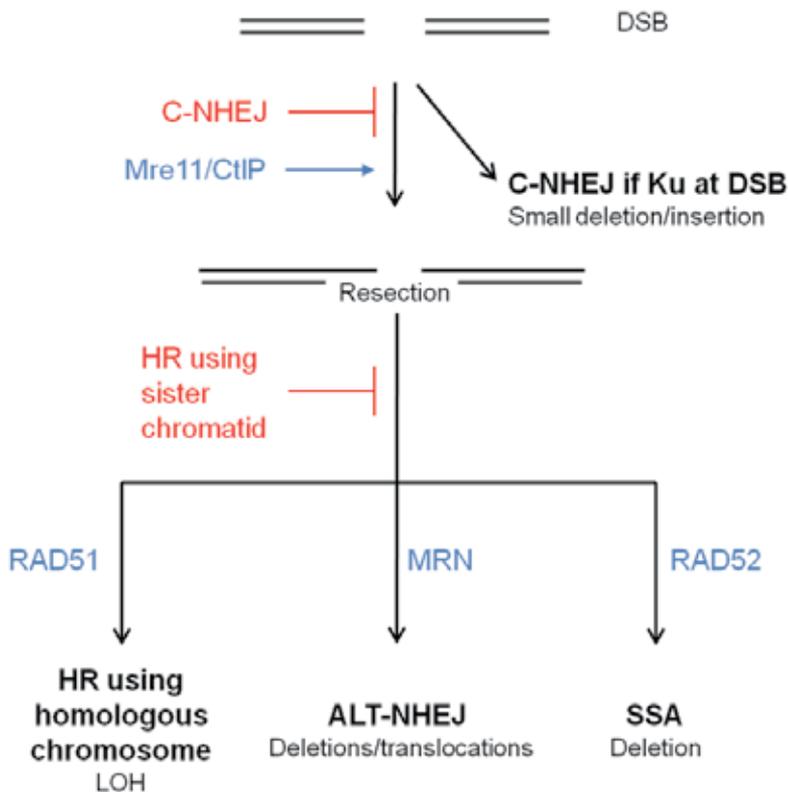


Fig. 2. The majority of the DSBs are repaired by C-NHEJ. If this pathway is inactive, DSBs can be repaired by HR using the homologous chromosome as template or by SSA or ALT-NHEJ. Positive regulators of specific stage as described in text are represented in blue while defective pathways are represented in red.

2.2.4 ALT-NHEJ at dysfunctional telomeres

Mammalian telomeres are regions of repetitive DNA sequences at the ends of chromosomes, which protect them from end-to-end fusion with neighbouring chromosomes. Critically shortened telomeres are recognized as DSBs and are highly susceptible to be repaired by HR or NHEJ pathways (Palm et al., 2009, Rai et al., 2010). However, unequal exchange of telomeric sequences by HR or misrepair by C- and/or ALT-NHEJ, can lead to loss of cell viability or can result in genomic instability and cancer. In mammals, telomeres form single-stranded G-rich overhangs that associate with and are protected by shelterin, a core complex of telomere-binding proteins that includes the double-stranded DNA-binding proteins TRF1 and TRF2 and protection of telomeres 1 (POT1a/b) that interacts with its binding partner TPP1 to protect them from resection, recombination and alteration (Palm et al., 2009). Telomeres are maintained by the enzyme telomerase, which is limited in human somatic cells, resulting in progressive telomere shortening. Celli et al., showed that Ku and TRF2 repress HR and represent an important aspect of telomere protection (Celli et al., 2006). Recent evidence suggests that dysfunctional telomeres that can no longer exert end-protective functions are recognized as DSBs by the DNA damage repair pathway. Thus, removal of TRF2 with retrovirus-mediated shTrf2, resulted in end-to-end chromosome fusions mediated by the C-NHEJ pathway (Rai et al., 2010). In addition, the data of Deng et al., indicated a critical role for the MRN complex in sensing these dysfunctional telomeres. They showed that in the absence of TRF2, MRE11 nuclease activity removes the 3' telomeric overhang to promote chromosome fusions. MRE11 can also protect newly replicated leading strand telomeres from NHEJ by promoting 5' strand resection to generate POT1a-TPP1-bound 3' overhangs (Deng et al., 2009). Rai et al. used also MEFs in which specific components of the C-NHEJ had been deleted to determine how dysfunctional telomeres are joined together (Rai et al., 2010). They showed that DSB marker 53BP1 (Schultz et al., 2000, Anderson et al., 2001) was necessary for end to end fusion in TRF2 deficient MEFs. Surprisingly, they showed that removal of *Tpp1-Pot1a/b* from *53BP1*^{-/-} MEFs or *DNA ligase IV*^{-/-} MEFs resulted in robust end to end fusions. They also examined chromosome fusion in MEFs from telomerase knock-out cells that generate naturally shortened telomeres, and which had also been knocked out for *53BP1*^{-/-}. Lymphomas derived from these mice demonstrated an increase in the number of fused chromosomes. These data suggested that fusion of naturally shortened telomeres do not require 53BP1 and occur through mechanisms independent of C-NHEJ. They concluded that telomeres engage distinct DNA repair pathways depending on how they are rendered dysfunctional, and that ALT-NHEJ is a major pathway for processing of dysfunctional telomeres (Rai et al., 2010).

2.2.5 NHEJ-defective mouse models of cancers and leukemias

2.2.5.1 NHEJ in V(D)J recombination and CSR

In addition to DSBs generated by exogenous and endogenous DNA damaging agents, DSBs also occur as a consequence of specific physiological processes such as the V(D)J recombination in B and T-lymphocytes and the immunoglobulin CSR within immunoglobulin variable domains in B-lymphocytes during the development and maturation of the immune system (Ferguson and Alt, 2001, Revy et al., 2005). The organism recruits stringent DNA repair machinery to ensure the efficient repair of the damage or the elimination of the damaged cells. Failure to properly repair the DNA damage may cause chromosomal abnormalities, which in turn may lead to genomic instability and predispose the cells to malignant transformation.

The immune system provides a unique platform for understanding the NHEJ pathway because of its requirement for V(D)J recombination and CSR for development and maturation. In these systems, DNA damage is initiated by recombination activating gene 1 and 2 (RAG1/RAG2) in the case of V(D)J recombination, activation-induced cytidine deaminase (AID) in the case of CSR (Oettinger et al., 1990, McBlane et al., 1995, Petersen et al., 2001, Manis et al., 2002), that is uniquely expressed in specialized B- or T-lymphocytes. The rejoining of the broken DNA ends is then completed by C- and/or ALT-NHEJ pathway (Bassing et al., 2002). Notably, V(D)J recombination specifically recruits the C-NHEJ pathway components (Corneo et al., 2007). In contrast, approximately 50% of CSR events are completed by the ALT-NHEJ pathways (Soulas-Sprauel et al., 2007b, Yan et al., 2007, Han and Yu, 2008). Animal models and human conditions have demonstrated that defects in any of the C-NHEJ pathway components may cause immunodeficiency. The resultant erroneous DNA repair may predispose the cells to genomic instability and the development of cancer.

2.2.5.2 Defective C-NHEJ in immunodeficiency

Spontaneous mutant and genetically engineered animal models deficient for the various C-NHEJ components have in common impaired V(D)J recombination and consequent immunodeficiency, together with increased sensitivity to ionizing radiation.

Severe combined immune deficiency (SCID) mouse is a naturally occurring mutant mouse strain (Bosma and Carroll, 1991) which harbors a non-sense mutation in their highly conserved C-terminal part of DNA PKcs gene (Blunt et al., 1996, Araki et al., 1997). These mice lack mature B and T lymphocytes (Bosma and Carroll, 1991), accompanied by an increased cellular radiosensitivity (RS-SCIDs), indicative of a defect in DNA repair. Similarly, DNA PKcs knockout mice do not show overt cellular growth defects but exhibit immunodeficiency and ionizing radiation hypersensitivity (Gao et al., 1998, Taccioli et al., 1998, Kurimasa et al., 1999). Artemis-deficient mice resemble DNA PKcs-deficient mice, including a leaky SCID and increased cellular ionizing radiation sensitivity, supporting the idea that Artemis cooperates with DNA PKcs in a subset of C-NHEJ functions (Rooney et al., 2002).

Like the DNA-PKcs mutant SCID mice, Ku70 and Ku86 knockout mice demonstrate “leaky” immunodeficiency and are hypersensitive to irradiation (Nussenzweig et al., 1996, Zhu et al., 1996, Gu et al., 1997, Ouyang et al., 1997). In addition, they also show signs of growth retardation and extensive apoptosis of the newly generated neurons. Mice lacking either XRCC4 or DNA ligase IV die *in utero* with massive neuronal apoptosis and a complete block in lymphocyte development, suggesting the requirement for Ku, XRCC4 and DNA ligase IV in growth control and neuron development (Barnes et al., 1998, Frank et al., 1998, Gao et al., 1998). Mice lacking XLF are also immunodeficient and hypersensitive to ionizing radiation. However, they have modestly reduced lymphocyte numbers, nearly normal V(D)J recombination and moderately defective immunoglobulin heavy chain CSR (Li et al., 2008). Combined deficiency of ATM and XLF severely impairs V(D)J recombination and nearly blocks mouse lymphocyte development, indicative of the compensatory roles of ATM and XLF in C-NHEJ pathway (Zha et al., 2011), as discussed earlier in this chapter.

2.2.5.3 Involvement of ALT-NHEJ pathways in leukemia/lymphoma in mouse models

In the absence of C-NHEJ, the microhomology-based ALT-NHEJ is thought to be employed to ligate the broken DNA ends generated during V(D)J recombination and CSR.

Mice defective for one or more C-NHEJ components show various degrees of genomic instability. The absence of Ku, XRCC4, DNA ligase IV, XLF, Artemis, or DNA PKcs leads to the accumulation of DNA breaks and translocations in ES cells, fibroblasts or stimulated B cells (Guidos et al., 1996, Nacht et al., 1996, Karanjawala et al., 1999, Difilippantonio et al., 2000, Ferguson and Alt, 2001, Zhu et al., 2002, Rooney et al., 2003, Yan et al., 2007, Franco et al., 2008, Li et al., 2008, Boboila et al., 2010a). In the presence of the p53-null background, deletion of any one of the key components of the C-NHEJ pathway invariably leads to the early onset of very aggressive tumors, mostly pro-B-cell lymphomas, which generally harbor chromosomal translocations. Mice defective for *P53* and *Ku86* develop pro-B-cell lymphoma at an early age (Difilippantonio et al., 2000). These tumors display a specific set of chromosomal translocations and gene amplifications involving the immunoglobulin heavy chain IgH/Myc locus, reminiscent of Burkitt lymphoma. Combined deficiency in p53/XRCC4 or p53/DNA ligase IV results in live births. However, the offspring are immunodeficient and develop pro-B cell lymphomas (Frank et al., 2000, Gao et al., 2000). Mice lacking both Artemis and p53 develop pro-B cell lymphomas harboring N-myc-IgH, but not the Myc-Igh translocations observed in tumors in other C-NHEJ/p53 deficient mice (Rooney et al., 2004). XLF/p53-double-deficient mice are not markedly prone to pro-B lymphomas. However, like other C-NHEJ/p53-deficient mice, they still develop medulloblastomas (Li et al., 2008).

Recent studies based on C-NHEJ deficient mutant models also revealed that Ku or DNA ligase IV/XRCC4 are not required for, but rather suppress chromosomal translocations (Corneo et al., 2007, Soulas-Sprauel et al., 2007a, Yan et al., 2007, Boboila et al., 2010a). It has recently been reported that translocation breakpoint junctions are similar in wild-type and Ku or XRCC4 deficient mutants, including an unchanged bias toward microhomology. Complex insertions at some breakpoint junctions show that joining can be iterative, encompassing successive processing steps before joining, implying that ALT-NHEJ contributes to the translocation formation in mammalian cells (Simsek and Jasin, 2010).

Altogether, the development of leukemia/lymphoma in C-NHEJ deficient mouse models suggests that ALT-NHEJ pathways play important roles in the DSB repair in V(D)J recombination and CSR. Its low fidelity predisposes the cells to genomic instability and the development of malignancy. Further investigations of the molecular mechanisms underlying these pathways will provide insights into the roles of ALT-NHEJ in the occurrence of genomic instability and the development of cancer.

2.2.6 ALT-NHEJ in human cancer and leukemia

Many of the studies characterizing ALT-NHEJ have been conducted in a background of experimentally induced deficiency of components of the C-NHEJ pathway. This has drawn criticism that the results demonstrating ALT-NHEJ are biased by artificial experimental conditions that do not exist in reality. Many human cancers are characterized by recurrent chromosome abnormalities and microhomologous sequences have been identified at the breakpoint junctions of these abnormalities. Therefore, human cancers may represent model systems in which to study ALT-NHEJ.

2.2.6.1 Leukemia and lymphoma cells as models for the study of ALT-NHEJ

Like the SCID phenotype observed in mice, defective V(D)J recombination in humans causes arrest of B and T lymphocyte maturation, conferring severe combined immune deficiency (T-B-SCID). 70% of T-B-SCID patients have mutations in RAG1 or RAG2, which disables the

initiation steps in V(D)J recombination. The remaining 30% patients also show hypersensitivity to ionizing radiation, and therefore referred to as RS-SCID. They are caused by defects in the C-NHEJ pathway (de Villartay et al., 2003). So far, most genetic defects reported are found in the Artemis gene. In other cases, mutations in DNA ligase IV, XLF and DNA PKcs are reported. EBV-associated B-cell lymphomas and leukemia have been reported in patients with Artemis and DNA ligase IV mutations, respectively, indicative of the genomic instability associated with the impaired C-NHEJ (Riballo et al., 1999, Moshous et al., 2003). Patients with mutations in the gene encoding XLF also have greater chromosomal instability (Dai et al., 2003, Buck et al., 2006). These findings suggest that RS-SCID patients defective for C-NHEJ have elevated genomic instability which may predispose the cells to cancer. It is also likely that ALT-NHEJ among other error-prone pathways may drive genomic instability in these cases.

2.2.6.2 Microhomologies at breakpoints junction of recurrent alterations in cancer and leukemia

In vivo and *in vitro* assays in cancer and leukemia cells demonstrate increased errors following repair, with the majority of errors resulting from large DNA deletions occurring at the repair sites characterized by sequence microhomologies. Using *in vitro* end-joining assays based on repair of pUC18 plasmids containing a DSB in cell lines derived from myeloid leukemias, Gaymes et al. demonstrated a significant increase in errors characterized by increased size of deletions and microhomologies at the repair junctions further suggestive of the importance of ALT-NHEJ repair in these malignancies (Gaymes et al., 2002). Analyzing actual genomic deletions in tumors, Canning and Dryja found genomic deletions involving the retinoblastoma gene in 12 of 49 tumors from patients with retinoblastoma or osteosarcoma. They mapped the deletion breakpoints and sequenced 200 base pairs surrounding each deletion breakpoint in DNA from 4 tumor samples. Three deletions had termini characterized by direct repeats ranging in size from 4 to 7 base pairs (Canning and Dryja, 1989).

Recurrent chromosome translocations characterize leukemia and lymphoma and are specifically associated with their classification and prognosis. They occur frequently in both *de novo* and therapy-related in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Cloning of the genomic breakpoints in the common chromosome translocations in leukemia reveal that most of the genomic breakpoints tend to cluster in a restricted intronic region (Zhang and Rowley, 2006). In addition, sequencing of the translocation junctions identified regions of microhomology, strongly indicative of the involvement of ALT-NHEJ in the repair of DSBs and the generation of these chromosomal abnormalities (Reichel et al., 1998, Gillert et al., 1999, Strissel et al., 2000, Rassool, 2003, Zhang and Rowley, 2006; Wiemels and Greaves, 1999, Xiao et al., 2001, Reiter et al., 2003, Zhang and Rowley, 2006). An important example of such a study is sequencing of the TEL AML1 gene fusions found in pediatric leukemias and approximately 25% of adult acute B cell lymphomas. Analysis of the DNA sequence and structure surrounding the breakpoints revealed clues to their possible formation (Wiemels and Greaves, 1999). A long-distance inverse PCR strategy was used to amplify *TEL-AML1* genomic fusion sequences from diagnostic DNA from nine patients. Breakpoints were scattered within the 14 kb of intronic DNA between exons 5 and 6 of *TEL* and in two putative cluster regions within intron 1 of *AML1*. DNA sequences containing the breakpoint junctions exhibited characteristic signs of C- and ALT-NHEJ, including microhomologies at the breakpoints, small deletions and

duplications. Wiemels and Greaves concluded that the data was compatible with the possibility that *TEL-AML1* translocations occur by nonhomologous recombination involving imprecise, constitutive repair processes following DSBs (Wiemels and Greaves, 1999, Zelent et al., 2004).

2.2.6.3 Origins of DNA damage?

Genes such as *AML1*, *TEL* or *mixed lineage leukemia (MLL)* have been found rearranged with different partner genes in lymphoid and myeloid leukemias (Zelent et al., 2004). These translocations have been shown to correlate with sites of double-strand DNA cleavage by agents to which the cells or patients have been exposed, including exogenous rare-cutting endonucleases, radiomimetic compounds, and topoisomerase inhibitors (Greaves and Wiemels, 2003). The nature of the DNA damage leading to *MLL* translocations in leukemia in infants have been examined by several investigators (Greaves and Wiemels, 2003). The finding of identical *MLL* rearrangements in the leukemias from pairs of monozygotic twins where both twins were affected, but not in their constitutional DNA, established that *MLL* translocations in infant leukemias are non-hereditary, non-constitutional, *in utero* events. Furthermore, the most likely explanation was that cells with the translocation were transferred from one twin to the other *via* the placenta (Felix et al., 2000). The retrospective finding of leukemia-associated *MLL* genomic breakpoint junction sequences by PCR analysis of genomic DNAs contained in bloodspots on neonatal Guthrie cards of infants who were diagnosed later with leukemia showed that *MLL* translocations also occur *in utero* in the non-twin cases (Gale et al., 1997). Molecular cloning and analysis of *MLL* genomic breakpoint junction sequences in infant leukemias suggested staggered and/or multiple sites of breakage as elements of damage and DNA repair by C- and/or ALT-NHEJ. This provided further evidence that DNA damage and repair underlie the formation of the translocations (Greaves and Wiemels, 2003, Gilliland et al., 2004). Because *MLL* translocations are much less frequent in *de novo* leukemias of older patients but frequent in leukemias following chemotherapeutic DNA topoisomerase II poisons, e.g., etoposide, it has been proposed that leukemia in infants may have an etiology resembling treatment-related cases (Gilliland et al., 2004). The chemotherapy-leukemia association in the treatment-related cases suggests that chromosomal breakage resulting from DNA topoisomerase II cleavage and attempted repair of DSBs may play a role in the formation of these translocations. The precision of the breakpoint junction sequences and the results of DNA topoisomerase II *in vitro* cleavage assays in treatment-related leukemias are consistent with the processing of 4-base, staggered DSB (Gilliland et al., 2004). In the infant leukemias, the breakpoint junction sequences and *in vitro* cleavage assays suggest a mechanism in which DNA topoisomerase II introduce separate single-stranded nicks in duplex DNA that are staggered by up to several hundred bases. This leads to a DNA damage-repair model in which various naturally occurring DNA topoisomerase II poisons induce DNA topoisomerase II-mediated damage in leukemia *in utero* (Gilliland et al., 2004). The large deleted regions observed in other infant cases are consistent with multiple sites of breakage or, alternatively, more extensive processing (Raffini et al., 2002).

2.2.6.4 Increased ALT-NHEJ activity in leukemia

In addition to increased DNA damage providing a substrate for error-prone repair and genomic instability, increased repair activity may also drive the acquisition of genomic alterations. Recently, Sallmyr et al. demonstrated increased activity of the ALT-NHEJ

pathway in chronic myeloid leukemia (CML) cells characterized by the oncogenic fusion tyrosine kinase, BCR-ABL (Gaymes et al., 2002, Sallmyr et al., 2008b). They showed that key proteins in the major C-NHEJ pathway, Artemis and DNA ligase IV, were down-regulated, whereas DNA ligase III α , and the protein deleted in Werner syndrome, WRN, are up-regulated in CML cells. Furthermore, they showed that DNA ligase III α and WRN form a complex that is recruited to DSBs, and that “knockdown” of either DNA ligase III α or WRN leads to increased accumulation of unrepaired DSBs, demonstrating that these DNA repair proteins contribute to their repair. To determine whether knockdown of either DNA ligase III α or WRN leads to differences in repair using DNA sequence microhomologies, Sallmyr et al. sequenced the breakpoint junctions of 15 repaired plasmids from each of the LacZ α reactivation experiments. The majority (80%) of plasmids in CML cell line, K562 were repaired using DNA microhomologies of 1 to 6 bp. In contrast, plasmids from cells with reduced levels of either DNA ligase III α or WRN had a reduction in the overall percentage of microhomologies and these constituted 1 to 3 bp in length (DNA ligase III α , 25%; WRN, 40%) at the breakpoint junctions in repaired plasmids. Notably, in cell lines established from normal lymphocytes, end-joining assays reveal that the DSBs are repaired mainly using the C-NHEJ pathway. Furthermore, sequencing of the rare DSBs that were misrepaired (1 in approximately 10,000) revealed deletions of only a few base pairs. These results indicate that while ALT-NHEJ is possibly operative at very low levels in normal cells, altered DSB repair in CML cells may be caused at least in part by the increased activity of ALT-NHEJ repair pathway, involving DNA ligase III α and WRN. In AML characterized by expression of the constitutively activated receptor tyrosine kinase Fms Like tyrosine 3/Internal tandem duplication (FLT3/ITD), Sallmyr et al. reported that this constitutively activated tyrosine kinase initiates a cycle of genomic instability that is likely to promote both aggressive disease and resistance to therapy (Sallmyr et al., 2008a). Specifically, Sallmyr et al. showed that expression of FLT3/ITD induces increased reactive oxygen species production and that cells transformed by FLT3/ITD, including primary AML cells and cell lines established from FLT3/ITD-positive AML patients, have increased endogenous DSBs (Sallmyr et al., 2008a). Furthermore, repair of DSBs by NHEJ is less efficient and more error-prone in FLT3/ITD-expressing cells (Sallmyr et al., 2008a). More recently, Fan et al. reported that the steady state levels of Ku86 and to a lesser extent, Ku70, are significantly reduced in FLT3/ITD-expressing cells (Fan et al., 2010). In turn, there is a concomitant increase in the steady state levels of ALT-NHEJ components, PARP-1 and DNA ligase III α (Fan et al., 2010). Similar alterations in Ku86 and PARP-1 are also observed in FLT3/ITD knock-in mice, but increased levels of DNA ligase III α are only seen in the homozygote mice (Fan et al., 2010). Similar changes in C-NHEJ and ALT-NHEJ components are observed at the transcript level (Li et al., 2011) (**Figure 3**). In the FLT3/ITD mouse model, the impairment of C-NHEJ decreases the ability of cells to complete post-cleavage DSB ligation, resulting in failure to complete V(D)J recombination inhibiting B-lymphocyte maturation (Li et al., 2011). As a consequence of these changes in NHEJ proteins, the frequency of DNA sequence microhomologies and the size of deletions at repair sites are increased, reflecting the increased contribution of ALT-NHEJ to DSB repair. This suggests that FLT3/ITD signaling is involved in regulating both C- and ALT-NHEJ, directly or indirectly (**Figure 3**). Importantly, they reported that reducing the levels of DNA ligase III α in AML cells not only reduces the frequency of DNA sequence microhomologies and the size of deletions at repair sites but also increases the steady state levels of unrepaired DSBs, indicating that the ALT-NHEJ pathway is particularly important for the survival of FLT3/ITD expressing AML cells (Li et al., 2011).

2.2.7 ALT-NHEJ as therapeutic targets

Several lines of evidence suggest that both hereditary and sporadic cancers have abnormal level of DNA damage and repair responses that lead to the generation of structural chromosomal abnormalities and genomic instability, which are critical for survival, disease progression and resistance. The identification of defects in the DNA damage response between normal and cancer cells at the molecular level will guide the development of more targeted therapies by identifying biomarkers that are indicative of the abnormal DNA repair in cancer cells. To exploit the differences in the DNA damage response between normal and cancer cells, it will be necessary to characterize the DNA repair abnormalities and develop agents that target the abnormal DNA repair pathways that are specific for cancer cells, thereby reducing survival of cancer but not normal cells. In addition to participating in base excision and SSB repair, PARP-1 and DNA ligase III α also appear to be involved in ALT-NHEJ. In recent studies, we and others have shown that PARP-1 and DNA ligase III α are upregulated in certain cancers and leukemias (Chen et al., 2008, Sallmyr et al., 2008b, Fan et al., 2010, Li et al., 2011) (Figure 4).

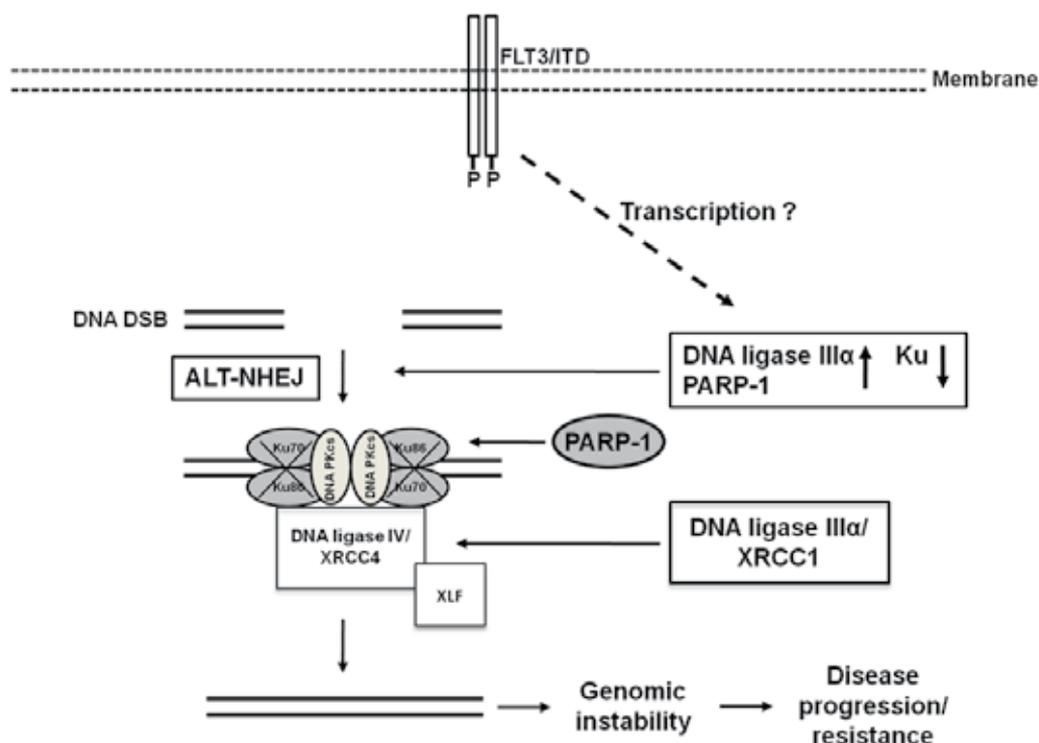


Fig. 3. Schematic for the mechanism of regulation of C- and ALT-NHEJ by FLT3/ITD. FLT3/ITD signalling leads to upregulation of DNA ligase III α and PARP-1 and downregulation of Ku70/86. The consequent increase in ALT-NHEJ activity promotes the acquisition of genomic changes that lead to disease progression or resistance to treatment.

2.2.7.1 PARP inhibitors

The abundant nuclear protein PARP-1 binds avidly to SSBs, an event that activates PARP-1 polymerase (Ame et al., 2004). Activated PARP-1 utilizes nicotinamide to synthesize

poly(ADP-ribose) polymers on itself and other nuclear proteins. Poly(ADP-ribosylated) PARP-1 serves as a recruitment factor for DNA ligase III α /XRCC1 and other factors involved in the repair of SSBs (Okano et al., 2003). Although there are other PARP family members, PARP-1 is the predominant enzyme that synthesizes poly(ADP-ribose) in response to DNA damage (Menissier de Murcia et al., 2003). The replication of DNA containing SSBs cause DSBs and so preventing the repair of SSBs by inhibiting PARP-1 results in an increase in DSBs. Since these replication-associated DSBs would normally be repaired by HR, cells that are defective in HR are hypersensitive to PARP inhibitors. Based on this rationale, potent and specific inhibitors of PARP were developed as therapeutic agents for inherited forms of breast and ovarian cancer as the PARP inhibitors should be cytotoxic for *BRCA* mutant tumors but not normal tissues with a functional *BRCA* allele (Bryant et al., 2005, Farmer et al., 2005). As expected, PARP inhibitors increased the cytotoxicity of a range of anti-cancer agents including temozolomide and ionizing radiation that cause SSBs (Tentori et al., 2002, Liu et al., 2008) and both *BRCA1*- and *BRCA2*-mutant cell lines were hypersensitive to PARP inhibitors in cell culture and mouse xenograft assays (Lord and Ashworth, 2008). These results formed the basis for a phase I clinical trial, which demonstrated that the PARP inhibitor AZD2281 exhibited antitumor activity in patients with ovarian and breast tumors resulting from either *BRCA1* or *BRCA2* mutations (Evers et al., 2008). The promising results from this clinical trial have prompted the evaluation of PARP inhibitors in combination with other cancer therapeutics in the treatment of different types of cancer.

Unfortunately, resistance to PARP-1 inhibitors has led to the failure of phase III clinical trials in triple negative breast cancers, and thus there is an urgency for elucidating the mechanisms by which resistance occurs in cells with defects in HR (Guha, 2011). One potential mechanism for resistance to PARP inhibitors in *BRCA*-deficient cells is that spontaneous or induced DSBs are rerouted for repair by error-prone mechanisms, including NHEJ, because the preferred mode of error-free repair by HR is unavailable (Venkitaraman, 2001). Patel et al. recently showed that in *BRCA2* deficient ovarian cancer cell lines PARP inhibitor treatment induces phosphorylation of DNA PK substrates and stimulates C-NHEJ selectively. Previous studies provided evidence for interplay between key C-NHEJ proteins and PARP-1: (i) PARP-1 can interact *in vitro* and *in vivo* with Ku (Galande and Kohwi-Shigematsu, 1999) and has been shown to compete with Ku80 for DNA ends *in vitro* (Wang et al., 2006), (ii) Ablation of C-NHEJ restores the survival of PARP-1-deficient cells treated with agents inducing DSBs (Hochegger et al., 2006). All together those results suggest that C-NHEJ and perhaps ALT-NHEJ could be involved in the genomic instability observed in HR-deficient cells treated with PARP inhibitors (Patel et al., 2011). Patel et al. showed that inhibiting DNA PK activity reverses the genomic instability induced by PARP inhibition in *BRCA2* deficient cells. Moreover, disabling C-NHEJ by using genetic or pharmacologic approaches diminished the toxicity of PARP inhibition in HR-deficient cells. These results not only implicate PARP-1 catalytic activity in the regulation of C-NHEJ and perhaps ALT-NHEJ in HR-deficient cells, but also indicate that deregulated C-NHEJ and perhaps ALT-NHEJ plays a major role in generating cytotoxicity and genomic instability in HR-deficient cells treated with PARP inhibitors (Patel et al., 2011). Recently Chen et al. showed that C-NHEJ protein DNA ligase IV was down regulated in cell lines derived from sporadic breast cancer (Chen et al., 2008). Thus, it would be important to evaluate C-NHEJ and ALT-NHEJ activity in *BRCA* deficient tumors in assessment of clinical response and resistance to PARP inhibitors.

2.2.7.2 DNA ligase inhibitors

DNA joining events are required for the completion of almost all DNA repair pathways. Thus, inhibitors of DNA ligase are predicted to sensitize cells to a variety of DNA damaging agents depending upon the inhibitor specificity for the three mammalian DNA ligases. Using computer-aided drug design based on the structure of human DNA ligase I in complex with nicked DNA, a series of small molecule inhibitors of human DNA ligases have been identified (Chen et al., 2008, Zhong et al., 2008). Briefly, an *in silico* data base of about 1.5 million commercially available small molecules was screened for candidates that were predicted to bind to a DNA binding pocket within the DNA binding domain (DBD) of human DNA ligase I. This binding pocket makes key contacts with nicked DNA (Chen et al., 2008). Out of 233 candidate molecules, 192 were assayed for their ability to inhibit human DNA ligase I but not T4 DNA ligase and for their ability to inhibit cell proliferation because human DNA ligase I is the major replicative DNA ligase. The *in vitro* DNA joining assays identified 10 small molecules that specifically inhibit human DNA ligase I by more than 50% at 100 mM, with 5 of these molecules also inhibiting the proliferation of cultured human cells. Since the amino acid sequences of the DBDs of human DNA ligases III and IV are closely related to that of the human DNA ligase I DBD, Chen et al. enquired whether the inhibitors of human DNA ligase I are also active against the other human DNA ligases. Molecules that inhibit DNA ligase I alone (L82), DNA ligase I and III (L67), and all three human DNA ligases (L189) *in vitro*, that were also active in cell culture assays, were further characterized. In accord with the screening strategy, all the ligase inhibitors with the exception of L82 act as competitive inhibitors with respect to nicked DNA. The structure of L67 and the other inhibitors consists of heterocyclic rings separated by a flexible linker. Interestingly, L67 and L189 are cytotoxic, whereas L82 is cytostatic. It is possible that this reflects the different mechanisms of inhibition. Alternatively, while inhibition of DNA ligase I alone is not toxic, inhibition of either DNA ligase III or DNA ligase IV alone or in combination with human DNA ligase I may be cytotoxic. Another interesting feature of the ligase inhibitors is that sub-toxic concentrations specifically potentiate the cytotoxicity of DNA-damaging agents in cancer cells (**Figure 4**). As in cell lines expressing BCR-ABL and FLT3/ITD (Sallmyr et al., 2008b, Fan et al., 2010), DNA ligase III α is also overexpressed in cancer cell lines, whereas the levels of DNA ligase IV are reduced compared to a non-cancerous breast epithelial cell line (Chen et al., 2008). Together these results suggests that ligase inhibitors will not only provide a novel approach to delineating the cellular functions of these enzyme, but may also serve as lead compounds for the development of therapeutic agents that target DNA replication and/or repair (Chen et al., 2008).

Notably, these compounds exhibit different specificities for the three human DNA ligases *in vitro* and a subset of these molecules preferentially sensitize cancer cells to DNA alkylating agents and ionizing radiation, suggesting that they may have utility as lead compounds for the development of novel therapeutic agents. Notably, the ligase inhibitors would constitute an extremely versatile group of agents in that, depending on their specificity, they can be used to target a variety of DNA repair pathways that would be chosen based on the DNA damaging agent. For example, a DNA ligase IV specific inhibitor would sensitize cells with a functional C-NHEJ pathway to ionizing radiation whereas a DNA ligase III specific inhibitor would sensitize cancer cells that are dependent upon ALT-NHEJ to ionizing radiation and other agents that cause DSBs, such as PARP inhibitors (**Figure 4**).

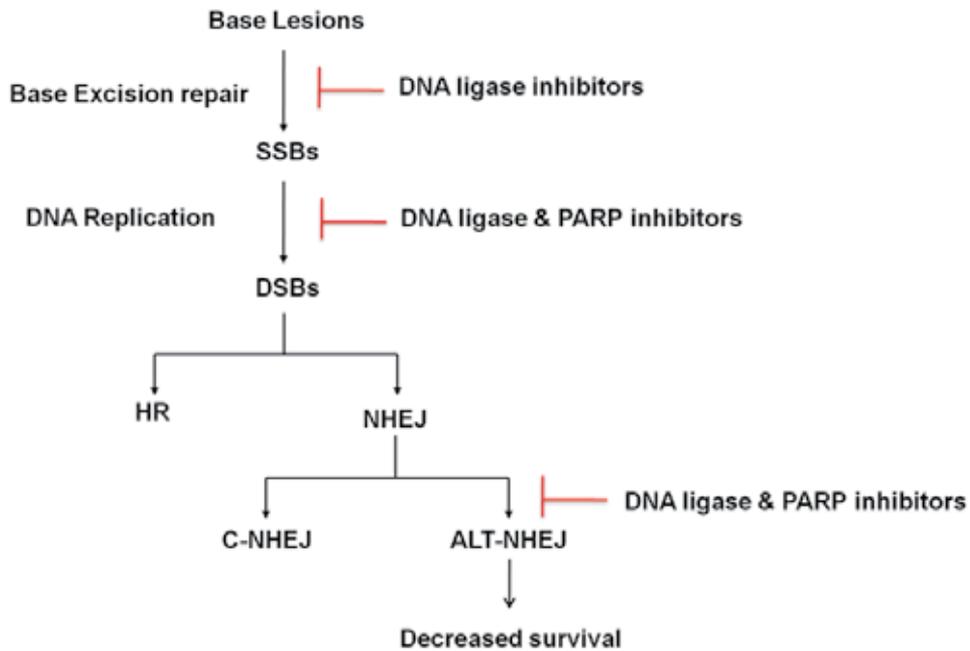


Fig. 4. Schematic of the effect of DNA ligase and PARP inhibitors on base excision repair, DNA replication and ALT-NHEJ in cancer and leukemia cells.

3. Conclusion

ALT-NHEJ is involved in the development of a variety of cancers, including leukemias, and is likely to play a key role in the generation of chromosomal abnormalities, including translocations, that drive cancer progression. It appears that cancer cells are more dependent on ALT-NHEJ for the repair of DSBs and survival, compared with normal cells. Thus targeting this pathway may be an attractive therapeutic strategy. Elucidation of the pathways components, and how they are regulated, will further guide the design of these therapies. Finally, investigation of the molecular mechanisms underlying abnormal DNA damage and repair in cancers and leukemias, together with the development of new animal models, will better our understanding of the complex relations between DNA repair and neoplastic transformation, which will provide new targets for the treatment of cancer.

4. References

- Ahnesorg, P., Smith, P. & Jackson, S.P. (2006). XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell*, 124, 2, 301-13.
- Ame, J.C., Spenlehauer, C. & de Murcia, G. (2004). The PARP superfamily. *Bioessays*, 26, 8, 882-93.
- Anderson, L., Henderson, C. & Adachi, Y. (2001). Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Molecular and cellular biology*, 21, 5, 1719-29.

- Araki, R., Fujimori, A., Hamatani, K., Mita, K., Saito, T., Mori, M., Fukumura, R., Morimyo, M., Muto, M., Itoh, M., Tatsumi, K. & Abe, M. (1997). Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice. *Proc Natl Acad Sci U S A*, 94, 6, 2438-43.
- Audebert, M., Salles, B. & Calsou, P. (2004). Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J Biol Chem*, 279, 53, 55117-26.
- Barnes, D.E., Stamp, G., Rosewell, I., Denzel, A. & Lindahl, T. (1998). Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. *Curr Biol*, 8, 25, 1395-8.
- Bassing, C.H., Swat, W. & Alt, F.W. (2002). The mechanism and regulation of chromosomal V(D)J recombination. *Cell*, 109 Suppl, S45-55.
- Bennardo, N., Cheng, A., Huang, N. & Stark, J.M. (2008). Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair. *PLoS Genet*, 4, 6, e1000110.
- Bennardo, N. & Stark, J.M. (2010). ATM limits incorrect end utilization during non-homologous end joining of multiple chromosome breaks. *PLoS genetics*, 6, 11, e1001194.
- Blunt, T., Gell, D., Fox, M., Taccioli, G.E., Lehmann, A.R., Jackson, S.P. & Jeggo, P.A. (1996). Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. *Proc Natl Acad Sci U S A*, 93, 19, 10285-90.
- Boboila, C., Jankovic, M., Yan, C.T., Wang, J.H., Wesemann, D.R., Zhang, T., Fazeli, A., Feldman, L., Nussenzweig, A., Nussenzweig, M. & Alt, F.W. (2010a). Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70. *Proc Natl Acad Sci U S A*, 107, 7, 3034-9.
- Boboila, C., Yan, C., Wesemann, D.R., Jankovic, M., Wang, J.H., Manis, J., Nussenzweig, A., Nussenzweig, M. & Alt, F.W. (2010b). Alternative end-joining catalyzes class switch recombination in the absence of both Ku70 and DNA ligase 4. *The Journal of experimental medicine*, 207, 2, 417-27.
- Bosma, M.J. & Carroll, A.M. (1991). The SCID mouse mutant: definition, characterization, and potential uses. *Annu Rev Immunol*, 9, 323-50.
- Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J. & Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, 434, 7035, 913-7.
- Buck, D., Malivert, L., de Chasseval, R., Barraud, A., Fondaneche, M.C., Sanal, O., Plebani, A., Stephan, J.L., Hufnagel, M., le Deist, F., Fischer, A., Durandy, A., de Villartay, J.P. & Revy, P. (2006). Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*, 124, 2, 287-99.
- Calsou, P., Frit, P., Humbert, O., Muller, C., Chen, D.J. & Salles, B. (1999). The DNA-dependent protein kinase catalytic activity regulates DNA end processing by means of Ku entry into DNA. *J Biol Chem*, 274, 12, 7848-56.
- Canning, S. & Dryja, T.P. (1989). Short, direct repeats at the breakpoints of deletions of the retinoblastoma gene. *Proc Natl Acad Sci U S A*, 86, 13, 5044-8.

- Celli, G.B., Denchi, E.L. & de Lange, T. (2006). Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. *Nat Cell Biol*, 8, 8, 885-90.
- Chappell, C., Hanakahi, L.A., Karimi-Busheri, F., Weinfeld, M. & West, S.C. (2002). Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining. *Embo J*, 21, 11, 2827-32.
- Chen, L., Trujillo, K., Sung, P. & Tomkinson, A.E. (2000). Interactions of the DNA ligase IV-XRCC4 complex with DNA ends and the DNA-dependent protein kinase. *J Biol Chem*, 275, 34, 26196-205.
- Chen, X., Zhong, S., Zhu, X., Dziegielewska, B., Ellenberger, T., Wilson, G.M., MacKerell, A.D., Jr. & Tomkinson, A.E. (2008). Rational design of human DNA ligase inhibitors that target cellular DNA replication and repair. *Cancer Res*, 68, 9, 3169-77.
- Cheng, Q., Barboulet, N., Frit, P., Gomez, D., Bombarde, O., Couderc, B., Ren, G. S., Salles, B. & Calsou, P. (2011). Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. *Nucleic acids research*, Epub ahead of print
- Corneo, B., Wendland, R.L., Deriano, L., Cui, X., Klein, I.A., Wong, S.Y., Arnal, S., Holub, A.J., Weller, G.R., Pancake, B.A., Shah, S., Brandt, V.L., Meek, K. & Roth, D.B. (2007). Rag mutations reveal robust alternative end joining. *Nature*, 449, 7161, 483-6.
- Dai, Y., Kysela, B., Hanakahi, L.A., Manolis, K., Riballo, E., Stumm, M., Harville, T.O., West, S.C., Oettinger, M.A. & Jeggo, P.A. (2003). Nonhomologous end joining and V(D)J recombination require an additional factor. *Proc Natl Acad Sci U S A*, 100, 5, 2462-7.
- de Villartay, J.P., Poinsignon, C., de Chasseval, R., Buck, D., Le Guyader, G. & Villey, I. (2003). Human and animal models of V(D)J recombination deficiency. *Curr Opin Immunol*, 15, 5, 592-8.
- DeFazio, L.G., Stansel, R.M., Griffith, J.D. & Chu, G. (2002). Synapsis of DNA ends by DNA-dependent protein kinase. *Embo J*, 21, 12, 3192-200.
- Deng, Y., Guo, X., Ferguson, D.O. & Chang, S. (2009). Multiple roles for MRE11 at uncapped telomeres. *Nature*, 460, 7257, 914-8.
- Deriano, L., Stracker, T.H., Baker, A., Petrini, J.H. & Roth, D.B. (2009). Roles for NBS1 in alternative nonhomologous end-joining of V(D)J recombination intermediates. *Mol Cell*, 34, 1, 13-25.
- Difilippantonio, M.J., Zhu, J., Chen, H.T., Meffre, E., Nussenzweig, M.C., Max, E.E., Ried, T. & Nussenzweig, A. (2000). DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature*, 404, 6777, 510-4.
- Dinkelmann, M., Spehalski, E., Stoneham, T., Buis, J., Wu, Y., Sekiguchi, J.M. & Ferguson, D.O. (2009). Multiple functions of MRN in end-joining pathways during isotype class switching. *Nat Struct Mol Biol*, 16, 8, 808-13.
- Dryja, T.P., Mukai, S., Petersen, R., Rapaport, J.M., Walton, D. & Yandell, D.W. (1989). Parental origin of mutations of the retinoblastoma gene. *Nature*, 339, 6225, 556-8.
- Evers, B., Drost, R., Schut, E., de Bruin, M., van der Burg, E., Derksen, P.W., Holstege, H., Liu, X., van Drunen, E., Beverloo, H.B., Smith, G.C., Martin, N.M., Lau, A., O'Connor, M.J. & Jonkers, J. (2008). Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. *Clin Cancer Res*, 14, 12, 3916-25.

- Falzon, M., Fewell, J.W. & Kuff, E.L. (1993). EBP-80, a transcription factor closely resembling the human autoantigen Ku, recognizes single- to double-strand transitions in DNA. *J Biol Chem*, 268, 14, 10546-52.
- Fan, J., Li, L., Small, D. & Rassool, F. (2010). Cells expressing FLT3/ITD mutations exhibit elevated repair errors generated through alternative NHEJ pathways: implications for genomic instability and therapy. *Blood*, 116, 24, 5298-305.
- Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., Martin, N.M., Jackson, S.P., Smith, G.C. & Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, 434, 7035, 917-21.
- Fattah, F., Lee, E.H., Weisensel, N., Wang, Y., Lichter, N. & Hendrickson, E.A. (2010). Ku regulates the non-homologous end joining pathway choice of DNA double-strand break repair in human somatic cells. *PLoS Genet*, 6, 2, e1000855.
- Felix, C.A., Lange, B.J. & Chessells, J.M. (2000). Pediatric Acute Lymphoblastic Leukemia: Challenges and Controversies in 2000. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, 285-302.
- Ferguson, D.O. & Alt, F.W. (2001). DNA double strand break repair and chromosomal translocation: lessons from animal models. *Oncogene*, 20, 40, 5572-9.
- Franco, S., Murphy, M.M., Li, G., Borjeson, T., Boboila, C. & Alt, F.W. (2008). DNA-PKcs and Artemis function in the end-joining phase of immunoglobulin heavy chain class switch recombination. *The Journal of experimental medicine*, 205, 3, 557-64.
- Frank, K.M., Sekiguchi, J.M., Seidl, K.J., Swat, W., Rathbun, G.A., Cheng, H.L., Davidson, L., Kangaloo, L. & Alt, F.W. (1998). Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. *Nature*, 396, 6707, 173-7.
- Frank, K.M., Sharpless, N.E., Gao, Y., Sekiguchi, J.M., Ferguson, D.O., Zhu, C., Manis, J.P., Horner, J., DePinho, R.A. & Alt, F.W. (2000). DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. *Mol Cell*, 5, 6, 993-1002.
- Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y. & et al. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. *Science*, 266, 5182, 120-2.
- Galande, S. & Kohwi-Shigematsu, T. (1999). Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. *The Journal of biological chemistry*, 274, 29, 20521-8.
- Gale, K.B., Ford, A.M., Repp, R., Borkhardt, A., Keller, C., Eden, O.B. & Greaves, M.F. (1997). Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 25, 13950-4.
- Gao, Y., Chaudhuri, J., Zhu, C., Davidson, L., Weaver, D.T. & Alt, F.W. (1998). A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. *Immunity*, 9, 3, 367-76.
- Gao, Y., Ferguson, D.O., Xie, W., Manis, J.P., Sekiguchi, J., Frank, K.M., Chaudhuri, J., Horner, J., DePinho, R.A. & Alt, F.W. (2000). Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature*, 404, 6780, 897-900.

- Gaymes, T.J., Mufti, G.J. & Rassool, F.V. (2002). Myeloid leukemias have increased activity of the nonhomologous end-joining pathway and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer. *Cancer Res*, 62, 10, 2791-7.
- Gillert, E., Leis, T., Repp, R., Reichel, M., Hosch, A., Breitenlohner, I., Angermuller, S., Borkhardt, A., Harbott, J., Lampert, F., Griesinger, F., Greil, J., Fey, G.H. & Marschalek, R. (1999). A DNA damage repair mechanism is involved in the origin of chromosomal translocations t(4;11) in primary leukemic cells. *Oncogene*, 18, 33, 4663-71.
- Gilliland, D.G., Jordan, C.T. & Felix, C.A. (2004). The molecular basis of leukemia. *Hematology (Am Soc Hematol Educ Program)*, 80-97.
- Goodarzi, A.A., Noon, A.T., Deckbar, D., Ziv, Y., Shiloh, Y., Lobrich, M. & Jeggo, P.A. (2008). ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol Cell*, 31, 2, 167-77.
- Gottlich, B., Reichenberger, S., Feldmann, E. & Pfeiffer, P. (1998). Rejoining of DNA double-strand breaks in vitro by single-strand annealing. *Eur J Biochem*, 258, 2, 387-95.
- Gottlieb, T.M. & Jackson, S.P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell*, 72, 1, 131-42.
- Greaves, M.F. & Wiemels, J. (2003). Origins of chromosome translocations in childhood leukaemia. *Nat Rev Cancer*, 3, 9, 639-49.
- Gu, Y., Seidl, K.J., Rathbun, G.A., Zhu, C., Manis, J.P., van der Stoep, N., Davidson, L., Cheng, H.L., Sekiguchi, J.M., Frank, K., Stanhope-Baker, P., Schlissel, M.S., Roth, D.B. & Alt, F.W. (1997). Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity*, 7, 5, 653-65.
- Guha, M. (2011). PARP inhibitors stumble in breast cancer. *Nat Biotechnol*, 29, 5, 373-4.
- Guidos, C.J., Williams, C.J., Grandal, I., Knowles, G., Huang, M.T. & Danska, J.S. (1996). V(D)J recombination activates a p53-dependent DNA damage checkpoint in scid lymphocyte precursors. *Genes Dev*, 10, 16, 2038-54.
- Haber, J.E. (2008). Alternative endings. *Proc Natl Acad Sci U S A*, 105, 2, 405-6.
- Han, L. & Yu, K. (2008). Altered kinetics of nonhomologous end joining and class switch recombination in ligase IV-deficient B cells. *J Exp Med*, 205, 12, 2745-53.
- Hartlerode, A.J. & Scully, R. (2009). Mechanisms of double-strand break repair in somatic mammalian cells. *Biochem J*, 423, 2, 157-68.
- Helleday, T., Lo, J., van Gent, D.C. & Engelward, B.P. (2007). DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA repair*, 6, 7, 923-35.
- Hohegger, H., Dejsuphong, D., Fukushima, T., Morrison, C., Sonoda, E., Schreiber, V., Zhao, G.Y., Saberi, A., Masutani, M., Adachi, N., Koyama, H., de Murcia, G. & Takeda, S. (2006). Parp-1 protects homologous recombination from interference by Ku and Ligase IV in vertebrate cells. *EMBO J*, 25, 6, 1305-14.
- Huertas, P. (2010). DNA resection in eukaryotes: deciding how to fix the break. *Nat Struct Mol Biol*, 17, 1, 11-6.
- Karanjawala, Z.E., Grawunder, U., Hsieh, C.L. & Lieber, M.R. (1999). The nonhomologous DNA end joining pathway is important for chromosome stability in primary fibroblasts. *Curr Biol*, 9, 24, 1501-4.
- Keller, K.L., Overbeck-Carrick, T.L. & Beck, D.J. (2001). Survival and induction of SOS in *Escherichia coli* treated with cisplatin, UV-irradiation, or mitomycin C are

- dependent on the function of the RecBC and RecFOR pathways of homologous recombination. *Mutat Res*, 486, 1, 21-9.
- Khanna, K.K. & Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet*, 27, 3, 247-54.
- Kurimasa, A., Ouyang, H., Dong, L.J., Wang, S., Li, X., Cordon-Cardo, C., Chen, D.J. & Li, G.C. (1999). Catalytic subunit of DNA-dependent protein kinase: impact on lymphocyte development and tumorigenesis. *Proc Natl Acad Sci U S A*, 96, 4, 1403-8.
- Lee-Theilen, M., Matthews, A.J., Kelly, D., Zheng, S. & Chaudhuri, J. (2011). CtIP promotes microhomology-mediated alternative end joining during class-switch recombination. *Nature structural & molecular biology*, 18, 1, 75-9.
- Lees-Miller, S.P., Chen, Y.R. & Anderson, C.W. (1990). Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. *Mol Cell Biol*, 10, 12, 6472-81.
- Li, G., Alt, F.W., Cheng, H.L., Brush, J.W., Goff, P.H., Murphy, M.M., Franco, S., Zhang, Y. & Zha, S. (2008). Lymphocyte-specific compensation for XLF/cernunnos end-joining functions in V(D)J recombination. *Mol Cell*, 31, 5, 631-40.
- Li, L., Zhang, L., Fan, J., Greenberg, K., Desiderio, S., Rassool, F.V. & Small, D. (2011). Defective nonhomologous end joining blocks B-cell development in FLT3/ITD mice. *Blood*, 117, 11, 3131-9.
- Lieber, M.R. (2008). The mechanism of human nonhomologous DNA end joining. *J Biol Chem*, 283, 1, 1-5.
- Lieber, M.R. (2010). NHEJ and its backup pathways in chromosomal translocations. *Nat Struct Mol Biol*, 17, 4, 393-5.
- Lieber, M.R., Ma, Y., Pannicke, U. & Schwarz, K. (2003). Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol*, 4, 9, 712-20.
- Lieber, M.R., Yu, K. & Raghavan, S.C. (2006). Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. *DNA Repair (Amst)*, 5, 9-10, 1234-45.
- Liu, S.K., Coackley, C., Krause, M., Jalali, F., Chan, N. & Bristow, R.G. (2008). A novel poly(ADP-ribose) polymerase inhibitor, ABT-888, radiosensitizes malignant human cell lines under hypoxia. *Radiother Oncol*, 88, 2, 258-68.
- Lobrich, M. & Jeggo, P.A. (2005). The two edges of the ATM sword: co-operation between repair and checkpoint functions. *Radiother Oncol*, 76, 2, 112-8.
- Lord, C.J. & Ashworth, A. (2008). Targeted therapy for cancer using PARP inhibitors. *Curr Opin Pharmacol*, 8, 4, 363-9.
- Ma, Y., Lu, H., Tippin, B., Goodman, M.F., Shimazaki, N., Koiwai, O., Hsieh, C.L., Schwarz, K. & Lieber, M.R. (2004). A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol Cell*, 16, 5, 701-13.
- Ma, Y., Pannicke, U., Schwarz, K. & Lieber, M.R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell*, 108, 6, 781-94.
- Manis, J.P., Tian, M. & Alt, F.W. (2002). Mechanism and control of class-switch recombination. *Trends Immunol*, 23, 1, 31-9.

- McBlane, J.F., van Gent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M. & Oettinger, M.A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*, 83, 3, 387-95.
- Menissier de Murcia, J., Ricoul, M., Tartier, L., Niedergang, C., Huber, A., Dantzer, F., Schreiber, V., Ame, J.C., Dierich, A., LeMeur, M., Sabatier, L., Chambon, P. & de Murcia, G. (2003). Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *Embo J*, 22, 9, 2255-63.
- Mimori, T. & Hardin, J.A. (1986). Mechanism of interaction between Ku protein and DNA. *J Biol Chem*, 261, 22, 10375-9.
- Mladenov, E. & Iliakis, G. (2011). Induction and repair of DNA double strand breaks: The increasing spectrum of non-homologous end joining pathways. *Mutation research*, 711, 1-2, 61-72.
- Moreno-Herrero, F., Holtzer, L., Koster, D.A., Shuman, S., Dekker, C. & Dekker, N.H. (2005). Atomic force microscopy shows that vaccinia topoisomerase IB generates filaments on DNA in a cooperative fashion. *Nucleic acids research*, 33, 18, 5945-53.
- Morris, T. & Thacker, J. (1993). Formation of large deletions by illegitimate recombination in the HPRT gene of primary human fibroblasts. *Proc Natl Acad Sci U S A*, 90, 4, 1392-6.
- Moshous, D., Pannetier, C., Chasseval Rd, R., Deist Fl, F., Cavazzana-Calvo, M., Romana, S., Macintyre, E., Canioni, D., Brousse, N., Fischer, A., Casanova, J.L. & Villartay, J.P. (2003). Partial T and B lymphocyte immunodeficiency and predisposition to lymphoma in patients with hypomorphic mutations in Artemis. *J Clin Invest*, 111, 3, 381-7.
- Nacht, M., Strasser, A., Chan, Y.R., Harris, A.W., Schlissel, M., Bronson, R.T. & Jacks, T. (1996). Mutations in the p53 and SCID genes cooperate in tumorigenesis. *Genes Dev*, 10, 16, 2055-66.
- Nohmi, T., Suzuki, M., Masumura, K., Yamada, M., Matsui, K., Ueda, O., Suzuki, H., Katoh, M., Ikeda, H. & Sofuni, T. (1999). Spi(-) selection: An efficient method to detect gamma-ray-induced deletions in transgenic mice. *Environ Mol Mutagen*, 34, 1, 9-15.
- Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzweig, M.C. & Li, G.C. (1996). Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature*, 382, 6591, 551-5.
- Nussenzweig, A. & Nussenzweig, M.C. (2007). A backup DNA repair pathway moves to the forefront. *Cell*, 131, 2, 223-5.
- Oettinger, M.A., Schatz, D.G., Gorka, C. & Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science*, 248, 4962, 1517-23.
- Okano, S., Lan, L., Caldecott, K.W., Mori, T. & Yasui, A. (2003). Spatial and temporal cellular responses to single-strand breaks in human cells. *Mol Cell Biol*, 23, 11, 3974-81.
- Ouyang, H., Nussenzweig, A., Kurimasa, A., Soares, V.C., Li, X., Cordon-Cardo, C., Li, W., Cheong, N., Nussenzweig, M., Iliakis, G., Chen, D.J. & Li, G.C. (1997). Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination In vivo. *J Exp Med*, 186, 6, 921-9.
- Palm, W., Hockemeyer, D., Kibe, T. & de Lange, T. (2009). Functional dissection of human and mouse POT1 proteins. *Mol Cell Biol*, 29, 2, 471-82.
- Patel, A.G., Sarkaria, J.N. & Kaufmann, S.H. (2011). Nonhomologous end joining drives poly(ADP-ribose) polymerase (PARP) inhibitor lethality in homologous

- recombination-deficient cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 8, 3406-11.
- Petersen, S., Casellas, R., Reina-San-Martin, B., Chen, H.T., Difilippantonio, M.J., Wilson, P.C., Hanitsch, L., Celeste, A., Muramatsu, M., Pilch, D.R., Redon, C., Ried, T., Bonner, W.M., Honjo, T., Nussenzweig, M.C. & Nussenzweig, A. (2001). AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature*, 414, 6864, 660-5.
- Raffini, L.J., Slater, D.J., Rappaport, E.F., Lo Nigro, L., Cheung, N.K., Biegel, J.A., Nowell, P.C., Lange, B.J. & Felix, C.A. (2002). Panhandle and reverse-panhandle PCR enable cloning of der(11) and der(other) genomic breakpoint junctions of MLL translocations and identify complex translocation of MLL, AF-4, and CDK6. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 7, 4568-73.
- Rai, R., Zheng, H., He, H., Luo, Y., Multani, A., Carpenter, P.B. & Chang, S. (2010). The function of classical and alternative non-homologous end-joining pathways in the fusion of dysfunctional telomeres. *EMBO J*, 29, 15, 2598-610.
- Rass, E., Grabarz, A., Plo, I., Gautier, J., Bertrand, P. & Lopez, B.S. (2009). Role of Mre11 in chromosomal nonhomologous end joining in mammalian cells. *Nat Struct Mol Biol*, 16, 8, 819-24.
- Rassool, F.V. (2003). DNA double strand breaks (DSB) and non-homologous end joining (NHEJ) pathways in human leukemia. *Cancer Lett*, 193, 1, 1-9.
- Rassool, F.V. & Tomkinson, A.E. (2010). Targeting abnormal DNA double strand break repair in cancer. *Cell Mol Life Sci*, 67, 21, 3699-710.
- Reichel, M., Gillert, E., Nilson, I., Siegler, G., Greil, J., Fey, G.H. & Marschalek, R. (1998). Fine structure of translocation breakpoints in leukemic blasts with chromosomal translocation t(4;11): the DNA damage-repair model of translocation. *Oncogene*, 17, 23, 3035-44.
- Reiter, A., Saussele, S., Grimwade, D., Wiemels, J.L., Segal, M.R., Lafage-Pochitaloff, M., Walz, C., Weisser, A., Hochhaus, A., Willer, A., Reichert, A., Buchner, T., Lengfelder, E., Hehlmann, R. & Cross, N.C. (2003). Genomic anatomy of the specific reciprocal translocation t(15;17) in acute promyelocytic leukemia. *Genes Chromosomes Cancer*, 36, 2, 175-88.
- Revy, P., Buck, D., le Deist, F. & de Villartay, J.P. (2005). The repair of DNA damages/modifications during the maturation of the immune system: lessons from human primary immunodeficiency disorders and animal models. *Adv Immunol*, 87, 237-95.
- Riballo, E., Critchlow, S.E., Teo, S.H., Doherty, A.J., Priestley, A., Broughton, B., Kysela, B., Beamish, H., Plowman, N., Arlett, C.F., Lehmann, A.R., Jackson, S.P. & Jeggo, P.A. (1999). Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. *Curr Biol*, 9, 13, 699-702.
- Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G.C., Recio, M.J., Reis, C., Dahm, K., Fricke, A., Krempler, A., Parker, A.R., Jackson, S.P., Gennery, A., Jeggo, P.A. & Lobrich, M. (2004). A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell*, 16, 5, 715-24.

- Robert, I., Dantzer, F. & Reina-San-Martin, B. (2009). Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. *J Exp Med*, 206, 5, 1047-56.
- Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. & Bonner, W.M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*, 273, 10, 5858-68.
- Rooney, S., Alt, F.W., Lombard, D., Whitlow, S., Eckersdorff, M., Fleming, J., Fugmann, S., Ferguson, D.O., Schatz, D.G. & Sekiguchi, J. (2003). Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. *J Exp Med*, 197, 5, 553-65.
- Rooney, S., Sekiguchi, J., Whitlow, S., Eckersdorff, M., Manis, J.P., Lee, C., Ferguson, D.O. & Alt, F.W. (2004). Artemis and p53 cooperate to suppress oncogenic N-myc amplification in progenitor B cells. *Proc Natl Acad Sci U S A*, 101, 8, 2410-5.
- Rooney, S., Sekiguchi, J., Zhu, C., Cheng, H.L., Manis, J., Whitlow, S., DeVido, J., Foy, D., Chaudhuri, J., Lombard, D. & Alt, F.W. (2002). Leaky Scid phenotype associated with defective V(D)J coding end processing in Artemis-deficient mice. *Mol Cell*, 10, 6, 1379-90.
- Roth, D.B., Porter, T.N. & Wilson, J.H. (1985). Mechanisms of nonhomologous recombination in mammalian cells. *Mol Cell Biol*, 5, 10, 2599-607.
- Roth, D.B. & Wilson, J.H. (1986). Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol*, 6, 12, 4295-304.
- Sallmyr, A., Fan, J., Datta, K., Kim, K.T., Grosu, D., Shapiro, P., Small, D. & Rassool, F. (2008a). Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML. *Blood*, 111, 6, 3173-82.
- Sallmyr, A., Tomkinson, A.E. & Rassool, F.V. (2008b). Up-regulation of WRN and DNA ligase IIIalpha in chronic myeloid leukemia: consequences for the repair of DNA double-strand breaks. *Blood*, 112, 4, 1413-23.
- Schultz, L.B., Chehab, N.H., Malikzay, A. & Halazonetis, T.D. (2000). p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *The Journal of cell biology*, 151, 7, 1381-90.
- Shibata, A., Conrad, S., Birraux, J., Geuting, V., Barton, O., Ismail, A., Kakarougkas, A., Meek, K., Taucher-Scholz, G., Lobrich, M. & Jeggo, P.A. (2011). Factors determining DNA double-strand break repair pathway choice in G2 phase. *EMBO J*, 30, 6, 1079-92.
- Simsek, D. & Jasin, M. (2010). Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. *Nat Struct Mol Biol*, 17, 4, 410-6.
- Singleton, B.K., Torres-Arzayus, M.I., Rottinghaus, S.T., Taccioli, G.E. & Jeggo, P.A. (1999). The C terminus of Ku80 activates the DNA-dependent protein kinase catalytic subunit. *Mol Cell Biol*, 19, 5, 3267-77.
- Smanik, P.A., Furminger, T.L., Mazzaferrri, E.L. & Jhiang, S.M. (1995). Breakpoint characterization of the ret/PTC oncogene in human papillary thyroid carcinoma. *Hum Mol Genet*, 4, 12, 2313-8.
- Soulas-Sprauel, P., Le Guyader, G., Rivera-Munoz, P., Abramowski, V., Olivier-Martin, C., Goujet-Zalc, C., Charneau, P. & de Villartay, J.P. (2007a). Role for DNA repair

- factor XRCC4 in immunoglobulin class switch recombination. *J Exp Med*, 204, 7, 1717-27.
- Soulas-Sprauel, P., Rivera-Munoz, P., Malivert, L., Le Guyader, G., Abramowski, V., Revy, P. & de Villartay, J.P. (2007b). V(D)J and immunoglobulin class switch recombinations: a paradigm to study the regulation of DNA end-joining. *Oncogene*, 26, 56, 7780-91.
- Strissel, P.L., Strick, R., Tomek, R.J., Roe, B.A., Rowley, J.D. & Zeleznik-Le, N.J. (2000). DNA structural properties of AF9 are similar to MLL and could act as recombination hot spots resulting in MLL/AF9 translocations and leukemogenesis. *Hum Mol Genet*, 9, 11, 1671-9.
- Taccioli, G.E., Amatucci, A.G., Beamish, H.J., Gell, D., Xiang, X.H., Torres Arzayus, M.I., Priestley, A., Jackson, S.P., Marshak Rothstein, A., Jeggo, P.A. & Herrera, V.L. (1998). Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. *Immunity*, 9, 3, 355-66.
- Tentori, L., Leonetti, C., Scarsella, M., d'Amati, G., Portarena, I., Zupi, G., Bonmassar, E. & Graziani, G. (2002). Combined treatment with temozolomide and poly(ADP-ribose) polymerase inhibitor enhances survival of mice bearing hematologic malignancy at the central nervous system site. *Blood*, 99, 6, 2241-4.
- Tutt, A., Bertwistle, D., Valentine, J., Gabriel, A., Swift, S., Ross, G., Griffin, C., Thacker, J. & Ashworth, A. (2001). Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. *Embo J*, 20, 17, 4704-16.
- Venkitaraman, A.R. (2001). Chromosome stability, DNA recombination and the BRCA2 tumour suppressor. *Curr Opin Cell Biol*, 13, 3, 338-43.
- Walker, J.R., Corpina, R.A. & Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature*, 412, 6847, 607-14.
- Wang, H., Perrault, A.R., Takeda, Y., Qin, W. & Iliakis, G. (2003). Biochemical evidence for Ku-independent backup pathways of NHEJ. *Nucleic Acids Res*, 31, 18, 5377-88.
- Wang, H., Rosidi, B., Perrault, R., Wang, M., Zhang, L., Windhofer, F. & Iliakis, G. (2005). DNA ligase III as a candidate component of backup pathways of nonhomologous end joining. *Cancer research*, 65, 10, 4020-30.
- Wang, M., Wu, W., Rosidi, B., Zhang, L., Wang, H. & Iliakis, G. (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res*, 34, 21, 6170-82.
- Wiemels, J.L. & Greaves, M. (1999). Structure and possible mechanisms of TEL-AML1 gene fusions in childhood acute lymphoblastic leukemia. *Cancer Res*, 59, 16, 4075-82.
- Wu, W., Wang, M., Mussfeldt, T. & Iliakis, G. (2008). Enhanced use of backup pathways of NHEJ in G2 in Chinese hamster mutant cells with defects in the classical pathway of NHEJ. *Radiat Res*, 170, 4, 512-20.
- Wu, X., Wilson, T.E. & Lieber, M.R. (1999). A role for FEN-1 in nonhomologous DNA end joining: the order of strand annealing and nucleolytic processing events. *Proc Natl Acad Sci U S A*, 96, 4, 1303-8.
- Xiao, Z., Greaves, M.F., Buffler, P., Smith, M.T., Segal, M.R., Dicks, B.M., Wiencke, J.K. & Wiemels, J.L. (2001). Molecular characterization of genomic AML1-ETO fusions in childhood leukemia. *Leukemia*, 15, 12, 1906-13.

- Xie, A., Kwok, A. & Scully, R. (2009). Role of mammalian Mre11 in classical and alternative nonhomologous end joining. *Nat Struct Mol Biol*, 16, 8, 814-8.
- Yan, C.T., Boboila, C., Souza, E.K., Franco, S., Hickernell, T.R., Murphy, M., Gumaste, S., Geyer, M., Zarrin, A.A., Manis, J.P., Rajewsky, K. & Alt, F.W. (2007). IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature*, 449, 7161, 478-82.
- Yaneva, M., Kowalewski, T. & Lieber, M.R. (1997). Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. *Embo J*, 16, 16, 5098-112.
- Zelent, A., Greaves, M. & Enver, T. (2004). Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. *Oncogene*, 23, 24, 4275-83.
- Zha, S., Guo, C., Boboila, C., Oksenysh, V., Cheng, H.L., Zhang, Y., Wesemann, D.R., Yuen, G., Patel, H., Goff, P.H., Dubois, R.L. & Alt, F.W. (2011). ATM damage response and XLF repair factor are functionally redundant in joining DNA breaks. *Nature*, 469, 7329, 250-4.
- Zhang, Y. & Jasin, M. (2011). An essential role for CtIP in chromosomal translocation formation through an alternative end-joining pathway. *Nat Struct Mol Biol*, 18, 1, 80-4.
- Zhang, Y. & Rowley, J.D. (2006). Chromatin structural elements and chromosomal translocations in leukemia. *DNA Repair (Amst)*, 5, 9-10, 1282-97.
- Zhong, S., Chen, X., Zhu, X., Dziegielewska, B., Bachman, K.E., Ellenberger, T., Ballin, J.D., Wilson, G.M., Tomkinson, A.E. & Mackerell, A.D., Jr. (2008). Identification and Validation of Human DNA Ligase Inhibitors Using Computer-Aided Drug Design. *J Med Chem*.
- Zhu, C., Bogue, M.A., Lim, D.S., Hasty, P. & Roth, D.B. (1996). Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell*, 86, 3, 379-89.
- Zhu, C., Mills, K.D., Ferguson, D.O., Lee, C., Manis, J., Fleming, J., Gao, Y., Morton, C.C. & Alt, F.W. (2002). Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. *Cell*, 109, 7, 811-21.

Nucleotide Excision Repair and Cancer

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1. Introduction

Cancer ranks as one of the most frequent causes of death worldwide and in Western society it is competing with cardiovascular disease as the number one killer. This high frequency in Western countries can be attributed to lifestyle and environmental factors, only 5-10% of all cancers are directly due to heredity. Common environmental factors leading to cancer include: tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation, lack of physical activity and environmental pollutants or chemicals (Anand et al.,2008). Exposure to these environmental factors cause or enhance abnormalities in the genetic material of cells (Kinzler KW et al.,2002). These changes in the DNA or hereditary predisposition can result in respectively uncontrolled cell growth, invasion and metastasis. Cancer cells can damage tissue and disturb homeostasis leading to dysfunctions in the body that can eventually lead to death. Under normal conditions cell growth is under strict conditions and control. Hereditary dysfunctions or introduced DNA damage in tumor suppressor genes, oncogenes or DNA repair genes can create an imbalance that may lead to cancer development. DNA repair and cell cycle arrest pathways are essential cellular mechanisms to prevent or repair substantial DNA damage which, if left unattended, can cause diseases.

Here, one of the most important and versatile DNA repair pathways, the Nucleotide Excision Repair (NER) pathway, will be discussed in relation to DNA damage accumulation and carcinogenesis together with its mechanistic mode of action.

2. DNA damage

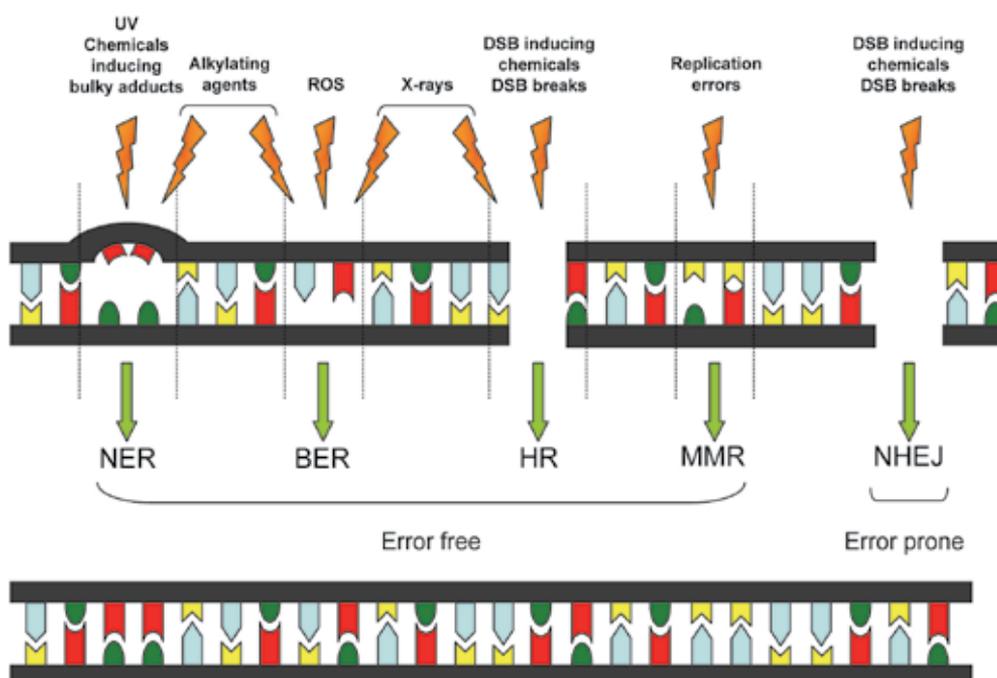
One of the initial steps in cancer development is the accumulation of DNA damage. These genomic assaults are abundant due to environmental factors and continuously ongoing metabolic processes inside the cell (Lodish et al.,2004). Endogenous DNA damage occurs at an estimated frequency of approximately 20,000 - 50,000 lesions per cell per day in humans (Lindahl,1993; Friedberg,1995), which roughly adds up to 10 - 40 trillion lesions per second in the human body. Endogenously generated lesions can occur through metabolic cellular processes and result in hydrolysis (e.g. depurination, depyrimidination and deamination), oxidation (8-oxoG, thymine glycol, cytosine hydrates and lipid peroxidation products) and non-enzymatic methylation of the DNA components (Cadet et al.,2003; Friedberg et al.,2006b). Besides these endogenous insults to the DNA, exogenous factors can play a significant role in

damaging the DNA. Examples of exogenous insults are ionizing radiation, ultraviolet (UV) radiation and exposure to chemical agents. One hour of sunbathing in Europe for example generates around 80,000 lesions per cell in the human skin (Mullaart et al.,1990). The endogenous and exogenous primary lesions can result in persistent DNA damage if left unattended. Therefore, repair pathways and cellular responses are of vital importance in the prevention of cancer and age-related diseases. DNA repair pathways come in many varieties, Figure 1 shows a schematic overview of biological responses to several types of DNA damage. Reversal of DNA damage and excision repair pathways are responsible for the fundamental repair of damaged nucleotides, resulting into the correct nucleotide sequence and DNA structure. Besides damaged nucleotides, cells often sustain fracture of the sugar-phosphate backbone, resulting in single- or double-strand breaks (SSB or DSB) (Friedberg et al.,2006b). Repairing the DNA damage can occur in an error-free (e.g. Nucleotide Excision Repair (NER), Base Excision Repair (BER), Homologous Recombination (HR)) or by an error-prone pathway like Non-Homologous End-Joining (NHEJ). Besides DNA repair pathways, DNA damage tolerance mechanisms are active to bypass lesion that normally block replication like Translesion Synthesis (TLS) or template switching. Template switching occurs in an error-free way, while TLS acts in an often error-prone manner (although a few polymerases of this pathway are able to handle the lesions in an error-free way). Even though error-prone mechanisms do not result in the original coding information they do enhance the chances of cell survival, which is preferred over correct genomic maintenance in these cases. In light of this, cell cycle checkpoint activation and scheduled cell death (apoptosis) also enhance chances of genomic stability and in some cases cell survival. The responses, in which tumor suppressor factor p53 plays a major role, greatly facilitate the efficiency of repair and damage tolerance. Arrested cell cycle progression will result in an increased time window for DNA repair or damage tolerance to occur. In addition, apoptosis will attenuate the risk of genomic instability by programming the cells with extensive DNA damage for cell death, thereby, annulling the possible negative effect of the DNA damage in those cells and hence maintaining homeostasis.

3. Nucleotide excision repair

The abundant targeting of bases and nucleotides in the genome makes the Nucleotide Excision Repair one of the most essential repair pathways. NER can restore the correct genomic information, but also replication and transcription after these types of damage. The pathway can deal with a broad spectrum of (mostly) structurally unrelated bulky DNA lesions, arisen from either endogenous or exogenous agents. NER for example removes DNA lesions from the genome such as photolesions, crosslinks, bulky aromatic hydrocarbon and alkylation adducts (Figure 1).

Nucleotide excision repair is a multistep pathway using over 30 proteins that eliminate the helix-distorting lesions. As mentioned, lesions of this matter can originate upon exposures to several damaging agents. For instance, UV radiation (sunshine) is a physical DNA damaging agent that mainly produces cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone products (6-4PP) but is also believed to induce oxidative DNA damage (Lo et al.,2005). Exposure to numerous chemicals can result in helix-distorting bulky adducts, for example polycyclic aromatic hydrocarbons (present in cigarette smoke or charcoaled meat) (de Boer et al.,2000). Interstrand crosslinks, alkylation adducts and oxygen free-radical induced minor base damage can trigger NER (Friedberg et al.,2006b).



Schematic overview of DNA repair pathways. Several types of induced DNA damage can trigger different repair pathways, which can repair the DNA in an error-free or an error-prone manner. NER (Nucleotide Excision Repair), BER (Base Excision Repair), HR (Homologous Recombination), MMR (Mismatch Repair), NHEJ (Non-Homologous End-Joining).

Fig. 1. DNA Repair pathways.

3.1 Global genome-NER and transcription coupled-NER

NER is divided into two subpathways which mechanistically initiate in a divergent manner, but after damage recognition both pathways proceed along the same molecular route (see Figure 2). The subpathways are designated Global Genome NER (GG-NER) and Transcription Coupled NER (TC-NER). GG-NER recognizes and removes lesions throughout the entire genome, and is considered to be a relatively slow and somewhat more inefficient process, since it scans the whole genome for DNA damage (Guarente et al.,2008). However, UV induced helix-distorting lesions like 6-4PPs, are rapidly cleared by GG-NER (Garinis et al.,2006). TC-NER is responsible for eliminating lesions in the transcribed strand of active genes. This repair process takes care of lesions blocking the transcription machinery and otherwise possible resulting dysfunctions. Since TC-NER is directly coupled to the transcription machinery it is considered to be faster acting and more efficient than GG-NER, but is only initiated when transcription of a gene is blocked.

3.2 DNA damage recognition

The difference between the two sub pathways is the initial damage recognition step (Figure 2). As mentioned previously, a helical distortion and alteration of DNA chemistry appears to be the first structural element that is recognized. For GG-NER, the XPC/hHR23B

complex (including centrin2), together with the UV-Damaged DNA Binding (UV-DDB) protein (assembled by the DDB1 (p127) and DDB2/XPE (p48) subunits), are involved in lesion recognition (Dip et al.,2004). The XPC/hHR23B complex is additionally essential for recruitment of the consecutive components of the NER machinery to the damaged site, also known as the preincision complex (Yokoi et al.,2000; Araujo et al.,2001).

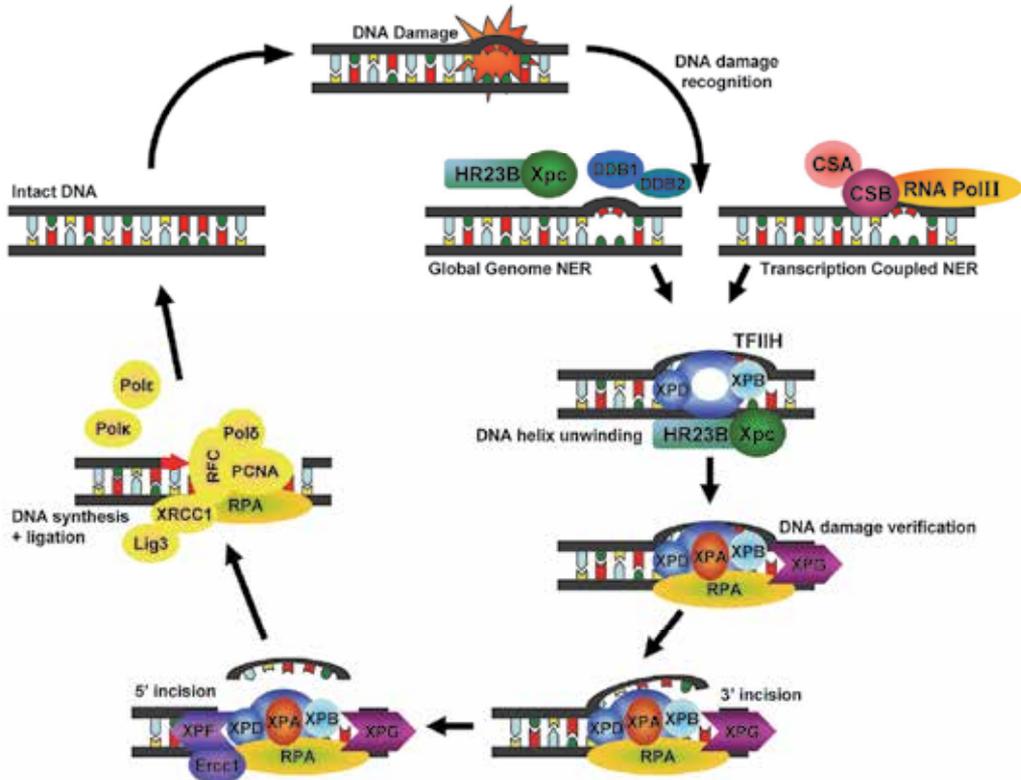


Fig. 2. Nucleotide Excision Repair.

Schematic overview of the Nucleotide Excision Repair (NER) pathway. Damaged DNA is recognized by either initial factors of the Global Genome Repair (e.g. Xpc) or Transcription Coupled Repair (CSA and CSB), which constitute the two different repair pathways in NER. After DNA damage recognition the repair route progresses along the same way. After helix unwinding and verification of the damage incisions are made to remove the faulty stretch of DNA. Finally, DNA synthesis and subsequent ligation reproduce the correct DNA sequence. It has been shown that XPC itself has affinity for DNA and can initiate GG-NER *in vitro*, but its functionality is enhanced when hHR23b and centrin2 are added (Nishi et al.,2005; Araki et al.,2001). The latter two are not able to bind to DNA themselves. Centrin2 as well as hHR23B stabilize the heterotrimer complex, putatively by inhibiting polyubiquitination of XPC and hence preventing subsequent degradation by the 26S proteasome (Nishi et al.,2005). XPC recognizes various helix-distorting base lesions that do not share a common chemical structure. Biochemical studies have revealed that XPC recognizes a specific secondary DNA structure rather than the lesions themselves and the presence of single-

stranded DNA seems a crucial factor (Sugasawa et al.,2001; Sugawawa et al.,2002; Min et al.,2007). XPC appears to scan the DNA for distortions by migrating over the DNA, repeatedly binding and dissociating from the helix. When XPC encounters a lesion the protein changes its conformation and aromatic amino acid residues stack with unpaired nucleotides opposite the lesion, thereby increasing its affinity and creating a conformation which makes it possible to interact with other NER factors (Hoogstraten et al.,2008).

Binding affinity of XPC to the DNA seems to correlate with the extent of helical distortion. 6-4PP products substantially distort the DNA structure and are therefore more easily recognized than CPDs, which only induce a minimal helical distortion (Sugasawa et al.,2005). More recent studies have indicated that UV-DDB facilitates recognition of lesions that are less well-recognized by the XPC-hHR23B complex, like CPDs, via ubiquitylation of XPC (Fitch et al.,2003).

The UV-DDB is able to recognize UV-induced photoproducts in the DNA and is now believed to precede the binding of XPC-hHR23B to the UV-damaged site. CPD repair is UV-DDB dependent (Fitch et al.,2003; Tang et al.,2000). Since affinity of the XPC-hHR23B to CPD sites is low, DDB2 is needed for efficient binding (Tang et al.,2000). Affinity of DDB2 for 6-4PP is also extremely high and the protein is furthermore able to bind to DNA lesions such as apurinic/aprimidinic (AP) sites and mismatches (Nichols et al.,2000; Wittschieben et al.,2005). DDB2 is also part of the E3 ubiquitin ligase complex which is further comprised of CUL4A, ROC1/RBX1, COP9 signalosome (CSN) and DDB1 (Groisman et al.,2003). Live cell imaging studies show prompt recruitment of DDB1, DDB2 and Cul4a to UV induced lesions (Alekseev et al.,2008). CUL4A displays ubiquitin ligase activity and was shown to ubiquitylate DDB2 (Chen et al.,2001; Nag et al.,2001; Matsuda et al.,2005). The CSN subunit contains deubiquitylation capacities. This interactive mechanism is thought to be responsible for (poly)ubiquitylation of XPC and DDB2, but not in a similar fashion and result. Upon ubiquitylation DDB2 is degraded by the 26S proteasome (Fitch et al.,2003; Ropic-Otrin et al.,2002). XPC is not degraded after UV DNA damage, hereby increasing its binding affinity to the DNA as well as stimulating the interaction with hHR23B (Araki et al.,2001; Ortolan et al.,2004; Ng et al.,2003). Degradation of UV-DDB enhances the binding of XPC-hHR23B to the DNA *in vitro* (Sugasawa et al.,2005). Timing of the programmed degradation of DDB2 determines the recruitment of XPC-hHR23B to the UV-damaged site (El Mahdy et al.,2006).

The XPC protein contains several binding domains, a DNA binding domain, a hHR23B binding domain, centrin2 binding domain and a TFIIH binding domain (Sugasawa,2008). TFIIH is a multifunctional transcription factor and NER complex and amongst others contains the helicases XPB and XPD (Figure 2). This complex is essential for the continuation of the NER pathway and is responsible for unwinding the DNA helix after damage recognition by XPC/hHR23B. XPC has been shown to physically interact with TFIIH and *in vivo* and *in vitro* studies have shown the recruitment of the NER complex to unwind the DNA is executed in a XPC-dependent manner (Sugasawa,2008; Friedberg et al.,2006b).

The XPC protein is redundant in TC-NER. Here a stalled RNA polymerase II (RNA polII) is the onset of the NER machinery. The proteins CSA and CSB play a crucial role in setting the transcription coupled repair in motion, but are also implicated in RNA polII transcription functions. The CSB protein interacts with RNA polII (Tantin et al.,1997), while CSA does not (Tantin,1998). CSA mainly interacts with CSB, XAB2 (XPA binding protein 2) and the p44 subunit of the TFIIH complex (Henning et al.,1995; Nakatsu et al.,2000). The function of CSA

remains to be elucidated but is implicated to be required for TC-NER during elongation of the transcription process (Groisman et al.,2003; Kamiuchi et al.,2002). CSA is also part of an E3 ubiquitin ligase complex in which CUL4A, CSN and DDB1 are involved. Both CSA and CSB are part of RNA PolII associated complexes, but for CSB additional functions are assigned outside NER (Sunesen et al.,2002).

In TC-NER, CSB is thought to be responsible for displacement of the stalled RNA polymerase. Additionally, as with XPC in GG-NER, the preincision complex of NER is recruited in a CSB-dependent manner (Fousteri et al.,2008; Fousteri et al.,2006). But first, as in GG-NER, the TFIIF complex is recruited after damage recognition.

3.3 DNA helix unwinding

From DNA damage recognition and subsequent recruitment of TFIIF on, GG-NER and TC-NER converge into the same pathway. The TFIIF complex consists of 10 proteins: XPB, XPD, p62, pP52, p44, p34, p8 and the CDK-activating kinase (CAK) complex: MAT1, CDK7 and Cyclin H. TFIIF forms an open bubble structure in the DNA helix (Giglia-Mari et al.,2004; Goosen,2010). The DNA helicases XPB and XPD facilitate the partial unwinding of the DNA duplex in an ATP-dependent manner, allowing the preincision complex to enter the site of the lesion (Figure 2) (Oksenyshyn et al.,2010). The preincision complex consists of the XPA, RPA and XPG proteins and is assembled at the damage site (Zotter et al.,2006) (Figure 2). The function of XPA is verification of the lesion; in addition, XPA acts, together with the single strand DNA binding complex RPA, as an organizational orchestrator, so that the repair machinery is positioned around the lesion. The arrival of XPA and RPA leads to complete opening of the damaged DNA and catalyzes the release of the CAK complex from the TFIIF complex. Data suggest this step is essential for the initiation of incision/excision of the damaged DNA (Andressoo et al.,2006; Coin et al.,2008).

XPA and RPA in the preincision complex bind to the damaged DNA. Specific binding properties for certain structural DNA distortions have been reported for XPA, which suggests the protein recognizes conformations of the DNA and is able to verify the damage (Lao et al.,2000; Krasikova et al.,2010). XPA has no enzymatic activity to attribute to the incision step, but nevertheless is indispensable for DNA incision (Miyamoto et al.,1992). Through zinc finger motifs of XPA interaction with RPA is established (Ikegami et al.,1998). RPA consists of 3 subunits and with high affinity binds to the undamaged strand (de Laat et al.,1998b) (Figure 2). The protein roughly covers 30 nucleotides (corresponding in size to the excised product later in NER) and acts as a wedge between the DNA strands. It is also believed to protect the undamaged intact strand from inappropriate nuclease activity (de Laat et al.,1998a; Hermanson-Miller et al.,2002). RPA is concerned as the major component in the preincision complex, thereby also protecting the native template strand. Furthermore, RPA interacts with several other factors of the nucleotide excision repair pathway, like the endonucleases XPG and the ERCC1-XPF dimer, which are required for the dual incision of the damaged strand (Figure 2). RPA hereby facilitates the correct positioning of the endonucleases and orchestrates the open complex formation (Krasikova et al.,2010; Park et al.,2006). Besides that, RPA later plays a role in the DNA repair synthesis and ligation steps (Shivji et al.,1995). XPA exhibits a moderate preference for binding to damaged DNA, as do other NER factors. Individually, the proteins XPA and RPA do not show sufficient selectivity to explain the high efficiency of NER lesion removal, which is most likely due to a discrimination cascade of recognition

and verification steps (Lin et al.,1992). XPA is thought to play a major role in verification, possibly by acting as a molecular sensor of aberrant electrostatic potential along the DNA substrate (Camenisch et al.,2008). Structural changes, like kinked backbones, in damaged DNA can induce deviant electrostatic potentials. Bulky adducts often result in sharply bent backbones due to the decrease of rigidity. Normally, base stacking supplies the DNA helix with strength and structure, but bulky lesions and hence loss of base stacking can weaken the sturdiness of the backbone. It is known that XPA has a higher affinity for kinked backbones. However how the exact recognition of the lesion is executed remains to be elucidated. But it is clear that the XPA-RPA complex is indispensable for the high efficiency of NER (Camenisch et al.,2008).

3.4 Incision, DNA repair synthesis and ligation

When the preincision complex is accurately positioned in relation to the damaged site by the XPA-RPA complex, single strand breaks are introduced by XPG and ERCC1-XPF (Figure 2). Several mechanistic theories were postulated over the years. A general consensus is that the combined actions of XPG and ERCC1-XPF result in excision of a 24-32 nucleotide long single strand fragment including the damaged site (Hess et al.,1997). XPG is responsible for the 3' incision and is putatively recruited by the TFIIH complex (Zotter et al.,2006). According to some studies its presence appears to be necessary for ERCC1-XPF activity, which is responsible for carrying out the 5' incision (Friedberg et al.,2006b; Wakasugi et al.,1997). Others propose a 'cut-patch-cut-patch' mechanism for the incision and resynthesis process within NER, where the 5' incision possibly precedes the 3' incision (Staresinic et al.,2009).

XPG is expected to have additional stabilization features, because of its ability to interact with XPB, XPD and several other subunits of the TFIIH complex(Friedberg et al.,2006b). Since loss of XPG results in very early death(Wijnhoven et al.,2007) the protein might be involved in systemic and important additional mechanisms, like transcription (Bessho,1999; Lee et al.,2002). Furthermore, XPG is suggested to have a role in oxidative damage removal (Dianov et al.,2000). The ERCC1-XPF seems to be a multifunctional complex as well, since it is also involved in interstand crosslink repair and homologous recombination (Niedernhofer et al.,2001; Al Minawi et al.,2009).

The excision of the damaged fragment is restored in original (undamaged) state by DNA synthesis and ligation steps (either by cut-patch-cut-patch mechanism or full excision followed by resynthesis and ligation). Both XPG and RPA are thought to be required for the transition between (pre)incision and post-incision events (Mocquet et al.,2008). XPG is thought to be involved in the recruitment of PCNA (Staresinic et al.,2009; Mocquet et al.,2008). Resynthesis of DNA requires PCNA because of its ability to interact with DNA polymerases (Mocquet et al.,2008). The mechanism of involvement of these polymerases in DNA resynthesis is not yet fully elucidated. Recent studies show at least three DNA polymerases are involved. Pol δ , Pol κ and Pol ϵ are recruited to damage sites (Figure 2). Recent *in vivo* studies show Pol β most likely plays no major role in NER (Ogi et al.,2010; Moser et al.,2007). To complete the repair of the damaged DNA site the resynthesized strand needs to be ligated. The primary participant in the subsequent ligation process of NER appears to be the XRCC1-Ligase 3 complex, which is shown to accumulate in both quiescent as well as proliferating cells after local UV irradiation (Moser et al.,2007). Ligase 1 appears to be involved in the ligation step in proliferating cells only (Moser et al.,2007). To date, the cross play of over 30 proteins in total is involved in NER to counteract DNA damage in the error free manner described above.

4. NER in cancer

DNA repair is vital to humans and other organisms and a defect in one of the genes can result in some severe syndromes or diseases by loss of genomic stability. Essential consequences of genomic instability can be cancer and other age-related diseases, such as neurological disorders as Huntington's disease and ataxias (Friedberg et al.,2006b). DNA damage for example can cause mutations that trigger (pre-)oncogenes, inactivate tumor suppressor genes or other indispensable genes which cause loss of homeostasis. Defects in the DNA repair machinery will inflate the mutational load, since DNA damage will be left unattended and subsequently gene mutations will accumulate. Therefore, organisms that harbor defective DNA repair are often more prone to develop cancer or (segmental) age-related diseases.

In humans, several syndromes have been identified which are the result of an impaired nucleotide excision repair pathway, of which Xeroderma Pigmentosum (XP), Cockayne syndrome (CS) and Trichothiodystrophy (TTD) are the most well-known. Since NER is the major defense against UV-induced DNA damage, all three syndromes are hallmarked by an extreme UV-sensitivity, of which XP ensues a highly elevated risk of developing skin cancer (Friedberg et al.,2006b; Cleaver et al.,2009).

The involvement of NER genes in rare and severe syndromes underscores the vital importance of this repair pathway. It is known that accumulative DNA damage is one of the most important causes in cancer development and loss of homeostasis in organisms (Mullaart et al.,1990; Lindahl,1993; Friedberg et al.,2006b; de Boer et al.,2000; Cleaver et al.,2009). Defects in DNA repair pathways are therefore also considered to accelerate aging and tumorigenesis. In defective NER both types of endpoints occur, XP patients are predisposed to cancer development while CS and TTD patients are not. The latter exhibit premature aging features which XP patients lack (Friedberg et al.,2006b; de Boer et al.,2000; Cleaver et al.,2009). Reason for this might be the involvement of several NER proteins in other significant cellular mechanisms. CSB is believed to be involved in (TC-)BER, while XPD is also associated with replication and transcription. Some of these affected mechanisms could overshadow the NER deficiency and ever increasing mutational load eventually predisposing an individual to cancer. Severely affected developmental and neurological systems could be more life threatening on the shorter term than tumor development is. This could be the rationale behind the fact that CS and TTD patients are extremely short-lived and not cancer prone.

5. Xeroderma pigmentosum

Xeroderma pigmentosum (XP), meaning parchment pigmented skin, was the first human causal NER-deficient disease identified (Cleaver et al.,2009). It is a rare, autosomal inherited neurodegenerative and skin disease in which exposure to sunlight (UV) can lead to skin cancer. In Western Europe and the USA the incidence frequency is approximately 1:250,000, rates are higher in Japan (1:40,000). XP-C and XP-A are the most common complementation forms of XP (Bhutto et al.,2008).

Early malignancies (from 1-2 years of age) in parts of the skin, eyes and the tip of the tongue develop due to sun-exposure (Table 1). Additionally, benign lesions like blistering, hyperpigmented spots and freckles are abundant (Figure 3). XP is associated with a more than 1,000-fold increase in risk of developing skin cancer. These cancers are mainly basal

Feature	%/age	Feature	%/age
<i>Cutaneous abnormalities</i>		<i>Neurological abnormalities</i>	
Median age of onset of symptoms	1.5 yr	Median age of onset	6 mo
Median age of onset of freckling	1.5 yr	Association with skin problems	33%
Photosensitivity	19%	Association with ocular abnormalities	36%
Cutaneous atrophy	23%	Low intelligence	80%
Cutaneous telangiectasia	17%	Abnormal motor activity	30%
Actinic keratoses	19%	Areflexia	20%
Malignant skin neoplasms	45%	Impaired hearing	18%
Median age of first cutaneous neoplasm	8 yr	Abnormal speech	13%
<i>Ocular abnormalities</i>		Abnormal EEG	11%
Frequency	40%	Microcephaly	24%
Median age of onset	4 yr	<i>Abnormalities associated with neurological defects</i>	
Conjunctival injection	18%	Slow growth	23%
Corneal abnormalities	17%	Delayed secondary sexual development	12%
Impaired vision	12%		
Photophobia	2%		
Ocular neoplasms	11%		
Median age of first ocular neoplasm	11 yr		

Adapted from Friedberg, E.C *et al.* 2006b

Table 1. Overview of some XP features and their average age of onset or frequency

and squamous cell carcinomas (45% of the XP patients) and to a lesser extent melanomas (Friedberg *et al.*,2006b) (Table 1). Besides skin cancers, XP patients have a 10-20 fold increased risk to develop internal cancers (Kraemer *et al.*,1984). The disease is mostly symptomatic during childhood. The mean latency time for cutaneous neoplasms is 8 years, this is in comparison to the general population in which the mean latency time is 50 years later (Kraemer,1997). Progressive neurological degeneration occurs in approximately 20% of the XP cases and can be correlated to deficiencies in specific XP genes (XPA, XPB, XPD and XPG) (Cleaver *et al.*,2009). XP-C and XP-F patients rarely develop neurological degeneration and if so with a later onset when compared for example to XP-A and XP-D patients (Kraemer,1997; Friedberg *et al.*,2006b). The heterogeneity in exhibited symptoms is correlated to the genetic heterogeneity in XP patients. XP-A, XP-B, XP-D and XP-G patients are in general the most severely affected and all these patients are defective in both GG-NER and TC-NER. Solely GG-NER is defective in XP-C and XP-E patients. XP-C and XP-E cells show higher survival rate after UV exposure than XP-A and XP-D cells for example (Friedberg *et al.*,2006b). This could be the reason that XP-C patients suffer less from sunburn. Most abundant XP variants in human are XP-A and XP-C (~50% of all XP cases) (Zeng *et al.*,1997).



Fig. 3. Xeroderma pigmentosum.

Photo of a 19 year old Xeroderma pigmentosum patient suffering from hyperpigment skin lesions and a tongue carcinoma (IARC).

6. NER mouse models in cancer research

To investigate the role of the proteins involved in NER on survival and cancer development several transgenic mouse models were created, mimicking the existing NER mutations or deletions in humans. Table 2 shows an overview of NER mouse models and their accompanying spontaneous phenotypes. Selected knockout mouse models (*Xpa*, *Xpc* and *Xpe*) are described in more detail further below. These three models show a decreased lifespan in comparison to their concurrent wild type controls, but not as extreme as several other NER-deficient mouse models in Table 2. Therefore the mouse models survive long enough to study the effect of impaired NER on cancer development. Others, like *Xpb*, *Xpf*, *Xpg* and *Ercc1* deficient models are too short-lived to study carcinogenesis.

6.1 *Xpa* deficient mouse model

The first DNA repair defective models were the *Xpa*-deficient mouse models, generated by de Vries et al. (de Vries et al.,1995) and independently by Nakane et al (Nakane et al.,1995). *Xpa*-deficient mice appeared more cancer prone compared to their heterozygous and wild type littermates when exposed to carcinogenic and genotoxic compounds (de Vries et al.,1997b; Takahashi et al.,2002; Ide et al.,2001; Hoogervorst et al.,2005; Hoogervorst et al.,2004). As in humans, the mouse model exhibited a marked predisposition to skin cancer upon UV treatment of shaved dorsal skin (de Vries et al.,1995).

Survival studies without directed exposure were performed initially but always in a mixed genetic background, C57BL/6J/Ola129 (de Vries et al.,1997b) and C3H/heN strains (Takahashi et al.,2002) and fairly small numbers. However, both studies indicated that *Xpa*^{-/-} mice (from here mentioned as *Xpa* mice) developed a significant number of spontaneous liver tumors. The C3H/heN strain wild type mice already showed 47% liver tumor incidence in the male mice within 16 months. The C57BL/6J/Ola129 mice were more resilient, no enhanced mortality was observed until the age of 1.5 years. The *Xpa* mice showed a 15% hepatocellular adenoma tumor incidence after 20 months, while there were no tumors in the wild type and heterozygous littermates. The lack of a pure genetic

Mouse model	Affected repair pathway	Enhanced spontaneous tumor response	Reference	Accelerated aging/developmental problems	Reference
<i>Xpa</i> ^{-/-}	GG-NER/TC-NER	Yes, liver	(de Vries et al.,1997b; Melis et al.,2008; Tanaka et al.,2001)	Shorter life span, no pathology	(Melis et al.,2008)
<i>Xpb</i> ^{-/-}	NER/transcription	n.a.		Impaired embryonic development	(Friedberg et al.,2006a)
<i>Xpc</i> ^{-/-}	GG-NER	Yes, lung	(Hollander et al.,2005; Melis et al.,2008)	Shorter life span	(Melis et al.,2008)
<i>Xpd</i> ^{TTD}	NER/transcription	No	(de Boer et al.,2002; Wijnhoven et al.,2005)	Shorter life span, aging and CR pathology	(de Boer et al.,2002; Wijnhoven et al.,2005)
<i>Xpd</i> ^{XPCS}	NER/transcription	n.d.			
<i>Xpe</i> (<i>DDB2</i>) ^{-/-}	GG-NER	Yes, various	(Ng et al.,2003; Yoon et al.,2005)		
<i>Xpf</i> ^{m/m}	NER/ICL	n.a.		Very short life span, maximum 3 weeks	(Tian et al.,2004)
<i>Xpg</i> ^{-/-}	TC-NER/transcription	n.a.		Very short life span, maximum 3 weeks	(Harada et al.,1999)
<i>mHR23B</i> ^{-/-}	GG-NER	n.a.		Very short life span/embryonic lethality	(Ng et al.,2002)
<i>Csa</i> ^{-/-}	TC-NER	No	(van der Horst et al.,2002)		
<i>Csb</i> ^{-/-}	TC-NER/transcription	No	(van der Horst et al.,1997)	Normal life span, mild pathology	(van der Horst et al.,1997), unpublished results
<i>Ercc1</i> ^{-/-}	NER/ICL	n.a.		Very short life span, maximum 4 weeks	(McWhir et al.,1993; Weeda et al.,1997)
<i>Ercc1</i> ^{Δ7/-}	NER/ICL	No	Personal communication van Steeg/Dollé	Short life span of 4-6 months	(Weeda et al.,1997)

ICL = interstrand cross link, CR = caloric restriction

n.a.: not applicable, mouse models are too short lived to develop tumors

n.d.: not determined

Table 2. Overview of spontaneous phenotypes of NER-deficient mouse models

background for this and other mouse models made it harder to investigate the underlying cause of the phenotypic responses in these mice. An *Xpa* mouse model in a pure genetic

C57BL/6J background more recently was investigated (Melis et al.,2008). C57BL/6J mice showed a low baseline tumor response and appear therefore suitable for studying mutagenesis and tumorigenesis. In a pure genetic background a significant increase in liver tumors was observed (10%). A small (but not significant) increase in lung tumors was also observed (6.6% of the *Xpa* mice) (Melis et al.,2008). Correspondingly, mutation accumulation in the C57BL/6J *Xpa* mice was significantly increased during survival compared to wild type mice in liver, implicating an *Xpa* repair defect and subsequent mutation induction in carcinogenesis (Melis et al.,2008).

Like human XP-A patients, *Xpa* mice appeared predisposed to skin cancer after UV light exposure to shaved dorsal skin of the mice (de Vries et al.,1995; Tanaka et al.,2001). Heterozygous *Xpa* mice did not show this cancer prone phenotype after UV exposure, not even when the *Xpa* mutation was crossed in in hairless mice (Berg et al.,1997). Skin cancer predisposition in XP mice might not only involve NER deficiency, but several reports indicate enhanced immunosuppression and impaired natural killer cell function are involved (Gaspari et al.,1993; Horio et al.,2001; Miyauchi-Hashimoto et al.,2001). *Xpa* mice were also predisposed to tumors of the cornea when exposed to UV radiation, see Table 3 (de Vries et al.,1998).

Chemical exposure of *Xpa* mice to 7,12-dimethyl-1,2-benz[a]anthracene (DMBA) also resulted in skin cancer (de Vries et al.,1995). Several chemical exposures in *Xpa* mice however shed some more light on the cancer development other than skin cancer, which in humans is the predominant tumor phenotype (Table 3). For example, oral treatment of *Xpa* deficient mice with genotoxic carcinogens like benzo[a]pyrene (B[a]P), 2-acetylaminofluorene (2-AAF), and 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP) resulted in lung tumors and lymphomas (B[a]P), liver and bladder tumors (2-AAF) and intestinal adenomas plus lymphomas (PhIP) (de Vries et al.,1997b; van Steeg et al.,1998; van Steeg et al.,2000; Ide et al.,2000). Other human carcinogens like cyclosporin A (CsA) and diethylstilbestrol (DES), although not directly mutagenic, showed to be carcinogenic in *Xpa* mice after 39 week exposure, but in contrary the low potent human carcinogen phenacetin did not result in a significant increase in tumors.

LacZ and *Hprt* mutation measurements in *Xpa* mice after B[a]P and 2-AAF treatment showed a 2-3 fold increase in mutations compared to wild type mice after only 12-13 weeks of exposure (Hoogervorst et al.,2005; van Oostrom et al.,1999; Bol et al.,1998; de Vries et al.,1997b). This increase in mutational load in comparison to wild type indicates *Xpa* mice are more sensitive to mutation accumulation, which consequently corresponds to the increased cancer susceptibility of *Xpa* mice.

The increased sensitivity towards cancer development of *Xpa* mice made it possible to identify genotoxic carcinogens even more accurate and faster when combined with heterozygosity for p53. This latter mouse model could be beneficial in reducing and refining *in vivo* carcinogenicity testing of compounds.

6.2 *Xpc* deficient mouse model

Two independent *Xpc*-deficient mouse models were also created in the mid-nineties (Cheo et al.,1997; Sands et al.,1995). As the *Xpa* mouse model, this model is informative for human XP and cancer development in general. The model is especially interesting since it is only defective for GG-NER and not for TC-NER. Hereby, differences between pathways can be investigated.

Mouse model	Treatment	Target	Enhanced tumor response*	References
<i>Xpa</i>	UV-B radiation	Skin	Yes	(de Vries et al.,1995; Nakane et al.,1995)
	DMBA paint + TPA	Skin	Yes	(de Vries et al.,1995; Nakane et al.,1995)
	B[a]P gavage	Multiple, lymphomas	Yes	(de Vries et al.,1997a; van Oostrom et al.,1999)
	B[a]P diet	Stomach, esophagus	Yes	(Hoogervorst et al.,2003)
	B[a]P intratracheal instillation	Lung	Yes	(Ide et al.,2000)
	AFB1 i.p. injection	Liver	Yes	(Takahashi et al.,2002)
	PhIP diet	Lymphoma, small intestine	No	(Klein et al.,2001)
	4NQO drinking water	Tongue	Yes	(Ide et al.,2001)
	2-AAF diet	Liver, bladder, gall bladder	Yes	(Hoogervorst et al.,2005; van Kreijl et al.,2001)
	CsA	Lymphoma	Yes	(van Kesteren et al.,2009)
	DES	Osteosarcoma, lymphoma	Yes	(McAnulty et al.,2005)
	Wy	Liver	Yes	(van Kreijl et al.,2001)
	DEHP	Liver	No	Unpublished results
	p-cres	Liver	Yes	Unpublished results
<i>Xpc</i>	UV-B radiation	Skin	Yes	(Sands et al.,1995; Berg et al.,1998)
	2-AAF diet	Liver, bladder	Yes	(Hoogervorst et al.,2005)
	AAF i.p. injection	Liver, lung	Yes	(Cheo et al.,1999)
	NOH-AAF i.p. injection	Liver, lung	Yes	(Cheo et al.,1999)
	DEHP	Liver	No	Unpublished results
	p-cres	Liver	Yes	Unpublished results
<i>Xpe/ DDB2</i>	UV-B radiation	Skin	Yes	(Itoh et al.,2004)
	DMBA paint	Skin	No	(Itoh et al.,2004)

* in comparison to the untreated controls

DEHP = Di(2-ethylhexyl) phthalate

AFB1 = Aflatoxin B1

4NQO = 4-Nitroquinoline 1-oxide

WY = Wyeth-14643

p-cres = p-cresidine

NOH-AAF = N-hydroxyacetylaminofluorene

Table 3. Tumor responses in *Xpa*, *Xpc* and *Xpe* mice upon exposure

As in human XP-C patients, *Xpc* mice are highly predisposed to UV radiation-induced skin cancer (Table 3) (Berg et al.,1998; Cheo et al.,1996; Cheo et al.,2000; Friedberg et al.,1999; Sands et al.,1995). Contrasting to *Xpa*^{+/-} mice the heterozygous *Xpc* mice are more susceptible to UV-induced skin cancer (but only at approximately from 1 year old) when compared to their wild type littermates. The haploinsufficient sensitivity could mean that XPC is a rate limiting factor in NER and since XPC is involved in damage recognition might explain the difference with *Xpa* heterozygous mice. Exposure studies with 2-AAF using *Xpc* mice showed a significant predisposition to liver and lung tumors compared to the heterozygous *Xpc* and wild type mice (Table 3) (Cheo et al.,1999; Friedberg et al.,2006b). Internal tumor incidence is higher in XP mice than in human XP, since patients normally develop skin cancer at a faster rate and die of resulting metastatic complications. NER is believed to be the sole pathway to remove CPD and 6-4PP lesions, while for chemical carcinogenic exposure other repair mechanisms are also present in the cell. In human, other types of cancer generally do not develop fast enough and are possibly overshadowed by skin cancers in XP.

In a mixed genetic background (C57BL/6J/129) no decrease in survival was found in relation to wild type mice, even though *Xpc* mice showed an extremely high and significantly increased lung tumor incidence (100%). However, in this study the wild type mice were not genetically related to the *Xpc* mice (Hollander et al.,2005). The spontaneous survival characteristics of *Xpc* mice in a pure genetic C57BL/6J background together with their related wild type littermates were also investigated. *Xpc* mice showed a significant decrease in survival, again exhibited a significant increase in lung and liver tumors and an increased mutation accumulation in these tissues compared to wild type mice (Melis et al., 2008). Here, *Xpc* mice showed a divergent tumor spectrum from *Xpa* mice in the same genetic C57BL/6J background. The additional increase in lung tumor development in two independent spontaneous survival studies indicate XPC is involved in other pathways besides NER. A corresponding strong increase in mutational load during aging was found in lungs of the C57BL/6J *Xpc* mice, which was not the case in *Xpa* mice (Melis et al.,2008). Uehara *et al.* have shown that enhanced spontaneous age-related mutation accumulation in *Xpc* mice is tissue dependent. Liver, lung, heart and spleen exhibited an increase in mutant frequency compared to wild type, while this difference was not visible in brain and small intestine. Mutant frequencies of liver, lung and spleen are higher in *Xpc* mice compared to *Xpa* mice, just as the tumor incidence in this study (Melis et al.,2008). The additional increase in mutational load in *Xpc* mice might be caused by increased sensitivity towards oxidative DNA damage. XPC functioning has been implied in other DNA repair pathways like base excision repair and non-homologous end joining or might be involved in redox homeostasis (D'Errico et al.,2006; Despras et al.,2007; Liu et al.,2010; Okamoto et al.,2008; Rezvani et al.,2010; Shimizu et al.,2003; Uehara et al.,2009).

Chemical exposures to B[a]P (Wickliffe et al.,2006), 3,4-epoxy-1-butene (EB) (Wickliffe et al.,2006), DMBA (Wijnhoven et al.,2001) and UV-B (Ikehata et al.,2007) also showed significantly enhanced mutant frequencies compared to wild type mice in several tissues. Direct comparisons to *Xpa* mice in these studies have not been made, however when *Xpa* and *Xpc* mice were exposed to pro-oxidants (DEHP and paraquat) for 39 weeks, *Xpc* again exhibited a higher mutant frequency than *Xpa*.

6.3 Xpe deficient mouse model

In 2004 and 2005 Itoh *et al.* and Yoon *et al.* independently generated a strain of *DDB2*^{-/-} mice (Itoh,2006; Itoh et al.,2004; Yoon et al.,2005). The latter group reported that *DDB2*^{-/-} mice

show a decrease in spontaneous survival (n=10) compared to wild type (Yoon et al.,2005). Also the heterozygous *DDB2*^{+/-} mice showed a decreased lifespan, although not as severe as the *DDB2*^{-/-} mice. Six out of 10 *DDB2*^{+/-} mice harbored tumors at the end of life, while 3 out of 10 *DDB2*^{+/-} mice were tumor bearing (Yoon et al.,2005). *DDB2*^{-/-} mice additionally showed to be cancer prone upon UV-B exposure, resulting in a significant increase in skin tumors (Table 3) (Itoh,2006; Itoh et al.,2004; Yoon et al.,2005). DMBA treatment however did not enhance tumor incidence compared to wild type (Table 3) (Itoh,2006; Itoh et al.,2004). *DDB2* deficiency is, due to these and other studies, since being classified as a XPE phenotype. Besides UV-B and DMBA exposure, other *in vivo* carcinogen exposures have not been reported in these models so far. *DDB2* is well conserved between humans and mice and appears to function as a tumor suppressor, at least in part, by controlling p53-mediated apoptosis after UV-irradiation (Itoh et al.,2004).

7. Conclusion

DNA repair has proven to be of vital importance and protects or at least delays cancer development and several age-related diseases. DNA damage accumulation and consequent mutation accumulation is considered pathogenic. NER has been shown to be a highly versatile and important DNA repair pathway, removing helix distorting DNA damages. Mutations in the XP genes of NER in human can result in the severe syndrome Xeroderma pigmentosum, which is accompanied by a cancer predisposition and severe UV sensitivity. Mouse models mimicking this human syndrome are important to study cancer development and the consequences of persistent DNA damage. Novel functionality of DNA repair proteins and implications of their deficiency in mutagenesis, cell cycle regulation, carcinogenesis and aging were discovered using NER-deficient models. Besides mechanistic insight these models can be used as a refined model in carcinogenicity testing, especially in combination with p53 heterozygosity. The increased cancer susceptibility can be beneficial towards a decrease in the number of animals used and the duration of carcinogenicity testing.

8. References

- Al Minawi, A. Z., Lee, Y. F., Hakansson, D., Johansson, F., Lundin, C., Saleh-Gohari, N., Schultz, N., Jenssen, D., Bryant, H. E., Meuth, M., Hinz, J. M., and Helleday, T. (2009). The ERCC1/XPF endonuclease is required for completion of homologous recombination at DNA replication forks stalled by inter-strand cross-links. *Nucleic Acids Res.* 37, 6400-6413.
- Alekseev, S., Luijsterburg, M. S., Pines, A., Geverts, B., Mari, P. O., Giglia-Mari, G., Lans, H., Houtsmuller, A. B., Mullenders, L. H., Hoeijmakers, J. H., and Vermeulen, W. (2008). Cellular concentrations of *DDB2* regulate dynamic binding of *DDB1* at UV-induced DNA damage. *Mol.Cell Biol.* 28, 7402-7413.
- Anand, P., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung, B., and Aggarwal, B. B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharm.Res.* 25, 2097-2116.
- Andressoo, J. O., Hoeijmakers, J. H., and Mitchell, J. R. (2006). Nucleotide excision repair disorders and the balance between cancer and aging. *Cell Cycle* 5, 2886-2888.

- Araki, M., Masutani, C., Takemura, M., Uchida, A., Sugawara, K., Kondoh, J., Ohkuma, Y., and Hanaoka, F. (2001). Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. *J.Biol.Chem.* 276, 18665-18672.
- Araujo, S. J., Nigg, E. A., and Wood, R. D. (2001). Strong functional interactions of TFIIH with XPC and XPG in human DNA nucleotide excision repair, without a preassembled repairosome. *Mol.Cell Biol.* 21, 2281-2291.
- Berg, R. J., de Vries, A., van Steeg, H., and de Gruijl, F. R. (1997). Relative susceptibilities of XPA knockout mice and their heterozygous and wild-type littermates to UVB-induced skin cancer. *Cancer Res.* 57, 581-584.
- Berg, R. J., Ruven, H. J., Sands, A. T., de Gruijl, F. R., and Mullenders, L. H. (1998). Defective global genome repair in XPC mice is associated with skin cancer susceptibility but not with sensitivity to UVB induced erythema and edema. *J.Invest Dermatol.* 110, 405-409.
- Bessho, T. (1999). Nucleotide excision repair 3' endonuclease XPG stimulates the activity of base excision repair enzyme thymine glycol DNA glycosylase. *Nucleic Acids Res.* 27, 979-983.
- Bhutto, A. M. and Kirk, S. H. (2008). Population distribution of xeroderma pigmentosum. *Adv.Exp.Med.Biol.* 637, 138-143.
- Bol, S. A., van Steeg, H., Jansen, J. G., Van Oostrom, C., de Vries, A., de Groot, A. J., Tate, A. D., Vrieling, H., van Zeeland, A. A., and Mullenders, L. H. (1998). Elevated frequencies of benzo(a)pyrene-induced Hprt mutations in internal tissue of XPA-deficient mice. *Cancer Res.* 58, 2850-2856.
- Cadet, J., Douki, T., Gasparutto, D., and Ravanat, J. L. (2003). Oxidative damage to DNA: formation, measurement and biochemical features. *Mutat.Res.* 531, 5-23.
- Camenisch, U. and Nageli, H. (2008). XPA gene, its product and biological roles. *Adv.Exp.Med.Biol.* 637, 28-38.
- Chen, X., Zhang, Y., Douglas, L., and Zhou, P. (2001). UV-damaged DNA-binding proteins are targets of CUL-4A-mediated ubiquitination and degradation. *J.Biol.Chem.* 276, 48175-48182.
- Cheo, D. L., Burns, D. K., Meira, L. B., Houle, J. F., and Friedberg, E. C. (1999). Mutational inactivation of the xeroderma pigmentosum group C gene confers predisposition to 2-acetylaminofluorene-induced liver and lung cancer and to spontaneous testicular cancer in Trp53^{-/-} mice. *Cancer Res.* 59, 771-775.
- Cheo, D. L., Meira, L. B., Burns, D. K., Reis, A. M., Issac, T., and Friedberg, E. C. (2000). Ultraviolet B radiation-induced skin cancer in mice defective in the Xpc, Trp53, and Apex (HAP1) genes: genotype-specific effects on cancer predisposition and pathology of tumors. *Cancer Res.* 60, 1580-1584.
- Cheo, D. L., Meira, L. B., Hammer, R. E., Burns, D. K., Doughty, A. T., and Friedberg, E. C. (1996). Synergistic interactions between XPC and p53 mutations in double-mutant mice: neural tube abnormalities and accelerated UV radiation-induced skin cancer. *Curr.Biol.* 6, 1691-1694.
- Cheo, D. L., Ruven, H. J., Meira, L. B., Hammer, R. E., Burns, D. K., Tappe, N. J., van Zeeland, A. A., Mullenders, L. H., and Friedberg, E. C. (1997). Characterization of defective nucleotide excision repair in XPC mutant mice. *Mutat.Res.* 374, 1-9.
- Cleaver, J. E., Lam, E. T., and Revet, I. (2009). Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. *Nat.Rev.Genet.* 10, 756-768.

- Coin, F., Oksenysh, V., Mocquet, V., Groh, S., Blattner, C., and Egly, J. M. (2008). Nucleotide excision repair driven by the dissociation of CAK from TFIIH. *Mol. Cell* 31, 9-20.
- D'Errico, M., Parlanti, E., Teson, M., de Jesus, B. M., Degan, P., Calcagnile, A., Jaruga, P., BJORAS, M., Crescenzi, M., Pedrini, A. M., Egly, J. M., Zambruno, G., Stefanini, M., Dizdaroglu, M., and Dogliotti, E. (2006). New functions of XPC in the protection of human skin cells from oxidative damage. *EMBO J.* 25, 4305-4315.
- de Boer, J., Andressoo, J. O., de Wit, J., Huijman, J., Beems, R. B., van Steeg, H., Weeda, G., van der Horst, G. T., van Leeuwen, W., Themmen, A. P., Meradji, M., and Hoeijmakers, J. H. (2002). Premature aging in mice deficient in DNA repair and transcription. *Science* 296, 1276-1279.
- de Boer, J. and Hoeijmakers, J. H. (2000). Nucleotide excision repair and human syndromes. *Carcinogenesis* 21, 453-460.
- de Laat, W. L., Appeldoorn, E., Jaspers, N. G., and Hoeijmakers, J. H. (1998a). DNA structural elements required for ERCC1-XPF endonuclease activity. *J. Biol. Chem.* 273, 7835-7842.
- de Laat, W. L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N. G., and Hoeijmakers, J. H. (1998b). DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. *Genes Dev.* 12, 2598-2609.
- de Vries, A., Dolle, M. E., Broekhof, J. L., Muller, J. J., Kroese, E. D., van Kreijl, C. F., Capel, P. J., Vijg, J., and van Steeg, H. (1997a). Induction of DNA adducts and mutations in spleen, liver and lung of XPA-deficient/lacZ transgenic mice after oral treatment with benzo[a]pyrene: correlation with tumour development. *Carcinogenesis* 18, 2327-2332.
- de Vries, A., Gorgels, T. G., Berg, R. J., Jansen, G. H., and van Steeg, H. (1998). Ultraviolet-B induced hyperplasia and squamous cell carcinomas in the cornea of XPA-deficient mice. *Exp. Eye Res.* 67, 53-59.
- de Vries, A., van Oostrom, C. T., Dortant, P. M., Beems, R. B., van Kreijl, C. F., Capel, P. J., and van Steeg, H. (1997b). Spontaneous liver tumors and benzo[a]pyrene-induced lymphomas in XPA-deficient mice. *Mol. Carcinog.* 19, 46-53.
- de Vries, A., van Oostrom, C. T., Hofhuis, F. M., Dortant, P. M., Berg, R. J., de Gruijl, F. R., Wester, P. W., van Kreijl, C. F., Capel, P. J., van Steeg, H., and . (1995). Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. *Nature* 377, 169-173.
- Despras, E., Pfeiffer, P., Salles, B., Calsou, P., Kuhfittig-Kulle, S., Angulo, J. F., and Biard, D. S. (2007). Long-term XPC silencing reduces DNA double-strand break repair. *Cancer Res.* 67, 2526-2534.
- Dianov, G. L., Thybo, T., Dianova, I. I., Lipinski, L. J., and Bohr, V. A. (2000). Single nucleotide patch base excision repair is the major pathway for removal of thymine glycol from DNA in human cell extracts. *J. Biol. Chem.* 275, 11809-11813.
- Dip, R., Camenisch, U., and Naegeli, H. (2004). Mechanisms of DNA damage recognition and strand discrimination in human nucleotide excision repair. *DNA Repair (Amst)* 3, 1409-1423.
- El Mahdy, M. A., Zhu, Q., Wang, Q. E., Wani, G., Praetorius-Ibba, M., and Wani, A. A. (2006). Cullin 4A-mediated proteolysis of DDB2 protein at DNA damage sites regulates in vivo lesion recognition by XPC. *J. Biol. Chem.* 281, 13404-13411.
- Fitch, M. E., Nakajima, S., Yasui, A., and Ford, J. M. (2003). In vivo recruitment of XPC to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. *J. Biol. Chem.* 278, 46906-46910.

- Fousteri, M. and Mullenders, L. H. (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell Res.* 18, 73-84.
- Fousteri, M., Vermeulen, W., van Zeeland, A. A., and Mullenders, L. H. (2006). Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. *Mol.Cell* 23, 471-482.
- Friedberg, E. C. (1995). Out of the shadows and into the light: the emergence of DNA repair. *Trends Biochem.Sci.* 20, 381.
- Friedberg, E. C., Cheo, D. L., Meira, L. B., and Reis, A. M. (1999). Cancer predisposition in mutant mice defective in the XPC DNA repair gene. *Prog.Exp.Tumor Res.* 35, 37-52.
- Friedberg, E. C. and Meira, L. B. (2006a). Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage Version 7. *DNA Repair (Amst)* 5, 189-209.
- Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., and Ellenberger, T. (2006b). *DNA Repair and Mutagenesis*. ASM Press).
- Garinis, G. A., Jans, J., and van der Horst, G. T. (2006). Photolyases: capturing the light to battle skin cancer. *Future.Oncol.* 2, 191-199.
- Gaspari, A. A., Fleisher, T. A., and Kraemer, K. H. (1993). Impaired interferon production and natural killer cell activation in patients with the skin cancer-prone disorder, xeroderma pigmentosum. *J.Clin.Invest* 92, 1135-1142.
- Giglia-Mari, G., Coin, F., Ranish, J. A., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N. G., Raams, A., Argentini, M., van der Spek, P. J., Botta, E., Stefanini, M., Egly, J. M., Aebersold, R., Hoeijmakers, J. H., and Vermeulen, W. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat.Genet.* 36, 714-719.
- Goosen, N. (2010). Scanning the DNA for damage by the nucleotide excision repair machinery. *DNA Repair (Amst)* 9, 593-596.
- Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., and Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113, 357-367.
- Guarente, L. P., Partridge, L., and Wallace, D. C. (2008). *Molecular Biology of Aging*. Cold Spring Harbor Laboratory Press).
- Harada, Y. N., Shiomi, N., Koike, M., Ikawa, M., Okabe, M., Hirota, S., Kitamura, Y., Kitagawa, M., Matsunaga, T., Nikaïdo, O., and Shiomi, T. (1999). Postnatal growth failure, short life span, and early onset of cellular senescence and subsequent immortalization in mice lacking the xeroderma pigmentosum group G gene. *Mol.Cell Biol.* 19, 2366-2372.
- Henning, K. A., Li, L., Iyer, N., McDaniel, L. D., Reagan, M. S., Legerski, R., Schultz, R. A., Stefanini, M., Lehmann, A. R., Mayne, L. V., and Friedberg, E. C. (1995). The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. *Cell* 82, 555-564.
- Hermanson-Miller, I. L. and Turchi, J. J. (2002). Strand-specific binding of RPA and XPA to damaged duplex DNA. *Biochemistry* 41, 2402-2408.
- Hess, M. T., Schwitter, U., Petretta, M., Giese, B., and Naegeli, H. (1997). Bipartite substrate discrimination by human nucleotide excision repair. *Proc.Natl.Acad.Sci.U.S.A* 94, 6664-6669.

- Hollander, M. C., Philburn, R. T., Patterson, A. D., Velasco-Miguel, S., Friedberg, E. C., Linnoila, R. I., and Fornace, A. J., Jr. (2005). Deletion of XPC leads to lung tumors in mice and is associated with early events in human lung carcinogenesis. *Proc.Natl.Acad.Sci.U.S.A* 102, 13200-13205.
- Hoogervorst, E. M., de Vries, A., Beems, R. B., van Oostrom, C. T., Wester, P. W., Vos, J. G., Bruins, W., Roodbergen, M., Cassee, F. R., Vijg, J., van Schooten, F. J., and van Steeg, H. (2003). Combined oral benzo[a]pyrene and inhalatory ozone exposure have no effect on lung tumor development in DNA repair-deficient Xpa mice. *Carcinogenesis* 24, 613-619.
- Hoogervorst, E. M., van Oostrom, C. T., Beems, R. B., van Benthem, J., Gielis, S., Vermeulen, J. P., Wester, P. W., Vos, J. G., de Vries, A., and van Steeg, H. (2004). p53 heterozygosity results in an increased 2-acetylaminofluorene-induced urinary bladder but not liver tumor response in DNA repair-deficient Xpa mice. *Cancer Res.* 64, 5118-5126.
- Hoogervorst, E. M., van Oostrom, C. T., Beems, R. B., van Benthem, J., van den, Berg J., van Kreijl, C. F., Vos, J. G., de Vries, A., and van Steeg, H. (2005). 2-AAF-induced tumor development in nucleotide excision repair-deficient mice is associated with a defect in global genome repair but not with transcription coupled repair. *DNA Repair (Amst)* 4, 3-9.
- Hoogstraten, D., Bergink, S., Ng, J. M., Verbiest, V. H., Luijsterburg, M. S., Geverts, B., Raams, A., Dinant, C., Hoeijmakers, J. H., Vermeulen, W., and Houtsmuller, A. B. (2008). Versatile DNA damage detection by the global genome nucleotide excision repair protein XPC. *J.Cell Sci.* 121, 2850-2859.
- Horio, T., Miyauchi-Hashimoto, H., Kuwamoto, K., Horiki, S., Okamoto, H., and Tanaka, K. (2001). Photobiologic and photoimmunologic characteristics of XPA gene-deficient mice. *J.Investig.Dermatol.Symp.Proc.* 6, 58-63.
- Ide, F., Iida, N., Nakatsuru, Y., Oda, H., Tanaka, K., and Ishikawa, T. (2000). Mice deficient in the nucleotide excision repair gene XPA have elevated sensitivity to benzo[a]pyrene induction of lung tumors. *Carcinogenesis* 21, 1263-1265.
- Ide, F., Oda, H., Nakatsuru, Y., Kusama, K., Sakashita, H., Tanaka, K., and Ishikawa, T. (2001). Xeroderma pigmentosum group A gene action as a protection factor against 4-nitroquinoline 1-oxide-induced tongue carcinogenesis. *Carcinogenesis* 22, 567-572.
- Ikegami, T., Kuraoka, I., Saijo, M., Kodo, N., Kyogoku, Y., Morikawa, K., Tanaka, K., and Shirakawa, M. (1998). Solution structure of the DNA- and RPA-binding domain of the human repair factor XPA. *Nat.Struct.Biol.* 5, 701-706.
- Ikehata, H., Saito, Y., Yanase, F., Mori, T., Nikaido, O., and Ono, T. (2007). Frequent recovery of triplet mutations in UVB-exposed skin epidermis of Xpc-knockout mice. *DNA Repair (Amst)* 6, 82-93.
- Itoh, T. (2006). Xeroderma pigmentosum group E and DDB2, a smaller subunit of damage-specific DNA binding protein: proposed classification of xeroderma pigmentosum, Cockayne syndrome, and ultraviolet-sensitive syndrome. *J.Dermatol.Sci.* 41, 87-96.
- Itoh, T., Cado, D., Kamide, R., and Linn, S. (2004). DDB2 gene disruption leads to skin tumors and resistance to apoptosis after exposure to ultraviolet light but not a chemical carcinogen. *Proc.Natl.Acad.Sci.U.S.A* 101, 2052-2057.
- Kamiuchi, S., Saijo, M., Citterio, E., de Jager, M., Hoeijmakers, J. H., and Tanaka, K. (2002). Translocation of Cockayne syndrome group A protein to the nuclear matrix: possible relevance to transcription-coupled DNA repair. *Proc.Natl.Acad.Sci.U.S.A* 99, 201-206.

- Kinzler KW and Vogelstein B (2002). The genetic basis of human cancer. (New York: McGraw-Hill).
- Klein, J. C., Beems, R. B., Zwart, P. E., Hamzink, M., Zomer, G., van Steeg, H., and van Kreijl, C. F. (2001). Intestinal toxicity and carcinogenic potential of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in DNA repair deficient XPA^{-/-} mice. *Carcinogenesis* 22, 619-626.
- Kraemer, K. H. (1997). Sunlight and skin cancer: another link revealed. *Proc.Natl.Acad.Sci.U.S.A* 94, 11-14.
- Kraemer, K. H., Lee, M. M., and Scotto, J. (1984). DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum. *Carcinogenesis* 5, 511-514.
- Krasikova, Y. S., Rechkunova, N. I., Maltseva, E. A., Petruseva, I. O., and Lavrik, O. I. (2010). Localization of xeroderma pigmentosum group A protein and replication protein A on damaged DNA in nucleotide excision repair. *Nucleic Acids Res.*
- Lao, Y., Gomes, X. V., Ren, Y., Taylor, J. S., and Wold, M. S. (2000). Replication protein A interactions with DNA. III. Molecular basis of recognition of damaged DNA. *Biochemistry* 39, 850-859.
- Lee, S. K., Yu, S. L., Prakash, L., and Prakash, S. (2002). Requirement of yeast RAD2, a homolog of human XPG gene, for efficient RNA polymerase II transcription. implications for Cockayne syndrome. *Cell* 109, 823-834.
- Lin, J. J. and Sancar, A. (1992). (A)BC excinuclease: the Escherichia coli nucleotide excision repair enzyme. *Mol.Microbiol.* 6, 2219-2224.
- Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* 362, 709-715.
- Liu, S. Y., Wen, C. Y., Lee, Y. J., and Lee, T. C. (2010). XPC silencing sensitizes glioma cells to arsenic trioxide via increased oxidative damage. *Toxicol.Sci.* 116, 183-193.
- Lo, H. L., Nakajima, S., Ma, L., Walter, B., Yasui, A., Ethell, D. W., and Owen, L. B. (2005). Differential biologic effects of CPD and 6-4PP UV-induced DNA damage on the induction of apoptosis and cell-cycle arrest. *BMC.Cancer* 5, 135.
- Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipurksy, S. L., and Darnell, J. (2004). Molecular Biology of the Cell. WH Freeman.
- Matsuda, N., Azuma, K., Saijo, M., Iemura, S., Hioki, Y., Natsume, T., Chiba, T., Tanaka, K., and Tanaka, K. (2005). DDB2, the xeroderma pigmentosum group E gene product, is directly ubiquitylated by Cullin 4A-based ubiquitin ligase complex. *DNA Repair (Amst)* 4, 537-545.
- McAnulty, P. A. and Skydsgaard, M. (2005). Diethylstilbestrol (DES): carcinogenic potential in Xpa^{-/-}, Xpa^{-/-} / p53^{+/-}, and wild-type mice during 9 months' dietary exposure. *Toxicol.Pathol.* 33, 609-620.
- McWhir, J., Selfridge, J., Harrison, D. J., Squires, S., and Melton, D. W. (1993). Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nat.Genet.* 5, 217-224.
- Melis, J. P., Wijnhoven, S. W., Beems, R. B., Roodbergen, M., van den, Berg J., Moon, H., Friedberg, E., van der Horst, G. T., Hoeijmakers, J. H., Vijg, J., and van Steeg, H. (2008). Mouse models for xeroderma pigmentosum group A and group C show divergent cancer phenotypes. *Cancer Res.* 68, 1347-1353.
- Min, J. H. and Pavletich, N. P. (2007). Recognition of DNA damage by the Rad4 nucleotide excision repair protein. *Nature* 449, 570-575.
- Miyamoto, I., Miura, N., Niwa, H., Miyazaki, J., and Tanaka, K. (1992). Mutational analysis of the structure and function of the xeroderma pigmentosum group A complementing

- protein. Identification of essential domains for nuclear localization and DNA excision repair. *J.Biol.Chem.* 267, 12182-12187.
- Miyachi-Hashimoto, H., Kuwamoto, K., Urade, Y., Tanaka, K., and Horio, T. (2001). Carcinogen-induced inflammation and immunosuppression are enhanced in xeroderma pigmentosum group A model mice associated with hyperproduction of prostaglandin E2. *J.Immunol.* 166, 5782-5791.
- Mocquet, V., Laine, J. P., Riedl, T., Yajin, Z., Lee, M. Y., and Egly, J. M. (2008). Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step. *EMBO J.* 27, 155-167.
- Moser, J., Kool, H., Giakzidis, I., Caldecott, K., Mullenders, L. H., and Foustieri, M. I. (2007). Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner. *Mol.Cell* 27, 311-323.
- Mullaart, E., Lohman, P. H., Berends, F., and Vijg, J. (1990). DNA damage metabolism and aging. *Mutat.Res.* 237, 189-210.
- Nag, A., Bondar, T., Shiv, S., and Raychaudhuri, P. (2001). The xeroderma pigmentosum group E gene product DDB2 is a specific target of cullin 4A in mammalian cells. *Mol.Cell Biol.* 21, 6738-6747.
- Nakane, H., Takeuchi, S., Yuba, S., Saijo, M., Nakatsu, Y., Murai, H., Nakatsuru, Y., Ishikawa, T., Hirota, S., Kitamura, Y., and . (1995). High incidence of ultraviolet-B-or chemical-carcinogen-induced skin tumours in mice lacking the xeroderma pigmentosum group A gene. *Nature* 377, 165-168.
- Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Kamiuchi, S., Yeo, J. P., Khaw, M. C., Saijo, M., Kodo, N., Matsuda, T., Hoeijmakers, J. H., and Tanaka, K. (2000). XAB2, a novel tetratricopeptide repeat protein involved in transcription-coupled DNA repair and transcription. *J.Biol.Chem.* 275, 34931-34937.
- Ng, J. M., Vermeulen, W., van der Horst, G. T., Bergink, S., Sugawara, K., Vrieling, H., and Hoeijmakers, J. H. (2003). A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. *Genes Dev.* 17, 1630-1645.
- Ng, J. M., Vrieling, H., Sugawara, K., Ooms, M. P., Grootegoed, J. A., Vreeburg, J. T., Visser, P., Beems, R. B., Gorgels, T. G., Hanaoka, F., Hoeijmakers, J. H., and van der Horst, G. T. (2002). Developmental defects and male sterility in mice lacking the ubiquitin-like DNA repair gene mHR23B. *Mol.Cell Biol.* 22, 1233-1245.
- Nichols, A. F., Itoh, T., Graham, J. A., Liu, W., Yamaizumi, M., and Linn, S. (2000). Human damage-specific DNA-binding protein p48. Characterization of XPE mutations and regulation following UV irradiation. *J.Biol.Chem.* 275, 21422-21428.
- Niedernhofer, L. J., Essers, J., Weeda, G., Beverloo, B., de Wit, J., Muijtjens, M., Odijk, H., Hoeijmakers, J. H., and Kanaar, R. (2001). The structure-specific endonuclease Ercc1-Xpf is required for targeted gene replacement in embryonic stem cells. *EMBO J.* 20, 6540-6549.
- Nishi, R., Okuda, Y., Watanabe, E., Mori, T., Iwai, S., Masutani, C., Sugawara, K., and Hanaoka, F. (2005). Centrin 2 stimulates nucleotide excision repair by interacting with xeroderma pigmentosum group C protein. *Mol.Cell Biol.* 25, 5664-5674.
- Ogi, T., Limsirichaiikul, S., Overmeer, R. M., Volker, M., Takenaka, K., Cloney, R., Nakazawa, Y., Niimi, A., Miki, Y., Jaspers, N. G., Mullenders, L. H., Yamashita, S., Foustieri, M. I., and Lehmann, A. R. (2010). Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells. *Mol.Cell* 37, 714-727.

- Okamoto, Y., Chou, P. H., Kim, S. Y., Suzuki, N., Laxmi, Y. R., Okamoto, K., Liu, X., Matsuda, T., and Shibutani, S. (2008). Oxidative DNA damage in XPC-knockout and its wild mice treated with equine estrogen. *Chem.Res.Toxicol.* 21, 1120-1124.
- Oksenyich, V. and Coin, F. (2010). The long unwinding road: XPB and XPD helicases in damaged DNA opening. *Cell Cycle* 9, 90-96.
- Ortolan, T. G., Chen, L., Tongaonkar, P., and Madura, K. (2004). Rad23 stabilizes Rad4 from degradation by the Ub/proteasome pathway. *Nucleic Acids Res.* 32, 6490-6500.
- Park, C. J. and Choi, B. S. (2006). The protein shuffle. Sequential interactions among components of the human nucleotide excision repair pathway. *FEBS J.* 273, 1600-1608.
- Rapic-Otrin, V., McLenigan, M. P., Bisi, D. C., Gonzalez, M., and Levine, A. S. (2002). Sequential binding of UV DNA damage binding factor and degradation of the p48 subunit as early events after UV irradiation. *Nucleic Acids Res.* 30, 2588-2598.
- Rezvani, H. R., Kim, A. L., Rossignol, R., Ali, N., Daly, M., Mahfouf, W., Bellance, N., Taieb, A., de Verneuil, H., Mazurier, F., and Bickers, D. R. (2010). XPC silencing in normal human keratinocytes triggers metabolic alterations that drive the formation of squamous cell carcinomas. *J.Clin.Invest.*
- Sands, A. T., Abuin, A., Sanchez, A., Conti, C. J., and Bradley, A. (1995). High susceptibility to ultraviolet-induced carcinogenesis in mice lacking XPC. *Nature* 377, 162-165.
- Shimizu, Y., Iwai, S., Hanaoka, F., and Sugasawa, K. (2003). Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase. *EMBO J.* 22, 164-173.
- Shivji, M. K., Podust, V. N., Hubscher, U., and Wood, R. D. (1995). Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. *Biochemistry* 34, 5011-5017.
- Staresincic, L., Fagbemi, A. F., Enzlin, J. H., Gourdin, A. M., Wijgers, N., Dunand-Sauthier, I., Giglia-Mari, G., Clarkson, S. G., Vermeulen, W., and Scharer, O. D. (2009). Coordination of dual incision and repair synthesis in human nucleotide excision repair. *EMBO J.* 28, 1111-1120.
- Sugasawa, K. (2008). XPC: its product and biological roles. *Adv.Exp.Med.Biol.* 637, 47-56.
- Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S., and Hanaoka, F. (2001). A multistep damage recognition mechanism for global genomic nucleotide excision repair. *Genes Dev.* 15, 507-521.
- Sugasawa, K., Okuda, Y., Saijo, M., Nishi, R., Matsuda, N., Chu, G., Mori, T., Iwai, S., Tanaka, K., Tanaka, K., and Hanaoka, F. (2005). UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* 121, 387-400.
- Sugasawa, K., Shimizu, Y., Iwai, S., and Hanaoka, F. (2002). A molecular mechanism for DNA damage recognition by the xeroderma pigmentosum group C protein complex. *DNA Repair (Amst)* 1, 95-107.
- Sunesen, M., Stevnsner, T., Brosh, R. M., Jr., Dianov, G. L., and Bohr, V. A. (2002). Global genome repair of 8-oxoG in hamster cells requires a functional CSB gene product. *Oncogene* 21, 3571-3578.
- Takahashi, Y., Nakatsuru, Y., Zhang, S., Shimizu, Y., Kume, H., Tanaka, K., Ide, F., and Ishikawa, T. (2002). Enhanced spontaneous and aflatoxin-induced liver tumorigenesis in xeroderma pigmentosum group A gene-deficient mice. *Carcinogenesis* 23, 627-633.
- Tanaka, K., Kamiuchi, S., Ren, Y., Yonemasu, R., Ichikawa, M., Murai, H., Yoshino, M., Takeuchi, S., Saijo, M., Nakatsu, Y., Miyauchi-Hashimoto, H., and Horio, T. (2001).

- UV-induced skin carcinogenesis in xeroderma pigmentosum group A (XPA) gene-knockout mice with nucleotide excision repair-deficiency. *Mutat.Res.* 477, 31-40.
- Tang, J. Y., Hwang, B. J., Ford, J. M., Hanawalt, P. C., and Chu, G. (2000). Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. *Mol.Cell* 5, 737-744.
- Tantin, D. (1998). RNA polymerase II elongation complexes containing the Cockayne syndrome group B protein interact with a molecular complex containing the transcription factor IIIH components xeroderma pigmentosum B and p62. *J.Biol.Chem.* 273, 27794-27799.
- Tantin, D., Kansal, A., and Carey, M. (1997). Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes. *Mol.Cell Biol.* 17, 6803-6814.
- Tian, M., Shinkura, R., Shinkura, N., and Alt, F. W. (2004). Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. *Mol.Cell Biol.* 24, 1200-1205.
- Uehara, Y., Ikehata, H., Furuya, M., Kobayashi, S., He, D., Chen, Y., Komura, J., Ohtani, H., Shimokawa, I., and Ono, T. (2009). XPC is involved in genome maintenance through multiple pathways in different tissues. *Mutat.Res.* 670, 24-31.
- van der Horst, G. T., Meira, L., Gorgels, T. G., de Wit, J., Velasco-Miguel, S., Richardson, J. A., Kamp, Y., Vreeswijk, M. P., Smit, B., Bootsma, D., Hoeijmakers, J. H., and Friedberg, E. C. (2002). UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. *DNA Repair (Amst)* 1, 143-157.
- van der Horst, G. T., van Steeg, H., Berg, R. J., van Gool, A. J., de Wit, J., Weeda, G., Morreau, H., Beems, R. B., van Kreijl, C. F., de Gruijl, F. R., Bootsma, D., and Hoeijmakers, J. H. (1997). Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. *Cell* 89, 425-435.
- van Kesteren, P. C., Beems, R. B., Luijten, M., Robinson, J., de Vries, A., and van Steeg, H. (2009). DNA repair-deficient Xpa/p53 knockout mice are sensitive to the non-genotoxic carcinogen cyclosporine A: escape of initiated cells from immunosurveillance? *Carcinogenesis* 30, 538-543.
- van Kreijl, C. F., McAnulty, P. A., Beems, R. B., Vynckier, A., van Steeg, H., Fransson-Steen, R., Alden, C. L., Forster, R., van der Laan, J. W., and Vandenberghe, J. (2001). Xpa and Xpa/p53[±] knockout mice: overview of available data. *Toxicol.Pathol.* 29 Suppl, 117-127.
- van Oostrom, C. T., Boeve, M., van den, Berg J., de Vries, A., Dolle, M. E., Beems, R. B., van Kreijl, C. F., Vijg, J., and van Steeg, H. (1999). Effect of heterozygous loss of p53 on benzo[a]pyrene-induced mutations and tumors in DNA repair-deficient XPA mice. *Environ.Mol.Mutagen.* 34, 124-130.
- van Steeg, H., Klein, H., Beems, R. B., and van Kreijl, C. F. (1998). Use of DNA repair-deficient XPA transgenic mice in short-term carcinogenicity testing. *Toxicol.Pathol.* 26, 742-749.
- van Steeg, H., Mullenders, L. H., and Vijg, J. (2000). Mutagenesis and carcinogenesis in nucleotide excision repair-deficient XPA knock out mice. *Mutat.Res.* 450, 167-180.
- Wakasugi, M., Reardon, J. T., and Sancar, A. (1997). The non-catalytic function of XPG protein during dual incision in human nucleotide excision repair. *J.Biol.Chem.* 272, 16030-16034.
- Weeda, G., Donker, I., de Wit, J., Morreau, H., Janssens, R., Vissers, C. J., Nigg, A., van Steeg, H., Bootsma, D., and Hoeijmakers, J. H. (1997). Disruption of mouse ERCC1 results in

- a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr.Biol.* 7, 427-439.
- Wickliffe, J. K., Galbert, L. A., Ammenheuser, M. M., Herring, S. M., Xie, J., Masters, O. E., III, Friedberg, E. C., Lloyd, R. S., and Ward, J. B., Jr. (2006). 3,4-Epoxy-1-butene, a reactive metabolite of 1,3-butadiene, induces somatic mutations in Xpc-null mice. *Environ.Mol.Mutagen.* 47, 67-70.
- Wijnhoven, S. W., Beems, R. B., Roodbergen, M., van den Berg J., Lohman, P. H., Diderich, K., van der Horst, G. T., Vijg, J., Hoeijmakers, J. H., and van Steeg, H. (2005). Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. *DNA Repair (Amst)* 4, 1314-1324.
- Wijnhoven, S. W., Hoogervorst, E. M., de Waard, H., van der Horst, G. T., and van Steeg, H. (2007). Tissue specific mutagenic and carcinogenic responses in NER defective mouse models. *Mutat.Res.* 614, 77-94.
- Wijnhoven, S. W., Kool, H. J., Mullenders, L. H., Slater, R., van Zeeland, A. A., and Vrieling, H. (2001). DMBA-induced toxic and mutagenic responses vary dramatically between NER-deficient Xpa, Xpc and Csb mice. *Carcinogenesis* 22, 1099-1106.
- Wittschieben, B. O., Iwai, S., and Wood, R. D. (2005). DDB1-DDB2 (xeroderma pigmentosum group E) protein complex recognizes a cyclobutane pyrimidine dimer, mismatches, apurinic/apyrimidinic sites, and compound lesions in DNA. *J.Biol.Chem.* 280, 39982-39989.
- Yokoi, M., Masutani, C., Maekawa, T., Sugasawa, K., Ohkuma, Y., and Hanaoka, F. (2000). The xeroderma pigmentosum group C protein complex XPC-HR23B plays an important role in the recruitment of transcription factor IIH to damaged DNA. *J.Biol.Chem.* 275, 9870-9875.
- Yoon, T., Chakraborty, A., Franks, R., Valli, T., Kiyokawa, H., and Raychaudhuri, P. (2005). Tumor-prone phenotype of the DDB2-deficient mice. *Oncogene* 24, 469-478.
- Zeng, L., Quilliet, X., Chevallier-Lagente, O., Eveno, E., Sarasin, A., and Mezzina, M. (1997). Retrovirus-mediated gene transfer corrects DNA repair defect of xeroderma pigmentosum cells of complementation groups A, B and C. *Gene Ther.* 4, 1077-1084.
- Zotter, A., Luijsterburg, M. S., Warmerdam, D. O., Ibrahim, S., Nigg, A., van Cappellen, W. A., Hoeijmakers, J. H., van Driel, R., Vermeulen, W., and Houtsmuller, A. B. (2006). Recruitment of the nucleotide excision repair endonuclease XPG to sites of UV-induced dna damage depends on functional TFIIH. *Mol.Cell Biol.* 26, 8868-8879.

Recombinant Viral Vectors for Investigating DNA Damage Responses and Gene Therapy of Xeroderma Pigmentosum

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1. Introduction

1.1 The dark side of the sun

The genome of all living organisms is constantly threatened by a number of endogenous and exogenous DNA damaging agents. Such damage may disturb essential cellular processes, such as DNA replication and transcription, thereby resulting in double-strand breaks (referred to as 'replication fork collapse'), which can lead to chromosomal aberrations and/or cell death, ultimately contributing to mutagenesis, early aging and tumorigenesis (Ciccio & Elledge, 2010). One of the most important exogenous sources of DNA damage is the ultraviolet radiation (UV) component of sunlight, since it is responsible for a wide range of biological effects, including alteration in the structure of biologically essential molecules, such as proteins and nucleic acids. Indeed, UV is one of the most effective and carcinogenic exogenous agents that act on DNA, threatening the genome integrity and affecting normal life processes in different aquatic and terrestrial organisms, ranging from prokaryotes to mammals (Rastogi et al., 2010). In addition, UV is the major etiologic agent in the development of human skin cancers (Narayanan et al., 2010).

Sunlight is the primary UV source, whose spectrum is usually classified according to its wavelength in UVA (320-400 nm; lowest energy), UVB (280-320 nm) and UVC (200-280 nm; highest energy). Although these three UV bands are present in sunlight, the stratospheric ozone layer entirely blocks the UVC and most of UVB, thus the solar UV spectrum that reaches the Earth's ground is composed by UVA and some UVB, even though ozone layer depletion can cause changes in this spectral distribution (Kuluncsics et al., 1999).

The chemical nature and efficiency in the formation of DNA lesions greatly depend on the wavelength of the incident photons. Despite its lowest energy, UVA light can deeply penetrate into the cells, mostly damaging DNA by indirect effects caused by the generation of reactive oxygen species which may react with nitrogen bases, resulting in base alterations and breaks in the DNA molecule. On the other hand, UVB can be directly absorbed by DNA bases, producing two main types of DNA damage, the cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone-(6-4)-photoproducts (6-4PPs), both resulting from

covalent linkages between adjacent pyrimidines located on the same DNA strand, which leads to severe structural distortions in the DNA double helix. Interestingly, it has been recently demonstrated that UVA can also be directly absorbed by the DNA molecule, efficiently generating both CPDs and 6-4PPs (Schuch et al, 2009).

CPDs correspond to the formation of a four-member ring structure involving carbons C5 and C6 of both neighboring bases, whereas 6-4PPs are formed by a non-cyclic bond between C6 (of the 5'-end) and C4 (of the 3'-end) of the involved pyrimidines. Since those lesions induce strong distortions in the DNA molecule, they may lead to severe consequences to the cell if not properly removed, such as transcription arrest and replication blockage, thus disturbing cell metabolism, interfering with the cell cycle and, eventually, inducing cell death. DNA mutations can also result from misleading DNA processing. Long term consequences may include even more deleterious events, such as photoaging and cancer (Sinha & Häder, 2002; Narayanan et al., 2010; Rastogi et al., 2010).

1.2 DNA repair of UV lesions and related human syndromes

To ensure the maintenance of the genome integrity, several mechanisms that counteract DNA damage have emerged very early in evolution, including an intricate machinery of DNA repair, damage tolerance, and checkpoint pathways (Figure 1).

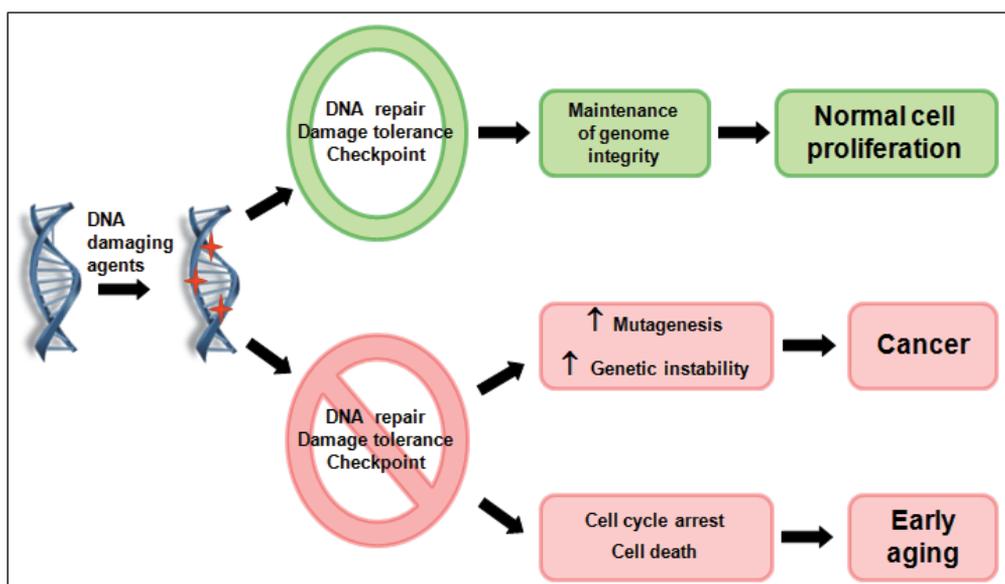


Fig. 1. Main consequences of DNA damage. DNA damage can be induced by a variety of endogenous and exogenous agents. Several mechanisms, including an intricate machinery of DNA repair, damage tolerance, and checkpoint pathways, counteract DNA damage, aiming for the maintenance of genome stability, and guaranteeing normal cell proliferation. When these mechanisms fail, errors in DNA replication and/or aberrant chromosomal segregations take place, increasing mutagenesis and genetic instability and contributing to a higher risk of cancer development. Alternatively, these damages may disturb the transcription and/or cause replication blockage, leading to cell death, thus contributing to early aging.

The nucleotide excision repair (NER) is one of the most versatile and flexible DNA repair systems, removing a wide range of structurally unrelated DNA double-helix distorting lesions, including UV photoproducts, bulky chemical adducts, DNA-intrastrand crosslinks, and some forms of oxidatively generated damage by orchestrating the concerted action of over 30 proteins, including the seven that are functionally impaired in xeroderma pigmentosum patients (XPA to XPG) (Costa et al., 2003; de Boer & Hoeijmakers, 2000). The NER pathway has been extensively studied at the molecular level in both prokaryotic and eukaryotic organisms. Depending on whether the damage is located in a transcriptionally active or inactive domain in the genome, its repair will be processed by one of two NER subpathways: global genome repair (GG-NER) or transcription-coupled repair (TC-NER). Indeed, while GG-NER is a random process, removing distorting lesions over the entire genome, TC-NER focus on those lesions which block RNA polymerases elongation, thus being highly specific and efficient (Fousteri & Mullenders, 2008; Hanawalt, 2002).

Briefly, the NER pathway involves a sequential cascade of events that starts with damage recognition, which defines the major difference between GG-NER and TC-NER. The latter is triggered upon blockage of RNA polymerase translocation at the DNA damage site, whereas GG-NER is evoked by specialized damage recognition factors, including the XPC-hHR23B heterodimer, and also XPE for certain lesions. The subsequent steps are carried out by a common set of NER factors that are shared by both subpathways and involve opening of the DNA helix around the lesion site by the concerted action of two helicases; dual incision of the damaged strand at both sides of the lesion by two endonucleases; removal of the damaged oligonucleotide (24-32 mer); gap filling of the excised patch using the undamaged strand as a template by the action of the replication machinery; and ligation of the new fragment to the chromatin by DNA ligase (Cleaver et al., 2009; Costa et al., 2003). Even though the core NER proteins that carry out damage recognition, excision, and repair reactions have been identified and extensively characterized, the regulatory pathways which govern the threshold levels of NER have not been fully elucidated (Liu et al., 2010). A schematic representation of this repair mechanism in humans is illustrated in Figure 2.

Several human autosomal recessive diseases are caused by dysfunction of the NER pathway, xeroderma pigmentosum (XP) being the prototype. Although this chapter will mainly focus on the XP syndrome, deficiencies in NER can also lead to other genetic diseases, such as trichothiodystrophy (TTD), Cockayne syndrome (CS), cerebro-oculo-facial-skeletal syndrome (COFS) and UV-sensitive syndrome (UVsS), all of which have photosensitivity as a common feature.

Xeroderma pigmentosum (XP) is a rare human disorder transmitted in an autosomal recessive fashion characterized by severe UV light photosensitivity, pigmentary changes, premature skin aging and a greater than 1,000-fold increase incidence of skin and mucous membrane cancer, including squamous and basal cell carcinomas and melanomas, with a 30-year reduction in life span (Cleaver et al., 2009; Karalis et al., 2011; Narayanan et al., 2010). In addition to cutaneous features, patients often develop ocular abnormalities, including neoplasms which may cause blindness. For most patients, often referred to as classical XP, this syndrome is caused by an impaired GG-NER activity, with or without deficiencies in TC-NER, determined by mutations in one of seven NER genes (*XPA* to *XPG*). When TC-NER is also affected (mutations in *XPA*, *XPB*, *XPD* and *XPG* genes), accelerated neurodegeneration may also occur in a substantial number of patients, suggesting increased neuronal cell death due to accumulated endogenous damage (Gerstenblith et al., 2010; Hoeijmakers, 2009). The eighth complementation group corresponds to the XP-variant

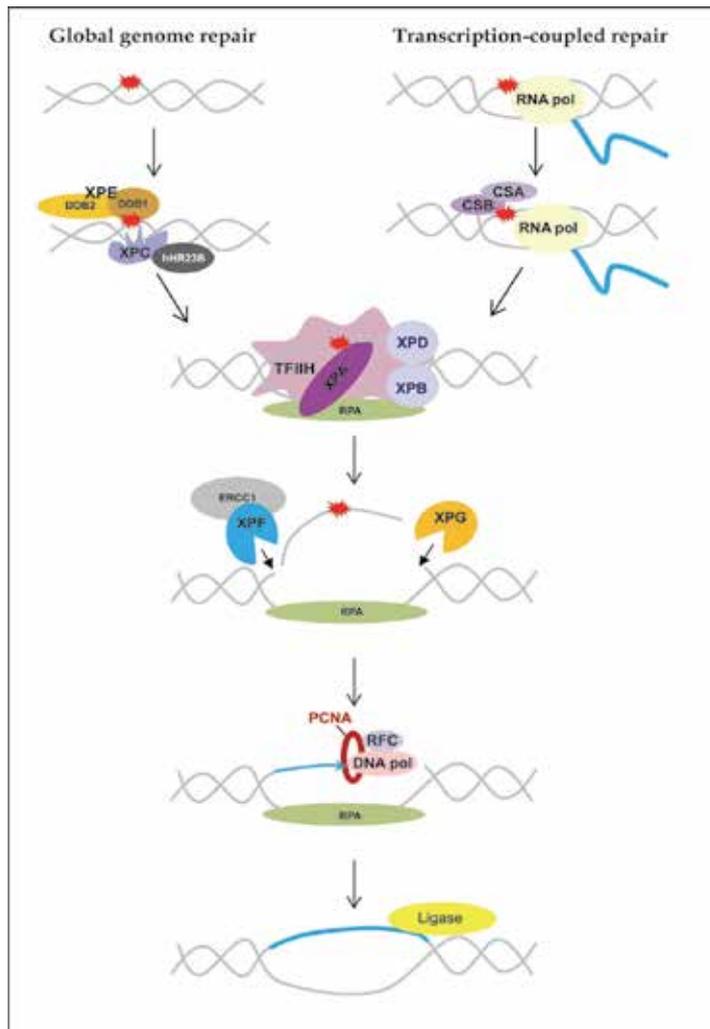


Fig. 2. Schematic representation of repair of DNA lesions by nucleotide excision repair (NER). Depending on where the DNA damage is located in the genome, it will be processed by one of the two NER subpathways: the global genome repair (GG-NER) or the transcription-coupled repair (TC-NER), that basically differ in the lesion recognition step. Lesions occurring randomly in the genome are recognized by the XPC-HR23B complex, with the participation of XPE (DDB1-DDB2) for certain lesions, both complexes are GG-NER-specific. On the other hand, lesions present in the transcribed strand of active genes that lead to the RNA polymerase arrest trigger the TC-NER subpathway, which involves the CSA and CSB proteins. The following steps are common to both subpathways. The DNA double helix around the lesion is opened by XPB and XPD (helicases belonging to the TFIIH complex) and the single strand region is stabilized by RPA, allowing damage verification by the XPA protein. The DNA around the damaged site is then cleaved by the XPF-ERCC1 and XPG endonucleases, excising an oligonucleotide of 24-32 mer, and this patch is resynthesized by the replication machinery using the undamaged strand as a template. Finally, the new fragment is sealed to the chromatin by the DNA ligase.

(XPV) patients, whose XP phenotype is related to mutations in the *POLH* gene, which encodes the translesion synthesis DNA polymerase eta responsible for the replication process on UV-irradiated DNA templates (Johnson et al., 1999; Masutani et al., 1999). A list of NER genes, which are related to XP syndrome, with their specific functions is given in Table 1.

Gene	Protein	Protein size (A.A.)	Function	Pathway
<i>XPA</i>	XPA	273	Interacts with RPA and other NER proteins, stabilizing ssDNA regions and also facilitating the repair complex assembly.	GG-NER TC-NER
<i>XPB</i>	XPB	782	Belongs to TFIIH complex, working as a 3' → 5' helicase.	GG-NER TC-NER
<i>XPC</i>	XPC	940	Responsible for lesion recognition in GG-NER.	GG-NER
<i>XPB</i>	XPB	760	Belongs to the TFIIH complex, working as a 5' → 3' helicase.	GG-NER TC-NER
<i>DDB2</i>	XPE/p48 subunit	428	Forms a complex with XPE/p127 subunit, which is believed to facilitate the identification of lesions that are poorly recognized by XPC-hHR23B.	GG-NER
<i>XPF</i>	XPF	905	Found as a complex with ERCC1, which functions as an endonuclease 5' of the lesion.	GG-NER TC-NER
<i>XPG</i>	XPG	1186	Functions as an endonuclease 3' of the lesion.	GG-NER TC-NER

*GG-NER- global genome repair; TC-NER- transcription-coupled repair.

Table 1. List of NER genes related to xeroderma pigmentosum and their roles in human DNA repair.

The Cockayne syndrome (CS) is predominantly a developmental and neurological disorder, caused by mutations leading to a defective TC-NER, which prevents recovery from blocked transcription after DNA damage. CS patients are characterized by early growth and development cessation, severe and progressive neurodysfunction associated with demyelination, sensorineural hearing loss, cataracts, cachexia, and frailty (Weidenheim et al., 2009). Curiously, although severe photosensitivity is a common feature reported for most CS patients, it is not linked to an increased frequency of skin cancers, like it is in XP patients. Interestingly, specific mutations in one of three XP genes (*XPB*, *XPB* and *XPG*) may result in a clinical phenotype which reflects a combination of the traits associated with XP and CS (XP/CS patients). This observation indicates that simultaneous defects in GG-NER and TC-NER can cause mutagenesis and cancer in some tissues and accelerated cell death and premature aging in others (Hoeijmakers, 2009).

The hallmark of trichothiodystrophy (TTD) is sulfur-deficient brittle hair, caused by a greatly reduced content of cysteine-rich matrix proteins in the hair shafts. In severe cases, mental abilities are also affected. Abnormal characteristics at birth and pregnancy complications are also common features of TTD, which may imply a role for DNA repair genes in normal fetal development (Stefanini et al., 2010). As CS patients, TTD patients do not present a high incidence of skin cancers. Genetically, three genes were identified for this disease (*XPB*, *XPD* and *TTDA*), but most TTD patients exhibit mutations on the two alleles of the *XPD* gene (Itin et al., 2001).

Cerebro-oculo-facial-skeletal syndrome (COFS) is a disorder determined by mutations in *CSB*, *XPD*, *XPG* and *ERCC1* genes, leading to a defective TC-NER (Suzumura & Arisaka, 2010). It is characterized by congenital microcephaly, congenital cataracts and/or microphthalmia, arthrogryposis, severe developmental delay, an accentuated postnatal growth failure and facial dysmorphism.

Photosensitivity and freckling are the main features of patients with UV-sensitive syndrome (UVsS), but these patients have mild symptoms and no neurological or developmental abnormalities or skin tumors. Although other genes may be involved, mutations in the *CSB* gene were found in some of these patients, leading to defective TC-NER of UV damage (Horibata et al., 2004; Spivak, 2005).

Therefore, the general relationship between defects in NER genes and clinical disease phenotypes is complex, since mutations in several genes can cause the same phenotype, and different mutations in the same gene can cause different phenotypes (Kraemer et al., 2007).

Even though DNA repair malfunctions are autosomal recessive diseases and their incidence is therefore relatively low (~1/100,000), many of the individuals with DNA repair deficiencies die in early childhood since there is no effective treatment, only palliative care. Therefore, the search for a long-term treatment has been intense. Several strategies using recombinant viral vectors are being used in order to improve the resistance of cells from these patients to DNA damaging agents (Lima-Bessa et al., 2009; Menck et al., 2007). Also, the studies of DNA repair mechanisms have yielded a better understanding of specific cell processes which lead to human diseases such as cancer, neurodegeneration and aging (Hoeijmakers, 2009). This review will focus on the use of recombinant viral vectors for the purposes of investigating both the cellular responses to DNA damage and the perspectives of providing therapy for XP patients.

2. Recombinant viral vectors as gene delivery tools

An ideal gene delivery tool should have the ability to transduce proliferating and fully differentiated cells with high efficiency; mediate high-level, prolonged and controlled transgene expression; have little toxicity (both at cellular and organism levels); elicit small immune responses *in vivo*; and be able to accommodate large DNA fragments for transgene transduction (Howarth et al., 2010). Unfortunately, there is no single tool that fulfills all these criteria.

Viruses have had million of years to improve their capacity to infect cells with the aid of evolutionary pressures. Researchers have been trying to take advantage of this ability creating recombinant viral vectors. In general, for that purpose, the viral genome is manipulated and sequences needed to form the infective virion are deleted, opening space to insert the transgene of interest.

Several viral vectors have been created and the most widely used are: adenovirus, retrovirus (including lentivirus) and adeno-associated virus. The main characteristics of these vectors are presented in Table 2.

Virus	Nucleic acid	Genome size (Kb)	Envelope	Virion size (nm)	Integration	Transgene size (Kb)	Immune response	Transgene expression
Adenovirus	dsDNA linear	36		90	episomal	8 - 25	***	days - months
Adeno-associated virus	ssDNA linear	4.7		25	site-specific	4.7 - 9	*	months-years
Retrovirus	ssRNA (homodimer)	7 - 12	*	100	random	<10	**	years
Lentivirus	ssRNA (homodimer)	9	*	100	random	10 - 16	**	years

Table 2. Main features of viruses currently used as recombinant vectors for gene delivery.

Searching for the perfect gene delivery tool, intense modifications have been added to the vectors' genomes, nucleocapsid and envelopes, always searching for less immunogenic vectors, with higher and more specific transduction properties. Currently, recombinant viruses are the vector of choice for research and clinical trials worldwide, but still only few phase II or III trials are being conducted (Atkinson & Chalmers, 2010). All viral vectors cited here have already been used in *in vitro*, *ex vivo* and *in vivo* experiments and in clinical trials.

2.1 Recombinant adenoviral vectors

Adenoviruses (Ad) are non-enveloped double-stranded DNA viruses with tropism for the respiratory and ocular tissues. The first generation recombinant vector can carry up to 8 Kbp of DNA, while the last generation, in which the viral DNA sequence is completely deleted (also named gutless), is able to efficiently transduce over 25 Kbp of DNA (Atkinson & Chalmers, 2010).

Despite the fact that the gutless vector needs the aid of helper viral proteins supplied *in trans*, adenoviral vectors are easily produced in high titers. Once the transgene has been delivered inside the nucleus it remains episomal, reducing the risk of tumorigenesis induced by insertional mutagenesis. On the other hand, the episomal DNA is not replicated and its segregation in mitosis leads to the eventual loss of the transgene in the daughter cells. Thus, the transgene expression is short-lived. A possible solution is to add a site-specific integration sequence next to the transgene, leading to a prolonged transgene expression (Atkinson & Chalmers, 2010). Another advantage of the adenoviral vectors is their ability to transduce post mitotic cells since the transgene is already delivered in its active form, as a double-stranded DNA. This property is of particular interest when aiming for gene therapy in neurons (Atkinson & Chalmers, 2010).

The biggest challenge for the use of adenoviral vectors *in vivo* is the immunological response it elicits. This strong response is not only due to the natural immunogenicity of its components, but also to pre-existing immunity caused by previous contact with at least one of the over 50 serotypes of human infecting adenovirus (Seregin & Amalfitano, 2009). Taking into consideration that these vectors are only capable of a transient expression of the transgene and that repeated dosage might be necessary, a strong immune response is very

undesirable. Possible alternatives to circumvent this issue are: manipulation of the viral capsid proteins and DNA, making them less immunogenic; the usage of a different serotype on each application; and the use of immunosuppressants (Atkinson & Chalmers, 2010; Seregin & Amalfitano, 2009).

The great importance of the immunological response against a gene therapy vector was brought to attention when, in 1999, a patient suffering from an ornithine transcarbamylase deficiency, died due to an unexpected inflammatory response reaction to the adenoviral vector used in a clinical trial (Edelstein et al., 2007). Still, adenoviral vectors are currently the most widely used viral vectors in clinical trials, accounting for approximately 24% of all vectors used in gene therapy clinical trials (Edelstein et al., 2007; Hall et al., 2010).

2.2 Recombinant adeno-associated viral (AAV) vectors

Adeno-associated viruses (AAV) are non enveloped, single-stranded DNA, with serotype-specific tropism viruses. To date, 12 serotypes have been identified in primate or human tissues (Schmidt et al., 2008) in a total of over 100 known serotypes (Wang et al., 2011). Their productive lytic infection depends on the presence of a helper virus, adeno or herpesvirus, that provide *in trans* the necessary genes for the AAV replication and virion production. In the absence of a helper virus, the AAV establishes its latent cycle integrating specifically in the 19q13.4 region of the human genome (Daya & Berns, 2008). The site-specific integration is mainly dependent on the virus internal terminal repeats (ITRs), the integration efficiency element (IEE) and Rep 68 and Rep 78 genes. In the 19q13.4 region, several muscle-related genes are present, including some responsible for actin organization. No significant side effects have been observed due to AAV genome integration in this chromosome region (Daya & Berns, 2008).

The onset of transgene expression delivered by an AAV vector is delayed, usually starting several days after the transduction, probably due to the time invested in the synthesis of the DNA second strand (Michelfelder & Trepel, 2009). Although late, the transgene expression is long lasting and there is a very low humoral response, mainly related to previous exposure to the viral antigens (Daya & Berns, 2008). Despite the small size of the AAV nucleocapsid and genome, it has been shown that transgenes up to 7.2 Kb can be delivered by AAV vectors, but the oversized genomes reduce at least 10 fold the transduction efficiency (Dong et al., 2010). Several strategies have been developed seeking to optimize the vector capacity, such as the *trans*-splicing vector. With the simultaneous usage of two AAV vectors, this technology takes advantage of the concatamers formed by the ITRs that can recombine to form the desired transgene inside the transduced cell. These trans-splicing vectors allow the final transgene to have up to 9 Kb (Daya & Berns, 2008).

Only recently adeno-associated viral vectors started being used in gene therapy research and account for less than 4% of all vectors used in gene therapy clinical trials (Edelstein et al., 2007; Hall et al., 2010). Although these vectors do not behave as the parental virus, since they do not integrate in the genome (due to the lack of the REP protein), gene expression can be very long and elicit low immunological responses, making AAV vectors promising in gene therapy investigations.

2.3 Recombinant retroviral vectors

The *Retroviridae* family is characterized by a single-stranded RNA genome which can only replicate inside the host cell with the aid of an RNA-dependent DNA polymerase, the reverse transcriptase. This enzyme transcribes the virus' RNA into a DNA sequence that the host cell machinery can transcribe and translate (Froelich et al., 2010).

Retroviral vectors are capable of transducing a wide range of cell types, are able to accommodate extensive changes in their genome, accept long transgenes, have low immunogenicity, can be produced in high titers, and promote a prolonged transgene expression due to their ability to integrate into the host cell genome (Froelich et al., 2010). On the other hand, most retroviral vectors can only transduce replicating cells since the transport of the transcribed viral DNA to the nucleus is mitosis-dependent. Additionally, there is always the risk of insertional mutagenesis due to the semi-random integration of the vector genome in the host cell's genome (Froelich et al., 2010). Nowadays, the most widely used retroviruses as gene therapy tools are the lentiviruses (LVs), such as the human immunodeficiency virus (HIV). These vectors have the same advantages as other retroviral vectors and are capable of transducing post mitotic cells. Moreover, the LVs tend not to integrate by transcription initiation sites, reducing the risk of insertional tumorigenesis (Froelich et al., 2010).

The retroviral vectors were the first vectors used in gene therapy clinical trials in 1989 (Edelstein et al., 2007, Rosenberg et al., 1990) and are extensively used in fundamental biological research, functional genomics and gene therapy (Mátraai et al., 2010). In 2004, 28% of the clinical trials involving viral vectors included retroviral vectors (Edelstein et al., 2007); in 2010 that number dropped to approximately 23% (Voigt et al., 2008). This drawback is due to the unfortunate events of the French severe combined immunodeficiency (SCID) trial in 2002, where two out of ten children died in consequence of a leukemia, which was related to the insertional mutagenesis of the retroviral vector used (Edelstein et al., 2007).

Since then, special attention has been paid to the safety of these vectors as many are known to derive from viruses that cause severe diseases, such as the acquired immunodeficiency syndrome (AIDS). Strategies are constantly developed to prevent the risk of insertional mutagenesis. For that purpose, in addition to the virions being replication-defective, generated by *trans*-complementation, several further manipulations of the viral genome were made. The development of a self-inactivating (SIN) vector (Iwakuma et al., 1999) prevents horizontal and vertical gene transfer and diminishes the probability of the production of a replicating virion or over-expression of a host cell oncogene (Edelstein et al., 2007).

3. Investigating DNA damage responses with adenoviral vectors in human cells

3.1 *In vitro* and *in vivo* adenoviral gene transduction for the correction of DNA repair defects

The knowledge of the molecular defects in XP cells was the starting point for understanding how human cells handle lesions in their genome. So far, different techniques have been used to study DNA repair mechanisms and reverse malfunctions in this essential system. One powerful tool employed in these studies has been the use of recombinant adenoviral vectors to transduce DNA repair genes directly into human skin cells, aiming to improve the knowledge of basic mechanisms that cells use to protect their genome.

Experiments using first generation recombinant adenoviral vectors have been successfully employed in the transduction of both SV40-transformed and primary fibroblasts derived from XP-A, XP-C, XP-D and XP-V patients (Armellini et al., 2007). The expression of the respective functional proteins in all transduced defective cell populations was significantly increased, reaching levels even higher than seen for wild type cells (Armellini et al., 2005;

Lima-Bessa et al., 2006; Muotri et al., 2002). Moreover, different phenotypical analyses, including cell cycle, apoptosis and cell survival assays, have been carried out, all indicating that the protein expression mediated by the recombinant adenoviruses was clearly accompanied by the recovery of the DNA repair ability and increased resistance to UV radiation, thereby demonstrating functional correction of the XP phenotype. It is worth mentioning that, even though transgene expression mediated by adenoviruses is typically short-lived, sustainable high expression of XPA and XPC proteins with parallel increased UV-irradiation resistance was obtained even two months after cell transduction (Muotri et al., 2002).

For XP-A, XP-C and XP-D transduced cell lines, phenotypic analyses also involved assays aiming to investigate their ability to perform DNA repair after UV irradiation. This has been measured through determination of unscheduled DNA repair synthesis (UDS), which corresponds to the incorporation of [methyl-³H] thymidine in cells that are not in S-phase, and is visualized by autoradiography as the presence of radioactive grains inside nuclei. Interestingly, UDS activity in all transduced deficient cell lines was restored to levels comparable to NER proficient cell lines, indicating those cells became able to efficiently remove UV lesions by restoring NER activity.

It is well known that UV radiation promotes DNA elongation delay as a result of replication blockage by UV photolesions (Cleaver et al., 1983), which can be easily seen by running pulse-chase experiments in alkaline sucrose gradients. Using this approach, it has been possible to show that XP-V transduced cells were able to elongate nascent DNA on UV-damaged DNA templates as efficiently as wild type cells (Lima-Bessa et al., 2006), once again demonstrating the great potential of recombinant adenoviruses in the transduction and expression of functional proteins.

One interesting conclusion came from the observation that even though *XPA*, *XPC* and *XPB* genes were over-expressed in all transduced cell lines when compared to NER proficient cells, this had no impact in the UV-resistance or NER capability, suggesting that neither of these proteins is limiting for NER in human cells. Another possible explanation is that once the NER pathway requires a coordinated action of several proteins, increasing only one of these proteins does not result in speeding up removal of the DNA lesions. Similarly, the excess of pol η (*XPV*) mediated by adenoviral transduction has not affected cell survival nor elongation of replication products in UV-treated XP-A human cells, suggesting not only that pol η is not a limiting factor for the efficient replication of the UV-damaged DNA in XP-A cells, but also demonstrating that the deleterious effects caused by the remaining DNA lesions in the genome cannot be mitigated by an efficient bypass mediated by pol η .

However, the potential of such vectors is not restricted to *in vitro* assays. Indeed, another real perspective is their use to investigate the molecular mechanisms of DNA repair and their consequences *in vivo*, thus opening new avenues for a better understanding of cellular and physiologic responses to DNA damage. *In vivo* experiments may also help to establish the relationship between DNA repair, cancer and aging, as mice models for different DNA repair syndromes have been developed by different groups worldwide. Despite the extensive use of these models to broaden the understanding of several DNA repair related disorders, little work has been done *in vivo* testing gene therapy strategies for these diseases. Indeed, up to the present moment, only one study showed an efficient *in vivo* gene therapy protocol for complementation of the XP phenotype (Marchetto et al., 2004).

Exciting results by Marchetto and co-workers showed that the administration of subcutaneous injections of an adenoviral vector carrying the *XPA* human gene directly into

the dorsal region of XP-A knockout mice led to an extensive expression of the heterologous protein in different skin cells, including dermal fibroblasts, cells of the hair follicle and basal replicating keratinocytes, which are believed to be the starting point of most skin tumors. As a result, the repair capability of these transduced cells was restored, thus preventing UVB-induced deleterious skin effects, such as persistent scars, skin hyperkeratosis and, ultimately, avoiding the formation of squamous cell carcinomas (Marchetto et al., 2004). Despite the promising results of this work, no others followed. Researchers are now aware of several possible limitations and complications of gene therapy after some unexpected severe events in clinical trials (Edelstein et al., 2007) and are spending more time improving gene targeting tools and techniques before risking *in vivo* approaches. In that sense, extreme progress has been made with experiments *in vitro*, as previously presented. A general panel showing the main uses of the recombinant adenoviral vectors carrying DNA repair genes is presented in Figure 3.

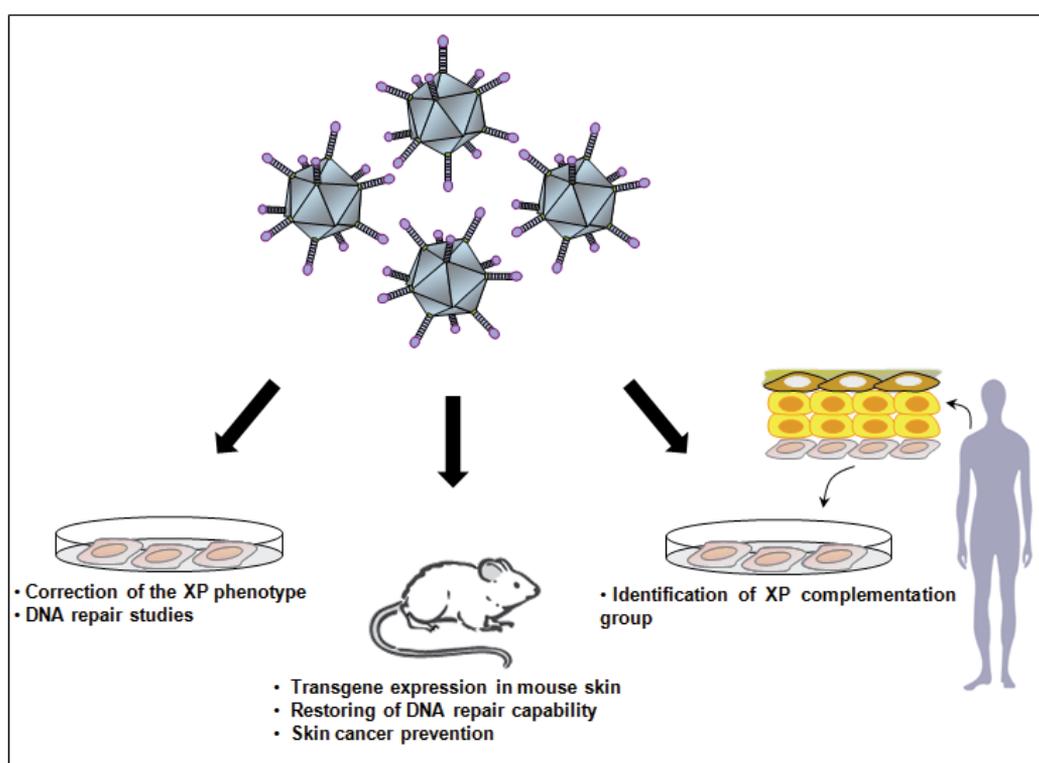


Fig. 3. DNA repair gene transduction by recombinant adenoviruses. Adenoviral vectors have been successfully employed to transduce human XP genes directly into established human cell lines (left), XP knockout mice skin (center), and fibroblasts from the skin of XP patients (right). Endpoints are indicated for each particular case.

Based on the successful complementation of the XP phenotype both *in vitro* and *in vivo*, adenoviral vectors could be proposed as an efficient tool for diagnosis and identification of XP patients' complementation groups. This hypothesis was recently tested and confirmed: with the use of adenoviruses carrying DNA repair genes, it has been possible to determine

the complementation group of three Brazilian XP patients, now characterized as XP-C patients. To that end, adenoviral transduced cells from these patients have been submitted to UV treatment and then analyzed by simple assays, such as cell survival and UDS (Leite et al., 2009). This diagnosis has been performed using the patients' skin fibroblasts but the potential use of adenoviral vectors for this purpose becomes even more exciting, considering that the adenoviral transduction could be held in cells present in the patients' blood, thus becoming a faster and less invasive technique. Besides scientific and epidemiological goals, the identification of the gene defect may help to predict clinical prognosis for the XP patients and guide appropriate genetic counseling for their families. Direct gene sequencing can be performed to identify the mutated genes, but as there are eight potential candidate genes for XP, functional complementation assays are still used for the genetic diagnosis of these patients.

3.2 Investigating UV-induced cell responses employing photolyases

Photoreactivation is a very efficient DNA repair mechanism, which specifically removes the two main UV photoproducts. Photoreactivation is carried out by flavoproteins known as photolyases. These enzymes recognize and specifically bind to UV lesions, thus reverting them back to the undamaged monomers, using a blue-light photon as energy source (Brettel & Byrdin, 2010; Sancar, 2008). Interestingly, photolyases demonstrate a great efficiency for discriminating the target lesion, either CPDs or 6-4PPs, and so far no photolyase has been shown to be able to repair both lesions. Thus, enzymes that repair CPDs are referred to as CPD-photolyases, while 6-4PP-photolyases specifically repair 6-4PPs (Müller & Carell, 2009). Both classes of photolyases are evolutionarily related, but functionally distinct (Lucas-Lledó & Lynch, 2009). Curiously, genes encoding genuine photolyases have been lost somehow in the course of the evolution of placental mammals, including humans. Instead, these organisms retain cryptochromes, photolyase-homologous proteins that participate in the maintenance of circadian rhythm, but that do not keep any residual activity related to DNA repair (Partch & Sancar, 2005).

Previous studies have confirmed that the CPD-photolyase is active when delivered to human cells, reducing mutagenesis (You et al., 2001), preventing UV-induced apoptosis (Chiganças et al., 2000) and recovering RNA transcription driven by RNA polymerase II (Chiganças et al., 2002). These successful studies have motivated the adenoviruses-mediated expression of the CPD-photolyase from the rat kangaroo *Potorous tridactylus* and the plant 6-4PP-photolyase from *Arabidopsis thaliana* in human cells aiming to discriminate the precise role of UV-induced cellular responses in both NER-deficient and NER-proficient human cells. Employing immunofluorescence, immunoblot and local UV experiments, it has been possible to see that these enzymes are not only very specific for their lesions, but are also really fast to find them, colocalizing with regions of damaged DNA and other DNA repair enzymes in less than two minutes (Chiganças et al., 2004; Lima-Bessa et al., 2008).

Adenoviral-mediated photorepair of CPDs substantially prevented apoptosis in all UV-irradiated cell lines (both NER-deficient and NER-proficient cells), confirming the involvement of these lesions in cell death signaling, as previously reported. On the other hand, 6-4PP repair by the 6-4PP-photolyase decreased UV-induced apoptosis only in those cell lines deficient for both NER subpathways, causing minimal effect, if any, in NER-proficient cells, including those lacking pol η . These results suggest that, when not efficiently repaired, 6-4PPs also have important biological consequences, triggering cell responses

leading to the activation of apoptotic cascades. Interestingly, in CS-A cells (TC-NER deficient), a substantial attenuation of apoptotic levels could be again detected when CPDs were removed from the genome by the means of CPD-photolyase, while no detectable effect was observed as a consequence of photorepair of 6-4PPs, indicating that CPD lesions are the major UV-induced DNA damage leading to cell death, also in cells that are only proficient in GG-NER, the main subpathway of NER responsible for the removal of 6-4PPs in humans (Lima-Bessa et al., 2008).

These results suggest that CPDs and 6-4PPs may play different roles in UV-induced apoptosis depending on the repair capacity of human cells. In GG-NER proficient cells, the harmful effects of UV light seem to be predominantly due to the prolonged remaining CPDs in the genome caused by their slow removal by NER, with the minor participation of 6-4PPs (Lima-Bessa et al., 2008). Indeed, it has been reported that about 80–90% of 6-4PPs are removed from the human genome in the first 4 hours following UV exposure, whereas 40–50% of CPDs still remain to be repaired 24 hours later, probably due to the higher affinity of the XPC/hHR23B complex for 6-4PPs (Kusumoto et al., 2001). Thus, the lack of noticeable effects on UV-induced apoptosis in NER-proficient cells after 6-4PPs photorepair may be simply due to their fast repair by GG-NER. On the other hand, as for CPDs, the remaining of 6-4PPs in the genome seems to cause major disturbances in cell metabolism that lead to cell death. A summary of these results is shown in Figure 4.

To further confirm the idea that the roles of CPDs and 6-4PPs in UV-killing are related to the cellular repair capacity, authors have expressed these photolyases in TTD1V1 cells, a particular TTD cell line with a slower kinetics of 6-4PPs repair, eliminating about 50% and 70% of 6-4PPs at 6 and 24 hours post-UV treatment, respectively. Once again, repair of both lesions by the respective photolyase notably reduced apoptosis in these cells, even though the 6-4PP photorepair was less effective than seen for NER-deficient cell lines (Lima-Bessa et al., 2008). These photolyases were also used to identify a defect in the recruitment of downstream NER factors on certain XPD/TTD mutated cells, slowing down the removal of UV-induced lesions. As this recruitment was recovered by treatment with the histone deacetylase inhibitor trichostatin A, the data indicated that this defect is partially related to the accessibility of DNA damage in closed chromatin regions (Chiganças et al., 2008).

Another interesting finding came from assays investigating the time-dependent kinetics of the apoptosis commitment after UV treatment. Transduced XP-A cells were UV-treated and photoreactivated (to allow photorepair of the respective UV lesions) at increasing periods of time. Surprisingly, the data suggests that the initial trigger event to cell death after UV irradiation is relatively delayed, since photorepair of CPDs or 6-4PPs was able to reduce apoptosis even when photoreactivation was performed up to 8 hours after UV irradiation. After that, photoreactivation did not prevent UV-killing in these cells, indicating a commitment by events that irreversibly lead to cell death. These results are also in agreement with the indications that fast removed lesions (such as 6-PPs) do not activate apoptosis in NER-proficient human cells (Lima-Bessa et al., 2008). The main implication of all these findings is the fact that skin carcinogenesis in XP patients may also have 6-4PP lesions as important players, suggesting that tumors from these individuals are not only quantitatively different from those of normal people, but may also have different causative lesions. Transduction of XP knockout mice with adenoviral vectors carrying photolyase genes may help to address this question.

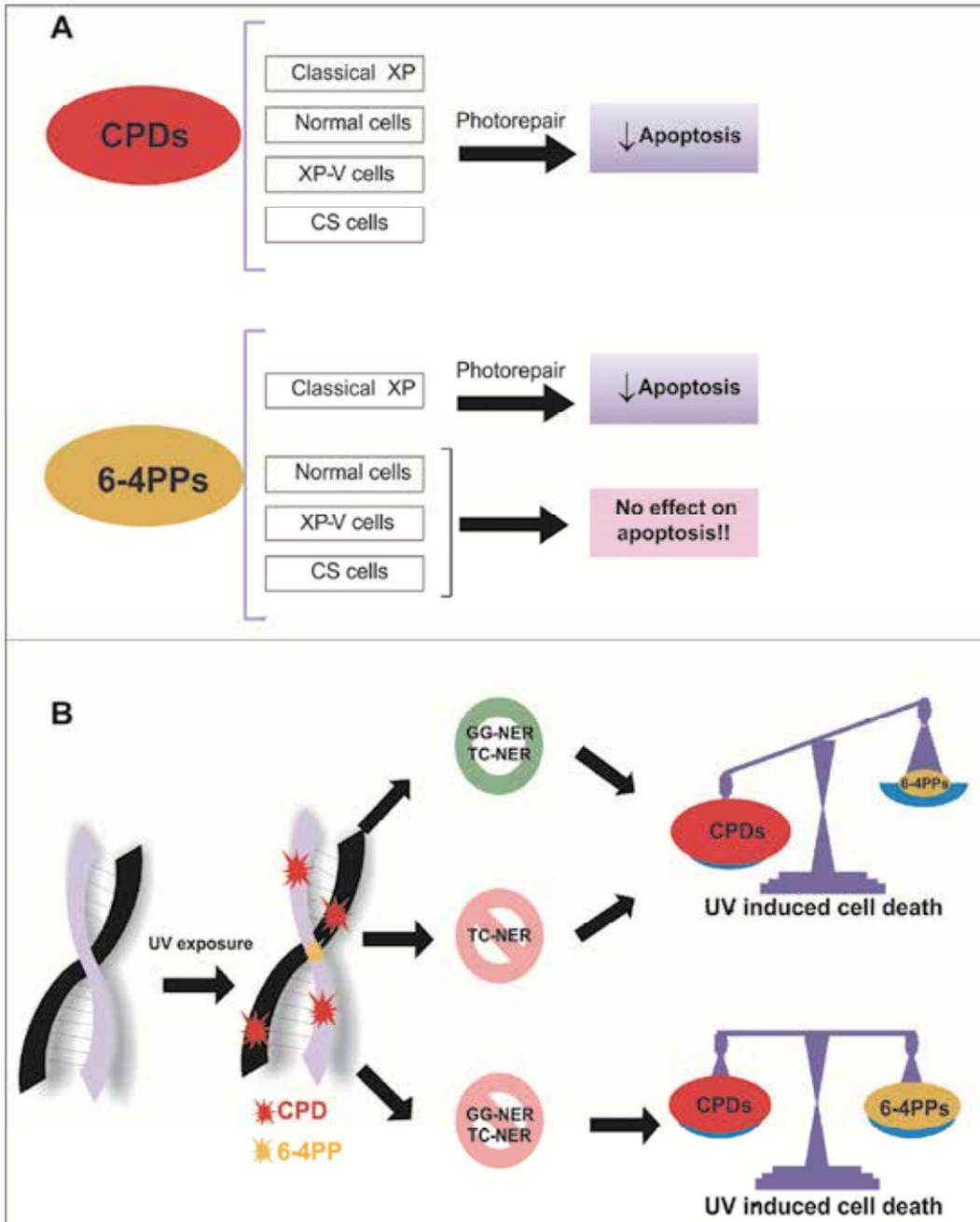


Fig. 4. Effects of photorepair of CPDs and 6-4PPs on UV-induced apoptosis. (A) Summary of the impact of the specific removal of CPDs and 6-4PPs by photorepair in human cell lines with different DNA repair capabilities. (B) Schematic representation of the main conclusions of the results shown in panel A. Those results clearly implicate that CPDs and 6-4PPs play different roles on UV-induced apoptosis depending on the cellular repair capacity.

4. Employing retroviral vectors for correcting XP phenotype

The first genetic analysis of XP patients was performed through somatic cell fusion followed by analysis of restoration of normal UDS. If somatic cell fusion complements XP genetic deficiency, it will then be positive for UDS activity. These experiments were able to identify the seven classical XP complementation groups and the variant group (Zeng et al., 1998). This implies that DNA repair deficiencies can, in fact, be corrected by the introduction of a normal copy of the affected gene, giving hope for the development of gene therapy protocols for XP patients. In fact, the introduction of a normal copy of the defective gene in XP cells can complement the DNA repair ability, as demonstrated by the delivery of conventional expression vectors, via calcium precipitation and microneedle injection (Mezzina et al., 1994).

In 1995, viral vectors were first used as gene delivery tools in DNA repair experiments (Carreau et al., 1995a). In this study, a LXPDSN retroviral vector carrying the wild-type XPD gene was capable of complementing primary fibroblasts of XPD patients with a long-term expression. A subsequent study showed that this complementation was gene-specific and that there was a long-term expression of the transgene (Quilliet et al., 1996). The use of retroviral vectors for DNA repair genes delivery was further validated in 1996 and 1997, when XP-A, XP-B, XP-C and TTD-D cells were also complemented with the aid of gene-specific retroviral vectors (Marionnet et al., 1996; Zeng et al., 1998).

The compilation of these results shows that the retroviral delivery of several DNA repair genes was able to specifically complement several deficiencies presented by XP, CS and TTD patients such as UDS, reduced catalase activity, UV-sensitivity, recovery of RNA synthesis, increased mutation frequency, stabilization of p53 (Dumaz et al., 1998) and deregulation of ICAM-1 (Ahrens et al., 1997).

Since XP patients already receive autologous graft transplants after massive skin tissue removal surgery (Atabay et al., 1991; Bell et al., 1983), most researches in the field of XP gene therapy focus on the three-dimensional skin reconstruction *in vitro*, using the patients' cells genetically corrected *ex vivo*. In this technique, the patients' fibroblasts and keratinocytes are cultured *in vitro* after a skin biopsy of a non-UV-irradiated area. Then, retroviral vectors are used to stably complement the genetic deficiency of these cells. Finally, the keratinocytes and the fibroblasts are used to three-dimensionally reconstruct the epidermis and dermis, respectively. This construct can then be used as a graft when the part of patient's damaged skin is removed in a necessary surgery. To that end, Arnaudeau-Bégard and co-workers managed to complement XP-C keratinocytes, recovering a wild-type phenotype and UV-resistance with the aid of a retroviral vector carrying a normal copy of the XPC gene (Arnaudeau-Bégard et al., 2003). Furthermore, Bergoglio and co-workers have also developed a selection method for genetically corrected keratinocytes that does not involve particles derived from microorganisms which could lead to immunological clearance of the transgene, using CD24 as an ectopic marker (Bergoglio et al., 2007).

In 2005, Bernerd and co-workers were able to reconstruct a three-dimensional skin model *in vitro* using fibroblasts and keratinocytes from a donor XP-C patient. With this model, they were able to see that the XP skin has peculiar characteristics: hypoplastic horny layers, decreased and delayed keratinocyte differentiation, epidermal invaginations, a generally altered proliferation control and fibroblasts with distinct morphology and orientation. Furthermore, the epidermal invaginations were proven to be related to alterations of both keratinocytes' and fibroblasts' functions and were characterized as epidermoid carcinoma-like structures (Bernerd et al., 2005). It is important to keep in mind that an XP skin biopsy

might give us further and more precise knowledge of the XP skin physiology, but this is a delicate procedure which requires the patients' agreement.

Since the use of common retroviral vectors in gene therapy can be dangerous due to semi-random insertional mutagenesis, researchers have developed several self-inactivating-lentiviral vectors carrying DNA repair genes. These vectors were shown to efficiently transduce primary and transformed fibroblasts, complementing in a gene-specific manner XP-A, XP-C and XP-D cells. Furthermore, the recovery of normal levels of UV-resistance in the transduced cells was shown to be persistent for at least 3 months (Marchetto et al., 2006). The reconstruction of a genetically corrected, three-dimensional XP skin followed by the implantation of the graft on a patient (Figure 5) is still an ongoing chore that has to be taken very cautiously, always prioritizing the patient's well-being.

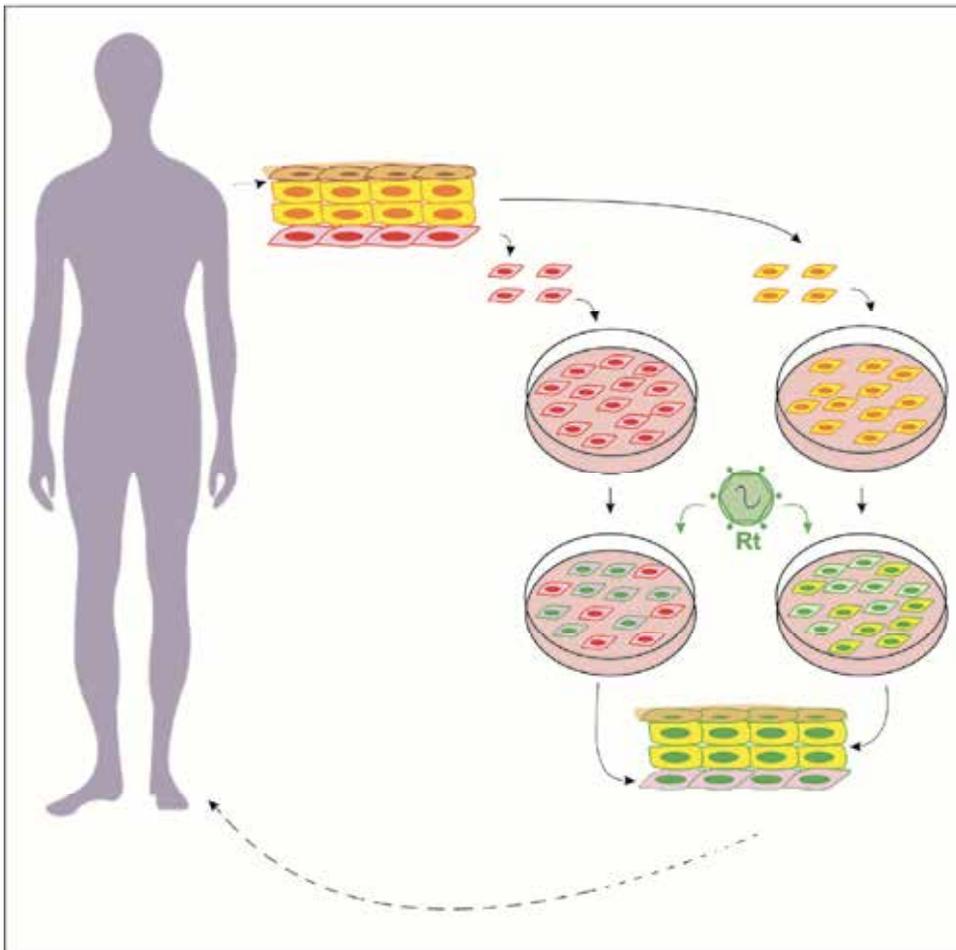


Fig. 5. Schematic representation of *ex vivo* gene therapy for XP patients using recombinant retrovirus (Rt). Skin-derived fibroblasts and keratinocytes from an XP patient are cultivated *in vitro*, and transduced with retroviral vector carrying the wild type XP cDNA. Transduced cells are then used to reconstruct the human skin *in vitro*, with a normal phenotype. Dashed line raises the possibility of engraftment of the reconstructed skin directly on XP patients.

It is also important to keep in mind that these grafts do not include melanocytes, responsible for the very common melanomas in these patients (Khavari, 1998), and that the skin will only be genetically complemented in the areas that receive the grafts, all the other areas of the body will still be extremely photosensitive since no paracrine effect is known for DNA repair proteins and that immunological clearance or gene silencing by cellular methylation can always prohibit a long-term transgene expression (Magnaldo & Sarasin, 2002). Importantly, several XP complementation groups also present other relevant symptoms, such as neurodegeneration, which will not be improved by the skin grafts. For those patients, another kind of gene therapy might be more efficient, such as the development of genetically corrected stem cells (ESs) (Magnaldo & Sarasin, 2002) or induced pluripotent cells (iPSCs, see below (Alison, 2009)). Unfortunately, there is still no reference on that kind of research for xeroderma pigmentosum.

5. Host cell reactivation (HCR) as a tool for DNA repair research

The host cell reactivation (HCR) technique was first described in human cells by Protic-Sabljić and co-workers in 1985 (Protic-Sabljić et al., 1985). In this first work, the technique consisted of transducing cells with a plasmid containing a putative cDNA with a selective gene into XP cells to look for a reversion of the UV sensitivity due to gene complementation, allowing identification of the genes responsible for that phenotype.

Other studies have refined the technique which is now widely used as an indirect measure of cellular DNA repair capacity. Mostly, a plasmid containing a reporter gene such as luciferase (LUC) or chloramphenicol acetyltransferase (CAT) is treated with a genotoxic agent such as UV radiation and introduced in the cell where DNA repair capacity is to be evaluated. If the cell is able to remove the lesions from the plasmid, the reporter gene will be expressed. Different DNA repair rates can be addressed by differences on the amount of gene reporter expression at a certain time (Merkle et al., 2004). A schematic representation of HCR is shown in Figure 6.

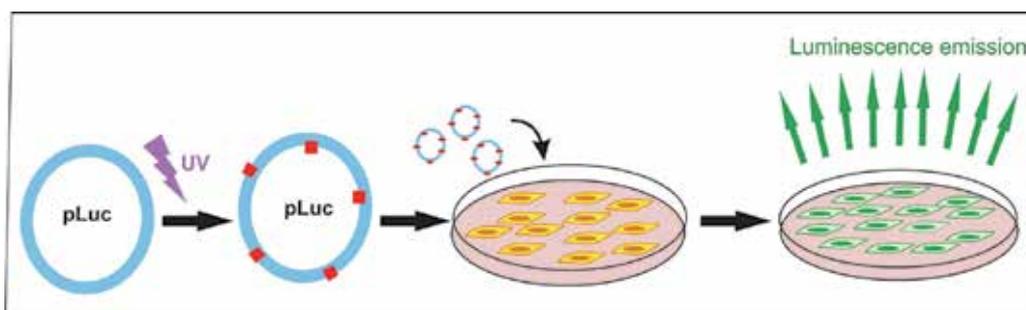


Fig. 6. Schematic representation of host cell reactivation (HCR) assay. A plasmid carrying a reporter gene (in this case, the luciferase gene) is UV-irradiated *in vitro* and then transfected into host cells. 48 hours later, the cellular DNA repair capacity is indirectly estimated by measurement of the reporter gene activity in the cellular extract.

In 1995, the HCR assay was further used to visualize the genetic complementation of mammalian expression vectors carrying the DNA repair genes *XPA*, *XPB*, *XPC*, *XPD* and *CSB*. In this study, plasmids containing LUC or CAT were UV irradiated and co-transfected with the plasmids containing each of the complementing genes of the DNA-repair deficient

cells. Again, only the cells with the correct complementation were capable of removing the DNA damage in the reporter gene, allowing the expression of that protein. This technique facilitates the identification of the complementation group of a given patient, being particularly useful in cases of CS, TTD and some XP patients such as XP-E that present a normal UDS after UV treatment (Carreau et al., 1995b).

Recent data using the HCR assay has shown that the CS proteins are essential for the reversion of oxidated lesions (Pitsikas et al., 2005; Spivak & Hanawalt, 2006; Leach & Rainbow, 2011) and evidence obtained with HCR suggests that, unlike what was previously shown with UDS assays, DNA repair capacity in fibroblasts does not decrease with aging (Merkle et al., 2004). This reduction may however be cell type-specific and DNA repair pathway-specific since blood cells repair capacity decreases approximately 0.6% per year of age (Moriwaki et al., 1996). This technique is still widely used and its great advantage is that the DNA plasmids or the viral vectors are treated in a controlled manner, not being subject to the cell's global response to the same treatment. Further technique improvements will surely allow HCR to be used in different assays such as *in vivo*, yielding a better knowledge of the DNA repair pathways and their interactions with other pathways and physiological events.

6. Other treatments for xeroderma pigmentosum

6.1 General care

There is no treatment that has been proven so far to be 100% effective in all XP cases. The only palliative measure that patients can rely on is complete sun avoidance. This includes not only avoiding going out even on cloudy days and covering all exposed body areas such as skin and eyes, but also using special artificial lights that emit no UV wavelengths (Kraemer, 2008). Premalignant lesions, such as actinic keratoses, and malignant lesions must be quickly treated with topical 5-fluoracil or liquid nitrogen, imiquimod cream, electrodesiccation and curettage, surgical excision or chemosurgery, as needed. When extensive areas are damaged and have to be surgically removed, skin grafts from sun unexposed areas of the same patient should be used. When eyes are affected, methylcellulose eye drops or contact lenses can help prevent trauma and corneal transplantations might be needed in extreme cases (Kraemer, 2008).

When caring for XP patients, it is very important to keep in mind that the total sun avoidance also prevents the production of vitamin D in the skin, so dietary supplementation might be needed. Furthermore, the DNA repair deficiencies which prevent the repair of photolesions may also make individuals sensitive to other mutagens such as cigarette smoke, so patients should be protected against these agents (Kraemer, 2008).

Aside from removal of local lesions and total sun avoidance, two other palliative treatments might help improving the XP patient's quality of life: topical use of T4 endonuclease (Yarosh et al., 2001) and oral intake of retinoids (Campbell & DiGiovanna, 2006).

6.2 Topical use of T4 endonuclease

In 1975, Tanaka and co-workers demonstrated that the bacteriophage T4 endonuclease V is capable of making an incision 5' within a CPD lesion. The resulting DNA flap is recognized and removed by a 5'→3' exonuclease, leaving a gap that is filled by a DNA polymerase, using the undamaged single strand as a template. A DNA ligase then joins the repaired fragment to the parental DNA (Tanaka et al., 1975).

In the 80's, Yarosh's laboratory discovered that the T4 endonuclease V could be delivered into cells using 200 nm liposomes as a delivery vehicle. The anionic liposomes not only protect the cationic enzyme inside, but also promote the escape from a clatrin-coated endosome after cellular intake by destabilizing the vesicle's membrane with an acid pH. By cleaving DNA at the site of UV-induced lesions, the enzyme reverses the DNA repair defect of XP cells (Yarosh, 2002). Further work by the same group also showed that these T4N5 liposomes in a 1% hydrogel lotion when applied in cultured human fibroblasts, mouse dorsal back or cultured human breast skin is capable of delivering the enzyme into cells in less than one hour, being almost entirely restricted to the epidermis (Ceccoli et al., 1989; Kibitel et al., 1991).

An inverse correlation was later shown between the T4N5 dose and the level of CPDs that remained in the epidermis. This curve reached a plateau (at 0.5 µg/ml), probably due to saturation of the cell machinery for further repairing the damage after the initial incision by the T4 enzyme (Yarosh et al., 1994). These studies also showed that even in the higher dose of T4N5 liposomes, only ~50% of the CPD lesions were removed but that was capable of reducing the mutagenesis rate by 99% in transformed fibroblasts and 30% in primary fibroblast cell culture. These numbers are probably not only related to the number of remaining lesions, but also to the smaller size of the repair patch filled by BER compared to that needed in NER (Yarosh, 2002; Cafardi & Elmets, 2008).

Finally, after two phase I clinical trials (Yarosh et al., 1996 as cited in Cafardi & Elmets, 2008) and three phase II clinical trials (Wolf et al., 2000 and Yarosh et al., 1996 as cited in Cafardi & Elmets, 2008), in 2001 the T4N5 liposomes were tested in XP patients. The patients were instructed to apply 4-5 ml of the lotion containing 1 mg/ml of endonuclease everyday for a year. Except for lesion removal when necessary, and daily use of sunscreens of 15 SPF or higher, no concomitant treatments were allowed. The treatment was shown to be efficient, reducing the rate of actinic keratoses and basal-cell carcinomas to 68% and 30% respectively in the placebo and treatment groups, reducing tumor promotion and progression. The treatment was also capable of reducing some immunosuppressant molecules, such as interleukin-10 (IL-10) and tumor necrosis factor- α (TNF- α). Unfortunately, the treatment was only effective for patients under 18 years-old. This might be because XP patients older than that already had too much DNA damage in their cells that could not be reversed (Yarosh et al., 2001). Despite the promising results, there are currently no topical DNA repair enzymes approved by the FDA. Clinical trials are still being conducted to analyze the application of T4N5 liposomes in other deficiencies and immunosuppressed patients (Cafardi & Elmets, 2008).

6.3 Oral use of retinoids

Despite interventions such as sunlight avoidance and tumor removal, most of the XP patients continue to develop a large number of skin cancers. These high-risk patients may suffer from field cancerization that may happen when a wide field of the epithelium has been exposed to the same genotoxic agent and adjacent but not contiguous areas present genetic and morphological alterations that may lead to a carcinogenesis process. As the whole skin area has been exposed to sunlight, inducing independent tumors with different growth rates, this hypothesis may explain why the patients have a 30% increase in the chances of having a second basal cell carcinoma (BCC) and then a 50% increase of a third BCC (Campbell & DiGiovanna, 2006).

In XP patients, the oral use of retinoids might be beneficial, regardless of the strong side effects. In chemoprevention the goal is to identify early biological events in the epithelium which may lead to a carcinogenesis process and intervene with chemicals which will help stop or reverse the process (Campbell & DiGiovanna, 2006). Retinoids, also known as vitamin A, are the most studied chemopreventive agent for skin cancers, upper aerodigestive tract and breast and cervical cancers. The exact mechanisms through which the retinoids are capable of reducing cancer incidence are still unclear, but it has been shown that they are capable of altering keratinocytes' growth, increasing their differentiation status, affecting their cell surface and immune modulation. Retinoids mediate gene transcription by binding to two families of nuclear receptors, the retinoid acid receptors (RARs) and the retinoid X receptors (RXRs). Retinoids have only a mild effect on existing tumors, but can suppress the development of new lesions (Campbell & DiGiovanna, 2006). In 1988, it was shown in a three year study that isotretinoin in a dose up to 2 mg/day/Kg was able to reduce skin cancers in XP patients by 63%. Unfortunately, in the year following the discontinuation of the treatment there was an increase of 8.5% of cancer incidence in those patients with reference to the two years of treatment (Kraemer et al., 1988).

Furthermore, the constant use of retinoids can have severe side effects ranging from inflammation in existing tumors, dry skin and mucosa and hair loss to pancreatitis, osteoporosis, hyperostosis and myalgia among others. The retinoids' toxicity is dose related and cumulative, but most of the side effects can be prevented with constant check-ups and use of local special moisturizers (Campbell & DiGiovanna, 2006). Indeed, several retinoids can be used as chemopreventives. The two most common are isotretinoin and acitretin, the first having a shorter half-life and being the drug of choice for women due to retinoids' theratogenic potential, especially in fetuses (Campbell & DiGiovanna, 2006).

6.4 Potential effects of DNA repair adjuvants

The use of DNA repair adjuvants and antioxidants may also help reducing skin cancer incidence in XP patients. Some known DNA repair adjuvants are selenium, aquosum extract of *Urcaria tomentosa* and Interleukin-12 (IL-12) (Emanuel & Scheinfeld, 2007).

Selenium seems to interact with Ref-1, activating p53, inducing the DNA repair branch of the p53 pathway, in a BRCA1-dependent manner, dealing mainly with oxidative stress (Fisher et al., 2006). On the other hand, it has been already reported that high levels of selenium can be mutagenic, carcinogenic and possibly teratogenic (Shamberger, 1985), probably due to non-specific sulfur substitution on proteins and consequent TC-NER activity decrease (Abul-Hassan et al., 2004). Thus, special attention should be taken regarding the dose of dietary selenium supplementation.

The aquosum extract of *Urcaria tomentosa* (cat's claw) seems to increase the removal of CPDs and reduce oxidative damage, either by an increase in base excision repair (BER) or by an antioxidant property, reducing erythema and blistering after UV. Despite several studies *in vitro* and *in vivo*, the precise mechanisms are still unknown (Emanuel & Scheinfeld, 2007).

Another interesting finding is that, besides IL-12 being a strong immunomodulatory molecule, able to prevent UV-induced immunosuppression through IL-10 inhibition (de Gruijl, 2008), it is also capable of increasing DNA repair by inducing NER, as shown by the RNA level increase in some NER molecules (Schwarz et al., 2005).

6.5 Gene therapy targeted approaches: The use of meganucleases for correcting XP-C cells

There are several techniques to specifically target, substitute, or correct a gene, diminishing the chances of insertional recombination, such as the use of recombinases, transposons, zinc-finger nucleases, endonucleases and meganucleases (Silva et al., 2011). Meganucleases can function as RNA maturases, facilitating the maturation of their own intron or as specific endonucleases that can recognize and cleave the exon-exon junction sequence wherein their intron resides, creating a specific double strand break (DSB), giving rise to the moniker "homing endonuclease". The meganuclease function is probably related to the current status of its lifecycle (Silva et al., 2011).

Meganucleases can be used as gene targeting tools in several ways. Ideally, they can provide a true reversion of the mutation, but the efficacy of correction is inversionally correlated to the distance of the initial DNA DSB. Alternatively, it can insert a functional gene upstream of the mutated one or in a safe location where it will not induce insertional mutagenesis. Also, meganucleases can be used for introducing specific mutations for research purposes such as understanding the role of a gene or of a specific point mutation. Furthermore, meganucleases capable of targeting viral sequences are being researched as antiviral agents (Silva et al., 2011).

Recently, the design of a specific I-CreI meganuclease targeting for the *XPC* gene was able to specifically target two *XPC* sequences, showing *in vitro* for the first time that extensive redesign of homing endonuclease can modify a specific chromosome region without loss of specificity or efficiency (Arnould et al., 2007). These results are very promising for the development of future gene therapy strategies for XP patients.

6.6 Induced Pluripotent Cells (iPSCs) as gene therapy agents

In 2006, the induction of pluripotent cells (iPSCs) by the expression of Oct3/4, Sox2, c-Myc and Klf4 in fibroblasts gave hope for a new gene therapy using pluripotent cells that would not elicit an immunological response in the patient, since his own cells would be used to induce the iPSCs and that would not be confronted by ethical issues like the use of embryonic stem cells (Takahashi & Yamanaka, 2006). Since then, this technology has been improved and iPSCs have been induced in a variety of cell types from different species. Also, iPSCs have been differentiated to several different cell types, from fibroblasts to neurons (Sidhu, 2011).

Fanconi Anemia (FA) is a DNA repair related disease, where mutations in one of fourteen genes lead to extreme sensitivity to interstrand crosslinking agents. Patients show progressive bone marrow failure, congenital developmental abnormalities and early onset of cancers, mostly acute myelogenous leukemia and squamous cell carcinomas. Bone marrow transplantation is a palliative treatment for the secondary leukemia but no cure is currently available for FA patients (Kitao & Takata, 2011). In 2009, Raya and co-workers were able to use lentiviral vectors to genetically correct fibroblast and keratinocytes from patients with various FA complementation group deficiencies and then induce their dedifferentiation into pluripotent stem cells. Interestingly, uncorrected FA cells did not generate iPSCs, indicating a role for DNA repair in nuclear reprogramming. Thus, the generated iPSCs had normal FA genes and have the potential of being used for gene therapy of the donor patients, with no risk of inducing immunological rejection (Raya et al., 2009). Hopefully soon FA patients and others will be able to benefit from this technology as a safe gene therapy approach.

7. Concluding remarks

Recombinant viral vectors were developed more than thirty years ago, and they have provided extremely useful tools to understand cell metabolism. This chapter focuses on their use to understand cells' responses to DNA damage, especially UV-irradiated DNA repair-deficient cells. These vectors provide means to interfere in these responses, affecting DNA metabolism and revealing important aspects of the DNA repair mechanisms. The discovery of RNA interference mechanisms in human cells offer still more opportunities to modify cells' responses by silencing specific DNA repair genes. Several libraries of viral vectors for the expression of small double-stranded RNA molecules (shRNA) targeting human genes are commercially available, and are already being used for understanding gene function. The use of such vectors to make cells deficient in more than one DNA repair pathway, using cells deficient in XP genes as hosts, for example, may help us to reveal the intricate network of interactions between the different metabolic pathways that contribute to genome maintenance after damage induction (Moraes, et al., 2011; in press). Moreover, the progress that has been made towards gene therapy for xeroderma pigmentosum, using these recombinant viral vectors is also discussed. Although the results indicate a series of limitations, and it is clear that there is still a long way to go, they make researchers go forward, giving a gleam of hope to these patients and their families.

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9. References

- Abul-Hassan, K.S., Lehnert, B.E., Guant, L., & Walmsley, R. (2004). Abnormal DNA repair in selenium-treated human cells. *Mutation research*, V. 565, N. 1, pp. 45-51, ISSN 0027-5107
- Alison, M.R. (2009). Stem cells in pathobiology and regenerative medicine. *The Journal of pathology*, V. 217, N. 2, pp. 141-143, ISSN 1096-9896
- Ahrens, C., Grewe, M., Berneburg, M., Grether-Beck, S., Quilliet, X., Mezzina, M., Sarasin, A., Lehmann, A.R., Arlett, C.F., & Krutmann, J. (1997). Photocarcinogenesis and inhibition of intercellular adhesion molecule 1 expression in cells of DNA-repair-defective individuals. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*., V. 94, N. 13, pp. 6837-6841, ISSN 1091-6490
- Armellini, M.G., Muotri, A.R., Marchetto, M.C., de Lima-Bessa, K.M., Sarasin, A., & Menck, C.F. (2005). Restoring DNA repair capacity of cells from three distinct diseases by XPD gene-recombinant adenovirus. *Cancer gene therapy*, V. 12, N. 4, pp. 389-396, ISSN 0929-1903
- Armellini, M.G., Lima-Bessa, K.M., Marchetto, M.C., Muotri, A.R., Chiganças, V., Leite, R.A., Carvalho, H., & Menck, C.F. (2007). Exploring DNA damage responses in human cells with recombinant adenoviral vectors. *Human , & experimental toxicology*, V. 26, N. 11, pp. 899-906, ISSN 0960-3271

- Arnaudeau-Bégard, C., Brellier, F., Chevallier-Lagente, O., Hoeijmakers, J., Bernerd, F., Sarasin, A., & Magnaldo, T. (2003). Genetic correction of DNA repair-deficient/cancer-prone xeroderma pigmentosum group C keratinocytes. *Human gene therapy*, V. 14, N. 10, pp. 983-996, ISSN 1043-0342
- Arnould, S., Perez, C., Cabaniols, J.P., Smith, J., Gouble, A., Grizot, S., Epinat, J.C., Duclert, A., Duchateau, P., & Pâques, F. (2007). Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *Journal of molecular biology*, V. 371, N. 1, pp 49-65, ISSN 0022-2836
- Atabay, K., Celebi, C., Cenetoglu, S., Baran, N.K., & Kiyamaz, Z. (1991). Facial resurfacing in xeroderma pigmentosum with monoblock full-thickness skin graft. *Plastic and reconstructive surgery*, V. 87, N. 6, pp. 1121-1125, ISSN 0032-1052
- Atkinson, H., & Chalmers, R. (2010). Delivering the goods: viral and non-viral gene therapy systems and the inherent limits on cargo DNA and internal sequences. *Genetica*, V. 138, N. 5, pp. 485-498, ISSN 0016-6707
- Bell, E., Sher, S., Hull, B., Merrill, C., Rosen, S., Chamson, A., Asselineau, D., Dubertret, L., Coulomb, B., Lapiere, C., Nusgens, B., & Neveux, Y. (1983). The reconstitution of living skin. *Journal of investigative dermatology*, V. 81, N. 1 (suppl.), pp. 2s-10s, ISSN 0022-202X
- Bergoglio, V., Larcher, F., Chevallier-Lagente, O., Bernheim, A., Danos, O., Sarasin, A., Rio, M.D., & Magnaldo, T. (2007). Safe selection of genetically manipulated human primary keratinocytes with very high growth potential using CD24. *Molecular therapy*, V. 15, N. 12, pp. 2186-2193, ISSN 1525-0016
- Bernerd, F., Asselineau, D., Frechet, M., Sarasin, A., & Magnaldo, T. (2005). Reconstruction of DNA repair-deficient xeroderma pigmentosum skin *in vitro*: a model to study hypersensitivity to UV light. *Photochemistry and photobiology*, V. 81, N. 1, pp. 19-24, ISSN 0031-8655
- Brettel, K., & Byrdin, M. (2010). Reaction mechanisms of DNA photolyase. *Current opinion in structural biology*, V. 20, N. 6, pp. 693-701, ISSN 0959-440X
- Cafardi, J.A., & Elmets, C.A. (2008). T4 endonuclease V: review and application to dermatology. *Expert opinion on biological therapy*, V. 8, N. 6, pp. 829-838, ISSN 1471-2598
- Campbell, R.M., & DiGiovanna, J.J. (2006). Skin cancer chemoprevention with systemic retinoids: an adjunct in the management of selected high-risk patients. *Dermatologic therapy*, V. 19, N. 5, pp. 306-314, ISSN 13960296
- Carreau, M., Quilliet, X., Eveno, E., Salvetti, A., Danos, O., Heard, J.M., Mezzina, M., & Sarasin, A. (1995a). Functional retroviral vector for gene therapy of xeroderma pigmentosum group D patients. *Human gene therapy*, V. 6, N. 10, pp. 1307-1315, ISSN 1043-0342
- Carreau, M., Eveno, E., Quilliet, X., Chevallier-Lagente, O., Benoit, A., Tanganelli, B., Stefanini, M., Vermeulen, W., Hoeijmakers, J.H., Sarasin, A., & Mezzina, M. (1995b). Development of a new easy complementation assay for DNA repair deficiency human syndromes using cloned repair genes. *Carcinogenesis*, V. 16, N. 5, pp. 1003-1009, ISSN 0143-3334
- Ceccoli, J., Rosales, N., Tsimis, J., & Yarosh, D.B. (1989). Encapsulation of the UV-DNA repair enzyme T4 endonuclease V in liposomes and delivery to human cells. *Journal of investigative dermatology*, V. 93, N. 2, pp. 190-194, ISSN 0022-202X

- Chigañças, V., Miyaji, E.N., Muotri, A.R., de Fátima-Jacysyn, J., Amarante-Mendes, G.P., Yasui, A., & Menck, C.F. (2000). Photorepair prevents ultraviolet-induced apoptosis in human cells expressing the marsupial photolyase gene. *Cancer research*, V. 60, N. 9, pp. 2458-2463, ISSN 1538-7445
- Chigañças, V., Batista, L.F., Brumatti, G., Amarante-Mendes, G.P., Yasui, A., & Menck, C.F. (2002). Photorepair of RNA polymerase arrest and apoptosis after ultraviolet irradiation in normal and XPB deficient rodent cells. *Cell death and differentiation*, V. 9, N. 10, pp. 1099-1107, ISSN 1350-9047
- Chigañças, V., Sarasin, A., & Menck, C.F. (2004). CPD-photolyase adenovirus-mediated gene transfer in normal and DNA-repair-deficient human cells. *Journal of cell science*, V. 117, N. Pt 16, pp. 3579-3592, ISSN 0021-9533
- Chigañças, V., Lima-Bessa, K.M., Stary, A., Menck, C.F., & Sarasin, A. (2008). Defective transcription/repair factor IIIH recruitment to specific UV lesions in trichothiodystrophy syndrome. *Cancer research*, V. 68, N. 15, pp. 6074-6083, ISSN 1538-7445
- Ciccía, A., & Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. *Molecular cell*, V. 40, N. 2, pp. 179-204, ISSN 1538-7445
- Cleaver, J.E., Kaufmann, W.K., Kapp, L.N., & Park, S.D. (1983). Replicon size and excision repair as factors in the inhibition and recovery of DNA synthesis from ultraviolet damage. *Biochimica et biophysica Acta*, V. 739, N. 2, pp. 207-215, ISSN 0304-4165
- Cleaver, J.E., Lam, E.T., & Revet, I. (2009). Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. *Nature reviews.Genetics*, V. 10, N. 11, pp. 756-768, ISSN 1471-0056
- Costa, R.M., Chigañças, V., Galhardo, Rda.S., Carvalho, H., & Menck, C.F. (2003). The eukaryotic nucleotide excision repair pathway. *Biochimie*, V. 85, N. 11, pp. 1083-1099, ISSN 0300-9084
- Daya, S., & Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. *Clinical microbiology reviews*, V. 21, N. 4, pp. 583-593, ISSN 0893-8512
- de Boer, J., & Hoeijmakers, J.H. (2000). Nucleotide excision repair and human syndromes. *Carcinogenesis*, V. 21, N. 3, pp. 453-460, ISSN 0143-3334
- de Gruijl, F. (2008). UV-induced immunosuppression in the balance. *Photochemistry and photobiology*, V. 84, N. 1, pp. 2-9, ISSN 0031-8655
- Dong, B., Nakai, H., & Xiao, W. (2010). Characterization of genome integrity for oversized recombinant AAV vector. *Molecular therapy*, V. 18, N. 1, pp. 87-92, ISSN 1525-0016
- Dumaz, N., Drougard, C., Quilliet, X., Mezzina, M., Sarasin, A., & Daya-Grosjean, L. (1998). Recovery of the normal p53 response after UV treatment in DNA repair-deficient fibroblasts by retroviral-mediated correction with the XPD gene. *Carcinogenesis*, V. 19, N. 9, pp. 1701-1704, ISSN 0143-3334
- Edelstein, M.L., Abedi, M.R., & Wixon, J. (2007). Gene therapy clinical trials worldwide to 2007- an update. *The journal of gene medicine*, V. 9, N. 10, pp. 833-842, ISSN 1521-2254
- Emanuel, P., & Scheinfeld, N. (2007). A review of DNA repair and possible DNA-repair adjuvants and selected natural anti-oxidants. *Dermatology online journal*, V. 13, N. 3, pp. 10, ISSN 1087-2108
- Fisher, J.L., Lancia, J.K., Mathur, A., & Smith, M.L. (2006). Selenium protection from DNA damage involves Ref1/p53/Brcal protein complex. *Anticancer research*, V. 26, N. 2A, pp. 899-904, ISSN 0250-7005

- Froelich, S., Tai, A., & Wang, P. (2010). Lentiviral vectors for immune cells targeting. *Immunopharmacology and immunotoxicology*, V. 32, N. 2, pp. 208-218, ISSN 0892-3973
- Fousteri, M., & Mullenders, L.H. (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell research*, V. 18, N. 1, pp. 73-84, ISSN 1001-0602
- Gerstenblith, M.R., Goldstein, A.M., & Tucker, M.A. (2010). Hereditary genodermatoses with cancer predisposition. *Hematology/oncology clinics of North America*, V. 24, N. 5, pp. 885-906, ISSN 0889-8588
- Hall, K., Blair Zajdel, M.E., & Blair, G.E. (2010). Unity and diversity in the human adenoviruses: exploiting alternative entry pathways for gene therapy. *The Biochemical journal*, V. 431, N. 3, pp. 321-336, ISSN 0264-6021
- Hanawalt, P.C. (2002). Subpathways of nucleotide excision repair and their regulation. *Oncogene*, V. 21, N. 58, pp. 8949-8956, ISSN 0950-9232
- Hoeijmakers, J.H. (2009). Molecular origins of cancer: DNA damage, aging, and cancer. *The New England journal of medicine*, V. 361, N. 15, pp. 1475-1485, ISSN 0028-4793
- Horibata, K., Iwamoto, Y., Kuraoka, I., Jaspers, N.G.J., Kurimasa, A., Oshimura, M., Ichihashi, M., & Tanaka, K. (2004). Complete absence of Cockayne syndrome group B gene product gives rise to UV-sensitive syndrome but not Cockayne syndrome. *PNAS*, V. 101, N. 43, pp. 15410-15415, ISSN 1091-6490
- Howarth, J.L., Lee, Y.B., & Uney, J.B. (2010). Using viral vectors as gene transfer tools. *Cell biology and toxicology*, V. 26, N. 1, pp. 1-20, ISSN 0742-2091
- Itin, P.H., Sarasin, A., & Pittelkow, M.R. (2001). Trichothiodystrophy: update on the sulfur-deficient brittle hair syndromes. *Journal of the American Academy of Dermatology*, V. 44, N. 6, pp. 891-920, ISSN 0190-9622
- Iwakuma, T., Cui, Y., & Chang, L.J. (1999). Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology*, V. 261, N. 1, pp. 120-132, ISSN 0042-6822
- Johnson, R.E., Kondratick, C.M., Prakash, S., & Prakash, L. (1999). hRAD30 mutations in the variant form of xeroderma pigmentosum. *Science*, V. 285, N. 5425, pp. 263-265, ISSN 0036-8075
- Karalis, A., Tischkowitz, M., & Millington, G.W. (2011). Dermatological manifestations of inherited cancer syndromes in children. *The British journal of dermatology*, V. 164, N. 2, pp. 245-256, ISSN 0007-0963
- Khavari, P.A. (1998). Gene therapy for genetic skin disease. *Journal of investigative dermatology*, V. 110, N. 4, pp. 462-467, ISSN 0022-202X
- Kibitel, J.T., Yee, V., & Yarosh, D.B. (1991). Enhancement of ultraviolet-DNA repair in denV gene transfectants and T4 endonuclease V-liposome recipients. *Photochemistry and photobiology*, V. 54, N. 5, pp. 753-60, ISSN 0031-8655
- Kitao, H., & Takata, M. (2011). Fanconi anemia: a disorder defective in the DNA damage response. *International journal of hematology*, V. 93, N. 4, pp. 417-424, ISSN 0925- 5710
- Kraemer, K.H., DiGiovanna, J.J., Moshell, A.N., Tarone, R.E , & Peck, G.L. (1988). Prevention of skin cancer in xeroderma pigmentosum with the use of isotretinoin. *The New England journal of medicine*, V. 318, N. 25, pp. 1633-1637, ISSN 0028-4793
- Kraemer, K.H., Patronas, N.J., Schiffmann, R., Brooks, B.P., Tamura, D., & DiGiovanna, J.J. (2007). Xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome: a complex genotype-phenotype relationship. *Neuroscience*, V. 145, N. 4, pp. 1388-1396, ISSN 0306-4522

- Kraemer, K. H. (2008). Xeroderma pigmentosum. Gene reviews-NCBI Bookshelf, NBK1397, PMID: 20301571.
- Kuluncsics, Z., Perdiz, D., Brulay, E., Muel B., & Sage, E. (1999). Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts. *Journal of Photochemistry and photobiology.B, Biology*, V. 49, N. 1, pp. 71-80, ISSN 1011-1344
- Kusumoto, R., Masutani, C., Sugasawa, K., Iwai, S., Araki, M., Uchida, A., Mizukoshi, T., & Hanaoka, F. (2001). Diversity of the damage recognition step in the global genomic nucleotide excision repair in vitro. *Mutation research*, V. 485, N. 3, pp. 219-227, ISSN 0027-5107
- Leach, D.M., & Rainbow, A.J. (2011). Early host cell reactivation of an oxidatively damaged adenovirus-encoded reporter gene requires the Cockayne syndrome proteins CSA and CSB. *Mutagenesis*, V. 26, N. 2, pp. 315-321, ISSN 1383-5718
- Leite, R.A., Marchetto, M.C., Muotri, A.R., Vasconcelos, Dde.M., de Oliveira, Z.N., Machado, M.C., & Menck, C.F. (2009). Identification of XP complementation groups by recombinant adenovirus carrying DNA repair genes. *The journal of investigative dermatology*, V. 129, N. 2, pp. 502-506, ISSN 0022-202X
- Lima-Bessa, K.M., Chiganças, V., Sary, A., Kannouche, P., Sarasin, A., Armelini, M.G., de Fatima Jacysyn, J., Amarante-Mendes, G.P., Cordeiro-Stone, M., Cleaver, J.E., & Menck, C.F. (2006). Adenovirus mediated transduction of the human DNA polymerase eta cDNA. *DNA repair*, V. 5, N. 8, pp. 925-934, ISSN 1568-7864
- Lima-Bessa, K.M., Armelini, M.G., Chiganças, V., Jacysyn, J.F., Amarante-Mendes, G.P., Sarasin, A., & Menck, C.F. (2008). CPDs and 6-4PPs play different roles in UV-induced cell death in normal and NER-deficient human cells. *DNA repair*, V. 7, N. 2, pp. 303-312, ISSN 1568-7864
- Lima-Bessa, K.M., Soltys, D.T., Marchetto, M.C., & Menck, C.F.M. (2009). Xeroderma pigmentosum: living in the dark but with hope in therapy. *Drugs of the Future*, V. 34, N. 8, pp. 665-672, ISSN 0377-8282
- Liu, L., Lee, J., & Zhou, P. (2010). Navigating the nucleotide excision repair threshold. *Journal of cellular physiology*, V. 224, N. 3, pp. 585-589, ISSN 1097-4652
- Lucas-Lledó, J.I., & Lynch, M. (2009). Evolution of mutation rates: phylogenomic analysis of the photolyase/cryptochrome family. *Molecular biology and evolution*, V. 26, N. 5, pp. 1143-1153, ISSN 0737-4038
- Magnaldo, T., & Sarasin, A. (2002). Genetic reversion of skin disorders. *Mutation research*, V. 509, N. 1-2, pp. 211-220, ISSN 0027-5107
- Marchetto, M.C., Muotri, A.R., Burns, D.K., Friedberg, E.C., & Menck, C.F. (2004). Gene transduction in skin cells: preventing cancer in xeroderma pigmentosum mice. *PNAS*, V. 101, N. 51, pp. 17759-17764, ISSN 1091-6490
- Marchetto, M.C., Correa, R.G., Menck, C.F., & Muotri, A.R. (2006). Functional lentiviral vectors for xeroderma pigmentosum gene therapy. *Journal of biotechnology*, V. 126, N. 4, pp. 424-430, ISSN 0168-1656
- Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., & Hanaoka, F. (1999). The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature*, V. 399, N. 6737, pp. 700-704, ISSN 0028-0836

- Menck, C.F., Armelini, M.G., & Lima-Bessa, K.M. (2007). On the search for skin gene therapy strategies of xeroderma pigmentosum disease. *Current gene therapy*, V. 7, N. 3, pp. 163-174, ISSN 1566-5232
- Marionnet, C., Quilliet, X., Benoit, A., Armier, J., Sarasin, A., & Stary, A. (1996). Recovery of normal DNA repair and mutagenesis in trichothiodistrophy cells after transduction of the XPD human gene. *Cancer research*, V. 56, N. 23, pp. 5450-5456, ISSN 1538-7445
- Mátrai, J., Chuah, M.K., & VandenDriessche, T. (2010). Recent advances in lentiviral vector development and applications. *Molecular therapy*, V. 18, N. 3, pp. 477-490, ISSN 1525-0016
- Merkle, T.J., O'Brien, K., Brooks, P.J., Tarone, R.E., & Robbins, J.H. (2004). DNA repair in human fibroblasts, as reflected by host-cell reactivation of a transfected UV-irradiated luciferase gene, is not related to donor age. *Mutation research*, V. 554, N. 1-2, pp. 9-17, ISSN 0027-5107
- Mezzina, M., Eveno, E., Chevallier-Lagente, O., Benoit, A., Carreau, M., Vermeulen, W., Hoeijmakers, J.H., Stefanini, M., Lehmann, A.R., Weber, C.A., & Sarasin, A. (1994). Correction by the ERCC2 gene of UV-sensitivity and repair deficiency phenotype in a subset of trichothiodistrophy cells. *Carcinogenesis*, V. 15, N. 8, pp. 1493-1498, ISSN 0143-3334
- Michelfelder, S., & Trepel, M. (2009). Adeno-associated viral vectors and their redirection to cell-type specific receptors. *Advances in genetics*, V. 67, pp. 29-60, ISSN 0065-2660
- Moraes, M.C.S., Cabral-Neto, J.B., & Menck, C.F. (2011). DNA repair mechanisms protect our genome from carcinogenesis. *Frontiers in bioscience*, in press, ISSN 0143-3334
- Moriwaki, S., Ray, S., Tarone, R.E., Kraemer, K.H., & Grossman, L. (1996). The effect of donor age on the processing of UV-damaged DNA by cultured human cells: reduced DNA repair capacity and increased DNA mutability. *Mutation research*, V. 364, N. 2, pp. 117-123, ISSN 0027-5107
- Müller, M., & Carell, T. (2009). Structural biology of DNA photolyases and cryptochromes. *Current opinion in structural biology*, V. 19, N. 3, pp. 277-285, ISSN 0959-440X
- Muotri, A.R., Marchetto, M.C., Zerbini, L.F., Libermann, T.A., Ventura, A.M., Sarasin, A., & Menck, C.F. (2002). Complementation of the DNA repair deficiency in human xeroderma pigmentosum group A and C cells by recombinant adenovirus-mediated gene transfer. *Human gene therapy*, V. 13, N. 15, pp. 1833-1844, ISSN 1043-0342
- Narayanan, D.L., Saladi, R.N., & Fox, J.L. (2010). Ultraviolet radiation and skin cancer. *International journal of dermatology*, V. 49, N. 9, pp. 978-986, ISSN 00119059
- Partch, C.L., & Sancar, A. (2005). Cryptochromes and circadian photoreception in animals. *Methods in enzymology*, V. 393, pp. 726-745, ISSN 0076-6879
- Pitsikas, P., Francis, M.A., & Rainbow, A.J. (2005). Enhanced host cell reactivation of a UV-damaged reporter gene in pre-UV-treated cells is delayed in Cockayne syndrome cells. *Journal of photochemistry and photobiology. B, Biology*, V. 81, N. 2, pp. 89-97, ISSN 1011-1344
- Protic-Sabljić, M., Whyte, D., Fagan, J., Howard, B.H., & Gorman, C. M., Padmanabhan, R., & Kraemer, K.H. (1985). Quantification of expression of linked cloned genes in a simian virus 40-transformed xeroderma pigmentosum cell line. *Molecular and cellular biology*, V. 5, N. 7, pp. 1685-1693, ISSN 0270-7306

- Quilliet, X., Chevallier-Lagente, O., Eveno, E., Stojkovic, T., Destée, A., Sarasin, A., & Mezzina, M. (1996). Long-term complementation of DNA repair deficient human primary fibroblasts by retroviral transduction of the XPD gene. *Mutation research*, V. 364, N. 3, pp. 161-169, ISSN 0027-5107
- Rastogi, R.P., Richa, Kumar, A., Tyagi, M.B., & Sinha, R.P. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *Journal of nucleic acids*, ISSN 2090-0201
- Raya, A., Rodríguez-Pizà, I., Guenechea, G., Vassena, R., Navarro, S., Barrero, M.J., Consiglio, A., Castellà, M., Río, P., Sleep, E., González, F., Tiscornia, G., Garreta, E., Aasen, T., Veiga, A., Verma, I.M., Surrallés, J., Bueren, J., & Izpisua Belmonte, J.C. (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature*, V. 460, N. 7251, pp. 53-59, ISSN 0028-0836
- Rosenberg, S.A., Aebersold, P., Cornetta, K. Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T. Yang, J.C., Topalian, S.L., Merino, M.J., Culver, K., Miller, D., Blaese, M., & Anderson, W.F. (1990). Gene transfer into humans - immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *The New England journal of medicine*, V. 323, N. 9, pp. 570-578, ISSN 0028-4793
- Sancar, A. (2008). Structure and function of photolyase and in vivo enzymology: 50th anniversary. *The Journal of biological chemistry*, V. 283, N. 47, pp. 32153-32157, ISSN 0021-9258
- Schmidt, M., Voutetakis, A., Afione, S., Zheng, C., Mandikian, D., & Chiorini, J.A. (2008). Adeno-associated virus type 12 (AAV12): a novel AAV serotype with sialic acid- and heparin sulfate proteoglycan-independent transduction activity. *Journal of virology*, V. 82, N. 3, pp. 1399-1406, ISSN 0022-538X
- Schuch, A.P., da Silva Galhardo, R., de Lima-Bessa, K.M., Schuch, N.J., & Menck C.F. (2009). Development of a DNA-dosimeter system for monitoring the effects of solar-ultraviolet radiation. *Photochemical , & photobiological sciences*, V. 8, N. 1, pp. 111-120, ISSN 1474-905X
- Schwarz, A., Maeda, A., Kernebeck, K., van Steeg, H., Beisert, S., & Schwarz, T. (2005). Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *The Journal of experimental medicine*, V. 201, N. 2, pp. 173-179, ISSN 0022-1007
- Seregin, S.S., & Amalfitano, A. (2009). Overcoming pre-existing adenovirus immunity by genetic engineering of adenovirus-based vectors. *Expert opinion on biological therapy*, V. 9, N. 12, pp. 1521-1531, ISSN 1471-2598
- Shamberger, R.J. (1985). The genotoxicity of selenium. *Mutation research*, V. 154, N. 1, pp. 29-48, ISSN 0027-5107
- Sidhu, K.S. (2011). New approaches for the generation of induced pluripotent stem cells. *Expert opinion on biological therapy*, V. 11, N. 5, pp. 569-579, ISSN 1471-2598
- Silva, G., Poirot, L., Galetto, R., Smith, J., Montoya, G., Duchateau, P., & Pâques, F. (2011). Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. *Current gene therapy*, V. 11, N. 1, pp. 11-27, ISSN 1566-5232
- Sinha, R.P., & Häder, D.P. (2002). UV-induced DNA damage and repair: a review. *Photochemical , & photobiological sciences*, V. 1, N. 4, pp. 225-236, ISSN 1474-905X

- Spivak, G. (2005). UV-sensitive syndrome. *Mutation research*, V. 577, N. 1-2, pp. 162-169, ISSN 0027-5107
- Spivak G., & Hanawalt, P.C. (2006). Host cell reactivation of plasmids containing oxidative DNA lesions is defective in Cockayne syndrome but normal in UV-sensitive syndrome fibroblasts. *DNA repair*, V. 5, N. 1, pp. 13-22, ISSN 1568-7864
- Stefanini, M., Botta, E., Lanzafame, M., & Orioli, D. (2010). Trichothiodystrophy: from basic mechanisms to clinical implications. *DNA repair*, V. 9, N. 1, pp. 2-10, ISSN 1568-7864
- Suzumura, H., & Arisaka, O. (2010). Cerebro-oculo-facio-skeletal syndrome. *Advances in experimental medicine and biology*, V. 685, pp. 210-214, ISSN 0065-2598
- Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, V. 126, N. 4, pp. 663-676, ISSN 0092-8674
- Tanaka, K., Sekiguchi, M., & Okada, Y. (1975). Restoration of ultraviolet-induced unscheduled DNA synthesis of xeroderma pigmentosum cells by the concomitant treatment with bacteriophage T4 endonuclease V and HJV (Sendai virus). *PNAS*, V. 72, N. 10, pp. 4071-4075, ISSN 1091-6490
- Voigt, K., Izsvák, Z., & Ivics, Z. (2008). Targeted gene insertion for molecular medicine. *Journal of molecular medicine*, V. 86, N. 11, pp. 1205-1219, ISSN 0946-2716
- Wang, J., Faust, S.M., & Rabinowitz, J.E. (2011). The next step in gene delivery: molecular engineering of adeno-associated virus serotypes. *Journal of molecular and cellular cardiology*, V. 50, N. 5, pp. 793-802, ISSN 0022-2828
- Weidenheim, K.M., Dickson, D.W., & Rapin, I. (2009). Neuropathology of Cockayne syndrome: evidence for impaired development, premature aging, and neurodegeneration. *Mechanisms of ageing and development*, V. 130, N. 9, pp. 619-636, ISSN 0047-6374
- Wolf, P. Maier, H., Mullegger, R.R., Chadwick, C.A., Hofmann-Wellenhof, R., Soyer, H.P., Hofer, A., Smolle, J., Horn, M., Cerroni, L., Yarosh, D., Klein, J., Bucana, C., Dunner K. Jr., Potten, C S., Hönigsmann, H., Kerl, H., & Kripke, M.L. (2000). Topical treatment with liposomes containing T4 endonuclease V protects human skin *in vivo* from ultraviolet-induced upregulation of interleukin-10 and tumor necrosis factor- α . *Journal of investigative dermatology*, V. 114, N. 1, pp. 149-156, ISSN 0022-202X
- Yarosh, D, Bucana, C., Cox, P., Alas, L., Kibitel, J., & Kripke, M. (1994). Localization of liposomes containing a DNA repair enzyme in murine skin. *Journal of investigate dermatology*, V. 103, N. 4, pp. 461-468, ISSN 0022-202X
- Yarosh, D., Klein, J., Kibitel, J., Alas, L., O'Connor, A., Cummings, B., Grob, D., Gerstein, D., Gilchrest, B.A., Ichihashi, M., Ogoshi, M., Ueda, M., Fernandez, V., Chadwick, C., Potten, C.S., Proby, C.M., Young, A.R., & Hawk, J.L. (1996). Enzyme therapy of xeroderma pigmentosum: safety and efficacy testing of T4N5 liposome lotion containing a prokaryotic DNA repair enzyme. *Photodermatology, photoimmunology , & photomedicine*, V. 12, N. 3, pp. 122-130, ISSN 0905-4383
- Yarosh, D., Klein, J., O'Connor, A.O., Hawk, J., Rafal, E., & Wolf, P. (2001). Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomized study. *Lancet*, V. 357, N. 9260, pp. 926-929, ISSN 0140-6736

- Yarosh, D.B. (2002). Enhanced DNA repair of cyclobutane pyrimidine dimers changes the biological response to UV-B radiation. *Mutation research*, V. 509, N. 1-2, pp. 221-226, ISSN 0027-5107
- You, Y.H., Lee, D.H., Yoon, J.H., Nakajima, S., Yasui, A., & Pfeifer GP. (2001). Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. *The Journal of biological chemistry*, V. 276, N. 48, pp. 44688-44694, ISSN 0021-9258
- Zeng, L., Sarasin, A., & Mezzina, M. (1998). Retrovirus-mediated DNA repair gene transfer into xeroderma pigmentosum cells: perspectives for a gene therapy. *Cell biology and toxicology*, V. 14, N. 2, pp. 105-110, ISSN 0742-2091

Part 2

DNA Repair and Cancer

DNA Damage, Repair and Misrepair in Cancer And in Cancer Therapy

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1. Introduction

The term cancer, which is derived from the Greek word "karkinos", meaning crab, encompasses over 100 distinct diseases that are characterized by an uncontrolled multiplication of abnormal cells. The oldest written description of cancer known to exist dates back to about 1600 BC, but is believed to be based on a much earlier document, from ca. 3000 BC. It is part of the Edwin Smith Papyrus, and describes eight cases of breast tumors or ulcers in Egypt and their treatment by cauterization. Signs of cancer can also be traced back as far as 3000 BC, on the bones of mummies from ancient Egypt and Peru. Humans, though, must have fought against this pathology throughout their existence, for which, according to the above-mentioned papyrus, there was no cure (American Cancer Society, 2010). This opinion was shared by the Greek physician Hippocrates (about 400 BC), known today as the "Father of Medicine", who believed that it was best to leave cancer alone, as those who received treatment did not survive long. Hippocrates claimed that cancer was due to an excess of black bile, one of the four fluids (or humors) that, according to the humoral theory that he developed, composed the body. Hippocrates was the first to use the words *carcinos* and *carcinoma* to describe non-ulcer forming and ulcer-forming tumors, probably to reflect similarities between certain aspects of the tumors' appearance and that of crabs (Feinberg *et al.*, 2006). In the first century BC, the Roman physician Celsus translated the Greek term into *cancer*, the Latin word for crab. Later, in the second century AD, Galen, another Roman physician, used the Greek word *oncos* (meaning swelling) to describe tumors.

From ancient times, cancer never failed to attract the attention of the medical community, leading to advances in several areas, most notably in the surgical removal of tumors.

Interest in oncology clearly intensified in the eighteenth century, leading to arguably the first true experiments in cancer research, namely the inoculation of a dog with cancer fluid from humans, reported by Bernard Peyrilhe, in 1774 (Triolo, 1965).

The multiple theories to explain neoplasia that accompanied the ever-increasing interest in oncological research were, almost inevitably, in line with the more general pathological theories of the time (Triolo, 1965). Accordingly, the vast majority of them have since fallen out of fashion. Understandably, the genetic basis of cancer, a cornerstone of modern cancer research, could only begin to unravel after the discovery of the chromosomes, those rod-like structures (*mitosen*) that formed from the nucleus fibrous network in the dividing nucleus, as observed by Walther Flemming, an acknowledged master of microscopy, during his landmark comprehensive investigation into cell division (Flemming, 1882). As the nuclear network could be easily stained, Flemming named it *chromatin* (i.e., stainable material), designating its metamorphosis as *mitosis* (from the Greek word for thread). In 1888, the term chromosome was finally coined, by Wilhelm Waldeyer (from *chroma* and *soma*, the Greek words for colored and body, respectively) (Waldeyer, 1888). Flemming's description of chromosomes was accompanied by a detailed account of the sequence of their movements during cell division, deduced from his many observations of cells in various stages of division (Flemming, 1882). He assumed that the chromosomes were longitudinally split during mitosis, the two halves partitioning into the two daughter cells at the end of the process. This account, which was confirmed, decades later, by microscopy of live dividing cells, is considered as a founding moment in genetic research. Likely unaware of Gregor Mendel's speculative work on heredity, which was published in an obscure journal in 1865 (Mendel, 1866), Flemming did not make a connection between the distribution of nuclear material during cell division and genetic inheritance. Nonetheless, he provided scientific evidence for a plausible mechanism of transmission of hereditary traits. Developed a little more than two decades later by Theodor Boveri and Walter Sutton, working independently, the chromosome theory of inheritance, also known as the Sutton-Boveri theory, rightly confirmed the chromosomes as the carriers of the genetic material (Harris, 2008; Sutton, 1903), thus expanding the science of genetics from the organismal level to the sub-cellular level. Still, almost another century had to elapse before cancer became widely recognized as a disease of the genes. In this section, a brief historical account is presented of the discoveries that, directly or indirectly, made a strong contribution to the establishment of the genetic basis of cancer.

1.1 The chromosome theories of cancer formation: From Hansemann and Boveri to the Philadelphia chromosome

In 1890, while studying mitosis in the human epidermis, David von Hansemann analyzed in detail 13 different carcinomas and consistently found examples of aberrant mitoses, namely multipolar mitoses and asymmetric distributions of chromosomes at anaphase. The occurrence of gross mitotic abnormalities in tumor cells had already been reported by several pathologists, but it was apparently Hansemann who first argued that aberrant cell divisions and the resulting abnormal (or aneuploid) karyotypes were essential determinants of malignancy (von Hansemann, 1890, as cited in Bignold *et al.*, 2006). Similarities between these mitoses and those occurring during oögenesis, namely the generation of daughter cells with a decreased chromatin content (the polar bodies, in the case of oögenesis), seemed to support the gametogenic ideas of tumor formation that were current in the nineteenth century. These theories linked certain features of tumors, such as their increased capacity for

independent growth (section 3), to those of germ cells. However, in the mid-1890s, Hansemann had already abandoned the oögenic component of his theories.

Hansemann's seminal hypothesis of a link between chromosomes and cancer was largely ignored and, still to this day, the chromosomal theory of cancer formation is frequently fully credited to Theodor Boveri (Harris, 2008), a zoologist who pursued Hansemann's theory a decade later (Harris, 2008). Nonetheless, it has to be acknowledged that Boveri took to a higher level this relationship between karyotypic disorders, which he proposed to be mostly initiated by multipolar mitosis, and malignancies. Namely, he provided experimental evidence that certain chromosome combinations lead to abnormal development and proposed mechanisms of malignancy based on novel concepts.

In the detailed studies that would culminate in his chromosome theory of inheritance, Boveri ingeniously devised experimental manipulations to artificially induce multipolar mitoses in sea urchin eggs (Harris, 2008). His apparently simple cytological procedures represented a major breakthrough: via aberrant chromosome segregations, cells with different chromosome complements were produced whose developmental prospects could be followed. As a result, pertinent links could be established between individual chromosomes and development. Boveri observed that, of the very few blastomeres that did not perish, most failed to follow their normal developmental pathways. From these different outcomes, he concluded that individual chromosomes were qualitatively dissimilar and that normal embryos could only develop when the right combination of chromosomes was present. Embryos perished possibly due to the loss of chromosomes involved in cellular housekeeping functions. Abnormal development resulted from loss of chromosomes involved in cellular functions that, although important, were not essential for the cells' viability. Particularly important in the context of tumorigenesis was the finding that, in the abnormal embryos that formed, blastula cells soon lost their epithelial contiguity, giving rise to irregular formations whose microscopic appearance was "strikingly similar to that of a medullary carcinoma". From this and from the previous recognition that malignant cells exhibit abnormal chromosome constitutions, Boveri surmised that "malignant tumors might be the consequence of a certain abnormal chromosome constitution, which in some circumstances can be generated by multipolar mitoses" (Harris, 2008).

Later, Boveri expanded his central hypothesis to include other concepts, some of which are, nowadays, basic tenets of cancer (Harris, 2008). After decades of intense research, it can now be appreciated that his ideas of "inhibitory chromosomes" and "activating chromosomes" anticipated the concepts of tumor suppressor genes and proto-oncogenes, respectively (section 1.2). He also foretold the existence of cell-cycle checkpoints (section 3), predicted the clonal origin of tumors and, implicitly, suggested that genomic instability drives the accumulation of chromosome aberrations and mutations in cancer. That many of Boveri's hypotheses were later proved correct is the more remarkable, considering that he never actually performed experiments with tumor tissue. Quite ironically, this same fact, together with his lack of medical training, may partly account for the great deal of skepticism with which the medical community met his views on the origin of malignant tumors.

Experimental proof of some of Boveri's predictions required the development of adequate cytogenetic techniques for counting and characterizing individual mammalian chromosomes. For instance, before the establishment of the correct chromosome number (i.e., 46), by Tjio and Levan, in 1956 (Tjio & Levan, 1956), the observation of 48 chromosomes in human tumors was accepted as normal, as this number was thought to represent the normal diploid number (Painter, 1921).

To a certain extent, the establishment of cancer as a genetic disease went hand in hand with the development of the emerging discipline of genetics. One crucial issue was the molecular nature of Mendel's factors or characters, these packets of hereditary information that passed discretely from one generation to another and that were responsible for the different characteristics of an organism. By the end of the 1880s, Hugo de Vries had developed the concept of *pangenes*, "special particles for every hereditary character" that composed the chromosomes (de Vries, 1910). In 1914, Boveri suggested, with remarkable foresight that if Mendel's hereditary units were located in the chromosomes, each chromosome had to contain a large number of units, probably arranged in a precise linear order. In his influential book "The Cell in Development and Inheritance", published in 1925, Edmund B. Wilson still referred to the gene, the term by which Mendel's factors became, by then, known, as "an hypothetical elementary entity that is essential to, or determines the development of a particular character" (Benson, 2001). In this context, the independent discoveries, by Hermann Muller (Muller, 1927) and Lewis Stadler (Stadler, 1928a, 1928b), in 1926-1927, that X-rays could induce mutations (in *Drosophila* and in barley and maize, respectively) were truly far-reaching, as they clearly proved genes to be susceptible to damage, transforming Mendel's abstractions into real biological entities.

In 1960, while studying chronic myelogenous (or myeloid) leukemia, Peter Nowell and David Hungerford made a tremendous discovery: tumor cells of this type of leukemia (but not of acute myelogenous leukemia) contained a "minute chromosome" that replaced one of the four smallest autosomes, a modification that was not present in normal cells of the same patients. They proposed the existence of a causal relationship between this chromosome modification and the development of this type of leukemia (Nowell & Hungerford, 1960). Moreover, the presence of this minute chromosome in all malignant cells of these patients lent strong support to Boveri's proposal that tumors originate from a single cell (the monoclonal origin of cancer).

Following the tradition to name each new chromosome after the city in which it was discovered, the minute chromosome was named the Philadelphia chromosome. Its true nature could finally be unraveled with the development of cytogenetic techniques of banding, particularly Giemsa banding (Nowell, 2007). In 1973, Janet Rowley demonstrated that it results from a translocation between the long arms of chromosomes 22 and 9 (Rowley, 1973). Rowley reported other types of translocations in hematopoietic cancers, including a translocation between chromosomes 8 and 21 in acute myeloblastic leukemia cells (Rowley & Potter, 1976). Later, the genes involved in the Philadelphia translocation chromosome were identified: the *v-abl* Abelson murine leukemia viral oncogene homolog (*ABL*), on chromosome 9, and the breakpoint cluster region gene (*BCR*), on chromosome 22. Their juxtaposition creates a fusion gene which encodes an abnormal tyrosine kinase (Groffen *et al.*, 1984; Lugo *et al.*, 1990).

1.2 From retroviruses to oncogenes and proto-oncogenes

The hypothesis that cancer could be caused by viruses was put forward in the beginning of the twentieth century. Although it is now apparent that the number of cancer types directly induced by viruses is rather small, several decades of intense work on the molecular mechanisms of viral oncogenesis have, nonetheless, produced significant discoveries in cancer research. A special mention must be made to Peyton Rous. His discovery, in 1910, of an avian sarcoma that could be successfully transplanted to another host of the same breed (Rous, 1910) was followed, a year later, by his discoveries that cell-free filtrates of this

tumor could cause cancer and that the cancer-causing agent was a "filterable agent", as viruses were then called (Rous, 1911). This virus was later named Rous sarcoma virus (RSV), after its discoverer. Now regarded as ground breaking, these discoveries were not fully appreciated for several decades. Nonetheless, RSV was distributed to many laboratories and the slow accumulation of knowledge on this and other viruses that took place in the decades that followed eventually led to the discovery of the first oncogene, *v-SRC*, as well as to that of its cellular precursor, *c-SRC*. In the late 1960s, Robert Huebner and George Todaro proposed the "oncogene" hypothesis of cancer, according to which both spontaneous cancers and those induced by chemical and physical agents resulted from de-repression of a transforming gene of covert C-type RNA viruses (Huebner & Todaro, 1969). Their suggestion was based on the observation that particles of this unique class of retroviruses, of which RSV is the most famous example, were found to be present in almost all vertebrate species and could be vertically (i.e., genetically) transmitted from cell to progeny cell and from animal to progeny animal. As the induced expression of this gene transformed normal cells into tumor cells, they named it an oncogene. In 1970, G. Steve Martin identified a RSV mutant that was temperature sensitive for transformation, but replicated at the non-permissive temperature. The discovery of this transformation-specific defect was an important step in the physical identification of the viral gene responsible for the transforming action of RSV (Martin, 1970), as it led to identification, by Peter Duesberg and Peter Vogt, of RNA sequences in the genome of RSV that were missing in the replication competent, but transformation-defective viral variants (Duesberg & Vogt, 1970). Finally, in 1976, Dominique Stehelin, Michael Bishop and Harold Varmus identified the first retroviral oncogene: *SRC* (Stehelin *et al.*, 1976a). Based on the intriguing fact that *SRC* was dispensable for virus replication, Bishop and Varmus speculated that it might be a cellular gene that was captured by the transforming virus. In subsequent studies using cDNA hybridization techniques, Bishop, Varmus and colleagues demonstrated the existence of *v-SRC* related sequences in the genome of birds (Stehelin *et al.*, 1976b). To emphasize the lack a direct transforming action, unless mutated or overexpressed, this new type of gene, *c-SRC*, precursor of viral oncogenes, was designated a proto-oncogene (Iba *et al.*, 1984; Parker *et al.*, 1984). The discovery of *c-SRC* triggered a frenzy of research into the roles of oncogenes, allowing for a better understanding of the signal-transduction pathways that control several biological processes. Of note was the finding that *SRC* is active in many human epithelial cancers (Bolen *et al.*, 1987; Jacobs & Rubsamen, 1983; Yeatman, 2004).

1.3 From hereditary cancers to tumor suppressor genes

The idea that multiple mutations on DNA, both in somatic and germ-line cells, and the formation of cancer are closely related can be traced back to the beginning of the twentieth century (Bignold *et al.*, 2006). In 1953, Carl Nordling analyzed the incidence of cancer in some countries and related it with the age and gender of the population, concluding that about six mutations (or hits) are concordant with the age when cancer usually emerges. He explained the high incidence of tumors in children by a higher rate of cell division during fetal development and, concomitantly, an enhanced accumulation of mutations (Nordling, 1953). However, it was just in 1971, with Alfred Knudson, that the multiple-hit theory gained firm ground. Knudson made a statistical analysis of cases of retinoblastoma, a tumor of the retina, which occurs both sporadically and as an inherited disease. He analyzed the occurrence of both unilateral and bilateral tumors (i.e., occurring in a single or in both eyes, respectively) and established that retinoblastoma was caused by two mutations. In bilateral

cases (familial cases), a mutation is inherited and the second mutation occurs after. In unilateral patients with sporadic cancers, both mutations are somatic. His hypothesis explained why a child born with the first hit in all cells was more likely to develop cancer in both eyes at an early stage and why a child who needed to have two mutations on somatic cells would probably just develop cancer in one eye (Knudson, 1971). Later, the retinoblastoma gene (*Rb* gene) was localized to chromosome 13 and unilateral and bilateral cancers were found to have the same second mutation in the *Rb* gene, indicating that cancer development occurred after inactivation of the second allele of the gene (loss of heterozygosity) (Cavenee *et al.*, 1983). The first tumor suppressor gene, *Rb*, was identified (Lee *et al.*, 1987). In 1988, Harbour found abnormalities in the *Rb* gene in small cell lung cancer (Harbour *et al.*, 1988). In the next years, multiple oncogenes (*NEU*, *c-MYC*, *c-MYB*, *RAS*) and tumor suppressor genes (*Tp53*, *Rb*) were found to be modified in different types of cancer.

2 DNA lesions: Types, origins and consequences

The genome is inherently unstable, undergoing spontaneous chemical reactions in the aqueous nuclear milieu, such as hydrolysis of nucleotide bases and non-enzymatic methylations. Genome integrity is also constantly compromised by occasional mismatches introduced by DNA polymerases during replication and by DNA strand breaks generated as a consequence of abortive activities of topoisomerases I and II. Finally, the genomes of all organisms are continuously exposed to a myriad of endogenous and exogenous agents that also produce DNA lesions (Jackson & Bartek, 2009). Altogether, these factors are responsible for the emergence of tens of thousands of DNA lesions per cell per day that corrupt our genetic information. These DNA lesions are varied and are frequently related to the nature of the genotoxic agent that produced them (Jackson & Bartek, 2009). They include adducts, oxidized bases, abasic sites, DNA crosslinks, single-strand breaks (SSBs) and, less frequently, double-strand breaks (DSBs). Although rather infrequent, these latter lesions are extremely toxic and difficult to repair. This section will briefly describe types, origins and consequences of DNA lesions that may be involved in tumorigenesis.

There are multiple examples of spontaneous hydrolytic reactions occurring at the level of the DNA molecule, such as the hydrolytic deamination of the DNA base cytosine, leading to the formation of the aberrant base uracil. This type of reaction has a high rate of occurrence (~100-500 times per human cell per day), particularly in regions of single-stranded DNA, such as replication forks, where protection of cytosines by the complementary strand is missing. Its incidence also increases with unmethylated cytosine. If this lesion stays unrepaired, point mutations C:G to T:A will occur upon DNA replication (Barnes & Lindahl, 2004; Parker & Stivers, 2011; Shen *et al.*, 1994). Misincorporated uracils can be removed at a high rate by DNA glycosylases of the base excision repair (BER) pathway (section 4), generating gaps in the DNA strands. This type of gap that does not contain any base, i.e., neither a pyrimidine nor a purine are known as AP (apurinic/apyrimidinic) or abasic sites. The main problem is that adenine and guanine can also be removed from the DNA strands at a similar high rate. As the resulting AP sites are identical in all cases, the repair machinery will randomly incorporate a new base. Fortunately, this probably does not occur to a significant extent *in vivo* (Barnes & Lindahl, 2004). Non-enzymatic hydrolysis of DNA bases (i.e., hydrolytic depurination) is another mechanism of production of abasic sites. Hydrolytic deamination is also responsible for the conversion of guanine, adenine and 5-methylcytosine to xanthine, hypoxanthine and thymine,

respectively, a process that can also be induced by X-rays, oxygen radicals or alkylating agents (Barnes & Lindahl, 2004; Hoeijmakers, 2001). Hypoxanthine forms a more stable base pair with cytosine than with thymine, leading to A:T to G:C transversions. Xanthine also pairs preferentially with cytosine, but with less coding specificity. So, the formation of hypoxanthine may be more dangerous from the point of view of formation of premutagenic lesions (Lindahl, 1993). The formation of thymine gives rise to G:T mispairs, frequently observed at CpG islands (Shen *et al.*, 1994).

Amongst endogenous genotoxic agents are certain by-products of physiological processes. Some of them, particularly reactive oxygen species (ROS) (Cooke *et al.*, 2003) produced during aerobic cellular respiration, represent a considerable threat to genome integrity. One damaging effect of these reactive species is the loss of DNA bases. ROS, as well as nitrogen reactive species, are also formed at sites of inflammation and infection by neutrophils and macrophages (Kawanishi *et al.*, 2006) and some of these reactive species can also be generated by environmental agents through redox-cycling processes, by Fenton reactions mediated by heavy metals (e.g., iron) and by ionizing radiation. The main modification that ROS introduce into the DNA backbone is the oxidation of guanine, generating 7,8-dihydro-8-oxoguanine (8-oxo-guanine). Contrary to its normal counterpart, the oxidized base interacts with cytosine and adenine nucleotides with almost the same affinity, eventually causing the transversion mutation from G:C to T:A (Bruner *et al.*, 2000). This same point mutation can result from the action of a variety of mutagens that also produce ROS, such as ultraviolet (UV) and ionizing radiation (see below). Not surprisingly, it is the second most frequently found somatic mutation in human cancers and is commonly found in the mutational spectrum of *Tp53* gene (Bruner *et al.*, 2000).

The modification of bases is by no means restricted to the formation of 8-oxo-guanine. For instance, the highly mutagenic O6-methylguanine can be formed by external alkylating agents (e.g., N-methylnitrosourea) through transfer of a methyl group to the oxygen atom of a guanine. The modified base can also pair with thymine, and not only with cytosine (Barnes & Lindahl, 2004).

UV light (Kapetanaki *et al.*, 2006), ionizing radiation and various chemicals (Kondo, 1977; Shrivastav *et al.*, 2010) are examples of exogenous genotoxic agents. Some of them, such as UV light, ionizing radiation and tobacco-derived chemicals, have been firmly established, by epidemiologic, animal and *in vitro* studies, as carcinogens. Exposure of the skin to UV radiation (both UV-A and -B; see below) is linked to skin cancer, both melanoma and non-melanoma. Cancers of the lung, oral cavity and adjacent tissues, amongst others, are known to be induced by tobacco-derived chemicals, probably the most prevalent environmental cancer-causing chemicals.

Under strong sunlight, UV radiation is a potent promoter of DNA lesions (Jackson & Bartek, 2009). According to its wavelength, this radiation can be classified into UV-A (320–400 nm), -B (280–320 nm) and -C (200–280 nm). Fortunately, the ozone layer in the upper atmosphere completely absorbs the most energetic component, i.e., UV-C, as well as a significant portion of the UV-B component, with only about 1-10% of this latter component actually reaching the earth. However, the major part (90–99%) of UV-A radiation crosses the atmosphere, reaching the earth surface (Bachelor & Bowden, 2004). Although this radiation is not energetic enough to produce direct DNA damage, it, nonetheless, produces indirect damage, mainly through the induction of free radicals and singlet oxygen (Wang *et al.*, 2001). On the contrary, the more energetic UV-B radiation can interact directly with DNA, thus presenting an enhanced mutagenic and carcinogenic action.

The main DNA lesions generated by UV-B radiation are pyrimidine dimers, which are both cytotoxic and mutagenic (Lindahl & Wood, 1999). Upon sunlight exposure, each cell of exposed skin may suffer 50-100 such lesions per second. This type of radiation can be absorbed by the 5-6 double bonds of DNA pyrimidines, allowing them to open. When two pyrimidines are adjacent in a DNA molecule, covalent bonds can form between them. The most frequent product of this reaction is a four-membered ring [a cyclobutane-type pyrimidine dimer (CPD)], resulting from the formation of two bonds between the neighboring bases. Less frequently, only one bond forms between the two pyrimidine molecules, giving rise to a 6,4-photoproduct (Goodsell, 2001). The formation of dimers induces local distortions in the DNA helix that weaken base pairing. As a result, these dimers can be misread during replication, introducing mutations. The signature mutations caused by UV light involve C to T mutations, caused when cytosines are mispaired with adenine bases during replication. The replacement of one cytosine by one thymine accounts for 70% of the UV-induced mutations and 10% of mutations involve the replacement of both cytosines by two thymines (Brash, 1997; Sage *et al.*, 1996). These signature mutations are frequently detected in the *Tp53* tumor suppressor gene of most human non-melanoma skin cancers (Bruner *et al.*, 2000), compromising its watchdog function (section 3), which strongly points for an important role in carcinogenesis.

Interstrand crosslinks (ICLs), which involve the formation of a covalent bond between nucleotides of both strands, are also highly dangerous lesions. Given that these crosslinks involve both strands of DNA, they inhibit strand separation during replication and, consequently, prevent transcription and translation. A variety of bi-functional alkylating agents, including platinum compounds, mitomycin C, nitrogen mustards and psoralen, can induce this type of lesion. The interstrand crosslink structure formed depends on which compound interacts with DNA and, consequently, the nature of its repair is diverse (Hlavín *et al.*, 2010).

SSBs on the DNA backbone can be produced by both endogenous and exogenous agents, namely ROS and alkylating compounds, respectively. The formation of this type of lesion is intrinsically linked to the presence of AP sites on the DNA molecule and with the activity of the BER system (Hoeijmakers, 2001). The major concern about SSBs is that they can be precursors for a foremost dangerous lesion, i.e., DSBs. Indeed, when two SSBs arise in close proximity, or when the DNA-replication apparatus encounters a SSB, DSBs are formed. DSBs can also be induced by some anti-tumoral agents that induce ROS generation. These lesions have a particularly high incidence in telomeric regions due to imperfect metabolism of chromosome ends (Khanna & Jackson, 2001).

The genome can also be modified on a large-scale basis through modifications in the structure and/or number of copies of chromosomes. Changes in chromosome structure, termed rearrangements, can occur by multiple processes, particularly by deletion or duplication of a chromosome portion, by inversion (modification of DNA orientation) or by translocation. The main cause of these chromosomal modifications is the breakage of DNA in two different locations, followed by a rejoining of the broken ends, which leads to a different chromosome organization (Nambiar & Raghavan, 2011). In the particular case of translocations, which exhibit a high prevalence in different types of cancer (section 1 and below), the lesion is formed when a segment of a given chromosome is moved to a different chromosome. The simplest type of translocation is the reciprocal translocation, usually involving change of genetic material between non-homologous chromosomes. As a consequence, translocations can cause recombination of normally separated genes (fusion

genes) or juxtaposition of the entire coding region next to an active promoter of other gene (Griffiths *et al.*, 2004).

Chromosome translocations are commonly observed on hematological cancers. About 90% of lymphomas and 50% of leukemias contain translocations (Nambiar & Raghavan, 2011). A translocation of genetic material between chromosomes 8 and 14 (t(8:14)(q24;q32)) is found on 80% of all cases of Burkitt lymphoma, resulting in the fusion of *c-MYC* (section 3), from chromosome 8, with a gene coding for a heavy-chain of a immunoglobulin (Hecht & Aster, 2000; Taub *et al.*, 1982). This translocation results on *c-MYC* overexpression, because it is placed under the control of 3' regulatory elements of the immunoglobulin (Hecht & Aster, 2000). The Philadelphia chromosome (section 1), which has a high incidence in chronic myelogenous leukemia, results from a translocation between the chromosomes 9 and 22, forming an aberrant *BCR-ABL* gene on chromosome 22. The protein expressed by this fusion gene has a constitutively tyrosine phosphokinase activity, which is essential for the oncogenic potential of *BRC-ABL*. This translocation is also found in acute lymphoblastic leukemia, but with a lower incidence (Kurzrock *et al.*, 2003). These cases exemplify the activation of a gene through its insertion next to a promoter and the overexpression of a fusion gene, respectively.

Translocations have also been found in solid tumors. For example, gene fusion was observed in a large number of prostate carcinomas, with *TMPRSS2-ERG* as the most common form. *ERG* overexpression is associated with *in vitro* invasiveness by activation of metalloproteinase pathways (Kumar-Sinha *et al.*, 2008). For a list of translocations associated with non-lymphoid malignancies, the reader is referred to (Nambiar *et al.*, 2008).

Despite their frequent occurrence in tumors, the exact mechanism of translocation formation is not fully understood. However, it is well known that formation of DSBs is essential (Khanna & Jackson, 2001). On hematopoietic tumors, chromosomal translocations usually involve the immunoglobulin locus on chromosome 14 and some data suggest that V(D)J recombination, a site-specific reaction necessary for the assembly of antigen receptor genes in developing B and T lymphocytes (Nussenzweig & Nussenzweig, 2010), may have an important role in this process (Nambiar & Raghavan, 2011).

3. The hallmarks of cancer and their genetic underpinnings

Advances in the last century allowed us to comprehend at the cellular and molecular levels tissue architecture and function in metazoans. During embryonic development, tailoring of new tissues is achieved through cell-cell competition: when two cell subpopulations with the same ancestry, but different growth potentials arise, faster-growing cells induce apoptosis in slower-growing ones and later engulf them, achieving a higher relative contribution to the adult tissue (Johnston, 2009). In adults, almost all organs possess niches harboring lineage-specific adult stem cells, which can be stimulated upon injury to produce progenitor (or transit amplifying) cells. These will then divide a certain number of times to regenerate the tissue, until they eventually become terminally differentiated (He *et al.*, 2009). Cell growth and division occur in a periodical manner throughout a cell's proliferative life. Experiments in plants carried out in the early 1950s established that each cell had DNA "units" characteristic of its particular strain. The amount of this DNA duplicated during growth and was halved during gamete production (Swift, 1950). Later studies using DNA labeled with radioactive phosphorus (^{32}P) permitted a more accurate analysis of the DNA content throughout the cell cycle (Howard & Pelc, 1953), which suggested a partition in

phases that still holds today (Sisken & Morasca, 1965). Cell cycle can be broadly divided in interphase and mitotic (M) phase. Interphase is a phase of growth during which metabolically active cells prepare themselves for cell division. It can be further divided in Gap 1 (G1), when cells receive external stimuli to grow, Synthesis (S), when DNA duplication occurs, and Gap2 (G2), which serves for cells to continue augmenting their size and ensuring that the mitotic machinery is ready. M phase can be further divided into mitosis, i.e., the division of the nucleus (and its chromosomes), and cytokinesis, i.e., division of the cytoplasm, giving rise to two daughter cells, genetically identical to each other and to the mother cell (Morgan, 2007).

Apoptosis is the programmed cell suicide that occurs following specific signals, which may be either intracellular stresses or external signals (intrinsic and extrinsic program of apoptosis, respectively). The main purpose of this cellular program is the protection of organisms from eventual deleterious effects of individual defective cells (Kerr *et al.*, 1972). It is always an unbalance between pro- and anti-apoptotic proteins (Adams & Cory, 2007) that triggers a cascade of cellular events, ultimately leading to activation of dormant cysteine proteases, the caspases, whose effector members provoke controlled cell disintegration (Danial & Korsmeyer, 2004).

Under normal conditions, cell cycle progression is tightly regulated (Rajewsky & Muller, 2002). When genomic integrity is compromised beyond a certain level, apoptosis ensues in order to guard the viability and function of the organism as a whole (Kerr *et al.*, 1972; Levine, 1997). In stark contrast, the high genomic instability existent in tumor cells, which may result, for instance, from overall DNA hypo-methylation (Eden *et al.*, 2003) or deficiency in mismatch repair (MMR; discussed in the next section) (Parsons *et al.*, 1993), gives rise to a set of mutations in growth-related genes that bestows cancer cells with enhanced growth and down-regulated apoptosis. These properties work in combination as a potent driving force of the aggressive capacity of neoplasias to rapidly evolve and proliferate (Campbell *et al.*, 2010).

Mutations with carcinogenic potential are mainly restricted to two classes of genes: proto-oncogenes and tumor suppressor genes. Proto-oncogene products are involved in development and tissue maintenance, usually having a stimulatory effect on cell growth; their mutated versions are called oncogenes and are, by definition, capable of inducing cancer through constitutive activation of proliferation (Adamson, 1987). On the other hand, tumor (or growth) suppressor gene products restrain cell growth and division and may even elicit apoptosis. The *Tp53* gene is a well-known example (Levine, 1997).

As mentioned before (section 1), the term cancer does not designate a single pathology, but rather over 100 distinct neoplastic diseases (or types of cancer) best known by an uncontrolled multiplication of abnormal cells. The observation, in pathological analyses, of particular lesions that seemed to represent intermediate, or premalignant, states between normalcy and invasive cancers strongly suggested that human tumorigenesis is a multistep process. Paradoxically, carcinogenesis and normal aging share more traits than previously suspected, including increasingly heterogeneous gene expression patterns (Bahar *et al.*, 2006; Campbell *et al.*, 2010) and modifications in DNA repair systems (Finkel *et al.*, 2007). The fact that the genomes of tumor cells invariably exhibit multiple alterations, ranging from the gross changes in chromosome complement, identified more than a century ago (section 1), to single base substitutions (i.e., point mutations; e.g., Kinzler & Vogelstein, 1996), led to the concept of cancer as a genetic disease. Thus, according to the dominant paradigm, carcinogenesis involves the successive acquisition of genetic alterations that confer certain

growth advantages (Fearon & Vogelstein, 1990). These alterations, when co-adjuvanted by chronic exposure to a growth promoter (Berenblum & Haran, 1955), act as a positive feedback loop, allowing further genetic alterations to accumulate. As a result, normal cell proliferation and homeostasis are progressively subverted, driving the stepwise transformation of normal human cells into increasingly malignant tumor masses. Taken together, these and other observations depict neoplastic transformation as a micro-evolutionary process: its inception depends on the *ab initio* transformation of a single cell and on the natural selection pressures that this transformed cell must overcome in order to thrive and proliferate in a specific hostile microenvironment, giving rise, through successive rounds of clonal selection, to a tumor mass, from which a subset of cells, possessing clonogenic potential, will invade surrounding and, ultimately, distant tissues (Aslakson & Miller, 1992).

In 2000, Hanahan and Weinberg, construing the seminal discoveries on the molecular basis of cancer achieved during the twentieth century, hypothesized that, in spite of their remarkable diversity, all neoplastic diseases might be rationalized in terms of a small number of underlying principles. At the time, they identified six well-defined traits (hallmarks) acquired by most, if not all, animal cells during their progressive transformation into fully malignant derivatives: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Very recently, and based on the intense research performed in this field during the last decade, the same authors updated this conceptual framework of cancer biology and included genomic instability as an instrumental, enhancing characteristic of tumorigenesis (Hanahan & Weinberg, 2011). Other emerging topics are tumor-associated cells and bioenergetics.

This section will briefly address the regulatory circuits that govern normal cell proliferation and homeostasis and then describe some of the genetic reprogramming that may underlie the hallmarks of cancer. It must be stressed that, although this chapter focuses on genetic alterations, it is now well established that epigenetic changes can be adjuvants or even surrogates of genetic mutations: for instance, specific DNA hypermethylation leads to silencing of tumor suppressor genes (Herman & Baylin, 2003) and hypo-methylation not only allows up-regulated transcription of oncogenes (Nishigaki *et al.*, 2005), but also, when generalized, genomic instability (Eden *et al.*, 2003). This additional layer of complexity sheds light on the conspicuous heterogeneity of cancer cell populations (Feinberg *et al.*, 2006) and accommodates the groundbreaking report of the generation of a whole mouse by transference of a melanoma cell nucleus into a normal oocyte. Thus, at least for some cancers, alterations that lead to aberrant development and malignization may be essentially perennial, rather than engraved in the genome (Hochedlinger *et al.*, 2004). Probably even more impressive is the fact that normal cells belonging to tumor stroma may behave as co-conspirators. For instance, cancer-associated fibroblasts may actively contribute to tumor initiation (Hayward *et al.*, 2001) and progression (Olumi *et al.*, 1999) and senescent fibroblasts were shown to foster tumorigenic potential of neoplastic cells (Krtolica *et al.*, 2001).

3.1 The regulatory circuits that govern normal cell proliferation and homeostasis

Eukaryotic cells are dependent on paracrine signaling to progress through the G1 phase of the cell cycle: growth factors produced by neighboring cells, such as endothelial growth factor (EGF) and platelet-derived growth factor (PDGF), are sensed by receptor tyrosine

kinases (RTKs) (Robinson *et al.*, 2000). These extracellular cues are internalized by RTKs through the activation of the highly conserved mitogen-activated protein kinase (MAPK) pathway, more specifically the RAS/RAF/MEK/ERK pathway (Mcubrey *et al.*, 2007). This protein kinase cascade is responsible for conveying and amplifying extracellular signals of growth factors to transcription factors that activate expression of genes essential for cell cycle progression (Aktas *et al.*, 1997), such as *c-FOS* and *c-MYC* (Armelin *et al.*, 1984; Kruijer *et al.*, 1984). When the environment is hostile, either due to deprivation of growth factors (Zetterberg & Larsson, 1985) or nutrients (Tobey & Ley, 1970), or upon contact inhibition (Nilausen & Green, 1965), cells stop dividing in a reversible manner. When this mechanism of adaptation was initially observed, termed negative pleiotypic response at that time (Hershko *et al.*, 1971), it was controversial whether cells were fixed somewhere in G1 or whether cell cycle was effectively abandoned. Later findings have shown that several cell growth inhibitory conditions lead to cell cycle exit to a quiescent viable state, or G0 phase, at a particular point of G1 (Pardee, 1974). This point, termed the restriction (R) point, is now known to be the checkpoint for entrance in S phase (Harbour & Dean, 2000). Tp53, widely known as caretaker of genome integrity (Levine, 1997), was attributed a central role in triggering and maintaining this quiescent state in human cells (Itahana *et al.*, 2002). Despite this apparent unity, it was found that different growth inhibitory conditions (e.g., serum withdrawal and high cell density) may trigger quiescence through distinct genetic mechanisms (Gos *et al.*, 2005).

What is, then, the molecular basis governing commitment to cell division? Curiously, a single locus in the short arm of chromosome (9p21), *CDKN2a*, codes two paramount proteins in R point regulation, p16^{INK4a} and p14^{ARF} (Quelle *et al.*, 1997). p16^{INK4a} inhibits cyclin-dependent kinases (CDKs) 4 and 6, responsible for the phosphorylation of retinoblastoma protein, Rb, the guardian of the R point. While in the hypo-phosphorylated state, Rb halts entry into S phase by imprisoning proteins of the elongation factor 2 (E2F) family necessary for the transcription of genes involved in DNA synthesis. In the presence of extracellular growth stimuli, p16^{INK4a} inhibition takes place, allowing entry into S phase (Harbour & Dean, 2000); it is noteworthy, however, that excessive mitogenic signaling can actually activate p16^{INK4a} (Lin *et al.*, 1998) and induce senescence. On the other hand, ARF represses HDM2, a negative regulator of Tp53, in turn responsible for promoting cell cycle arrest via enhanced transcription of GADD45, a PCNA-binding protein (Levine, 1997). This frugality of nature in allocating in the same locus two cell cycle inhibitors acting upstream the most preponderant cell division regulators, *Rb* and *Tp53*, is very rare in mammalian genomes. Interestingly, insertion of cancer-associated *p16INK4a/p14ARF* locus mutations in mice, whilst compromising G1 arrest through p16^{INK4a}, did not affect ARF function (Quelle *et al.*, 1997). These findings are biologically sound: though the action of Rb as a downstream element of Tp53-driven cell cycle arrest (Hahn & Weinberg, 2002) might suggest the existence of a single pathway involving these two proteins, it is long known that, whereas p16^{INK4a} induces G1 arrest, p14^{ARF} can block cell cycle both at G1 and G2 phases (Quelle *et al.*, 1995). In fact, the number of independent functions ascribed to these tumor suppressors has been growing remarkably: Tp53 functions as a cell death arbiter, triggering apoptosis in response to genotoxic (Levine, 1997), acidic (Williams *et al.*, 1999) and hypoxic (Hammond & Giaccia, 2005) stresses, acting also as a bioenergetic switch (Ma *et al.*, 2007), while Rb has been shown to carry out its tumor suppressor activity also via cell cycle-independent transcriptional promotion of differentiation (Sellers *et al.*, 1998), being neuronal migration a recently described example (McLellan *et al.*, 2007). Finally, it is important to note that much

remains to be known about the integration of Tp53 and Rb pathways at the organismal level, as mice genetically engineered to harbor individual cells lacking either Tp53 (Ghebranious & Donehower, 1998) or Rb (Lipinski & Jacks, 1999) displayed no neoplasias, exhibiting only a greater tendency to develop malignancies late in life.

Apart from growth factors, normal cells need also to be anchored on a substratum in order to proceed in cell cycle (Schulze *et al.*, 1996). This is due to the dependence of *Cyclin A* gene expression, a crucial event for DNA replication (Girard *et al.*, 1991), on cell anchorage. Indeed, forced expression of *Cyclin D1* was sufficient to ablate blockade of *Cyclin A* expression and, concomitantly, cell cycle progression (Schulze *et al.*, 1996). This cell cycle regulator associates with CDKs 4 and 6 (Sherr & Roberts, 1999), eliciting Rb phosphorylation and, consequently, entrance into S phase, as described above (Harbour & Dean, 2000). Actually, the sustenance of a feature classically associated with tumorigenic and metastatic potential, anchorage-independent growth (Shin *et al.*, 1975), is achieved through constitutive activation of *Cyclin D1* proto-oncogene via mutations, translocations or amplification (Moreno-Bueno *et al.*, 2003). Furthermore, in that state of anchorage-independent growth, the expression profiles of surface adhesion molecules, essential for the establishment of anchorage-dependent growth, are tightly regulated to favor growth and metastasis: AKT, activated through direct phosphorylation by up-regulated integrin-linked kinase (ILK), increases integrin expression, leading to malignization and invasiveness (Mizejewski, 1999; Persad *et al.*, 2001; Persad & Dedhar, 2003), whereas loss of vinculin fosters anchorage-independent growth (Rodriguez Fernandez *et al.*, 1993; Rodriguez Fernandez *et al.*, 1992) and downregulates the tumor suppressor phosphatase and tensin homolog (PTEN) (Subauste *et al.*, 2005).

3.2 Subversion of growth signaling pathways

One of the characteristics of cancer cells that have long intrigued scientists is their autonomy relatively to the surrounding tissues. Cancer cells have developed several strategies to outwit normal proliferation homeostasis within an organism, namely self-sufficiency in growth signals and insensitivity to growth-inhibitory signals. Although much remains to be known concerning the precise cellular and molecular mechanisms underlying these capabilities, likely to be cancer-type specific, it is now clear that several cancer cell types engage in autocrine growth signaling: by synthesizing and extruding to the extracellular medium growth factors that are recognized by their own surface receptors, these cells trigger their own proliferation (Grivennikov & Karin, 2008; Pandey *et al.*, 2008). A classical example is transforming growth factor alpha (TGF- α), initially discovered in retrovirus-transformed cells (de Larco & Todaro, 1978), which competes with EGF for its receptor, EGFR, due to structural similarity (Marquardt *et al.*, 1984). TGF- α autocrine overactivity is a common feature of cancers, being TGF- α amplification one of the underlying mechanisms (Yung *et al.*, 1990), revealed essential for growth initiation of cancer cells (Jiang *et al.*, 1998). Additionally, some tumors display mutated versions of EGFR that chronically impinge on signaling pathways, independently of any stimulation by their respective ligands (Boerner *et al.*, 2003).

But how do these cells sustain the resultant constitutive growth? It is now evident that, during carcinogenesis, cells undergo a profound reset of their signaling pathways downstream of this unchecked stimulatory input, usually also through mutations in proto-oncogenes. Paradigmatic examples are *RAS* and *RAF* (Davies & Samuels, 2010), as mutations in these genes lead to production of abnormal proteins that drive constitutive

stimulation of cell growth and proliferation in cancer cells through chronic activation of the MAPK cascade (Mcubrey *et al.*, 2007). Furthermore, cancer cells are also reprogrammed to be intrinsically resistant to antigrowth signaling. This is achieved either through downregulation or deleterious mutations of transforming growth factor (TGF)- β receptors (Stover *et al.*, 2007), whose ligand, TGF- β suppresses *c-MYC* expression (Pietenpol *et al.*, 1990). *c-MYC* is a proto-oncogene whose product, c-MYC, orchestrates the expression of a myriad of genes involved in cell growth and metabolism, being mutated in ca. 30% of all human cancers (Dang *et al.*, 2008). Strikingly, c-MYC transcription factor is a keystone in malignant dedifferentiation since it has the unique capacity to activate a core set of genes associated with embryonic stem cells in human epithelial primary and tumor cells alone (Wong *et al.*, 2008).

3.3 Getting immortal: Proliferation enhancement and apoptosis short-circuiting

At the beginning of the last century, it was believed that cultured mammalian cells were immortal if the proper culture conditions were provided. This belief was based on claims by the Nobel laureate Alexis Carrel that chick fibroblasts had been continuously maintained in his laboratory for more than 30 years (Carrel & Ebeling, 1921). However, this remarkable achievement could never be reproduced by other researchers, and it was found later that it had been merely the result of a regular addition of chick embryonic stem cells to the culture by technicians, unbeknownst to him (Witkowski, 1980). In 1961, Leonard Hayflick clearly established that cells have a limited replicative span, independently of culture conditions or surrounding cells (Hayflick & Moorhead, 1961), after which they completely stop dividing, irreversibly entering a state called replicative senescence (RS) (Pardee, 1974). With the cells used (human diploid fibroblasts, the number of cell divisions necessary for RS to be reached (later called the Hayflick) was approximately 50. The molecular basis of this limit has been already established. After each round of replication, chromosomes get shorter, due to an ineptitude of the replication complex to fully replicate chromosome ends (the end-replication problem (Watson, 1972)). Briefly, during DNA replication, DNA polymerase needs an RNA primer to start copying DNA. As it works in the 5'-3' direction, the only perfect template is the 3'-5' (leading) mother strand. The other (lagging strand) loops back on it and can be copied using multiple RNA primers. After the synthesis of DNA between RNA primers, which are later degraded, the replication complex ligates those DNA fragments, but is incapable of synthesizing in the 3'-5' direction (Johnson & O'donnell, 2005). Hence, to prevent the erosion of genes (and also to avoid the end-joining of chromosomes (Blackburn, 2000)), the natural ends of eukaryotic chromosomes are protected by telomeres. These are nucleoprotein structures containing repetitive DNA sequences capped by a multiprotein complex named shelterin. Thus, telomere shortening following each cell division functions as a hardwired program to limit the cellular life span, independently of growth stimuli, and may be considered as a biological "replicometer" (Hayflick, 2000). The hypothesis that the gradual diminution of telomeres could ultimately lead to replicative senescence was first put forward by Olovnikov (Olovnikov, 1973). Evidence strutting this theory came later from studies in human fibroblasts showing that telomere length strongly correlates with replicative span (Allsopp *et al.*, 1992), possessing a threshold value below which replicative senescence is triggered (Steinert *et al.*, 2000). Telomerase, a reverse transcriptase capable of synthesizing telomeric DNA using an internal RNA template (Blackburn, 2000), is not expressed in human somatic cells (Kim *et al.*, 1994).

Recently, a spectacular reversal of the aging process of severely aged transgenic mice deficient in telomerase was observed upon reactivation of telomerase and concomitant telomere lengthening (Jaskelioff *et al.*, 2010). A less mentioned, but also relevant, telomere maintenance mechanism, occurring in both normal (Dunham *et al.*, 2000) and cancer human cells (Muntoni & Reddel, 2005), is alternative telomere lengthening (ALT). ALT relies on homologous recombination (HR), where telomeric DNA is synthesized using another telomere as a template. Interestingly, HR, a cell cycle-dependent DNA repair strategy usually employed when sister chromatids are available, i.e. after DNA duplication during S phase, is central for cancer aggressiveness and the acquisition of drug resistance (Helleday, 2010). Still, the relation between telomere length and cellular replicative span is not clear cut, as immortalization of human fibroblasts via ectopic expression of telomerase did not augment telomeres (Zhu *et al.*, 1999). In primary human bronchial fibroblasts, this procedure actually bestowed immortality to cells with net telomere shortening (Wise *et al.*, 2004). This is in harmony with other cell regulatory roles recently ascribed to telomerase, namely interaction with β -catenin, an important inducer of adult stem cell proliferation (Reya & Clevers, 2005), to activate WNT-dependent genes (Park *et al.*, 2009).

Unlike their normal somatic counterparts, but in common with germline cells, the vast majority of cancer cells express high levels of telomerase, which allows them to overcome replicative senescence and gain immortality (Kim *et al.*, 1994). Still, this strategy may also be interpreted as a hurdle for the generation of chromosomal aberrations, a hallmark of carcinogenesis (Hanahan & Weinberg, 2011). In fact, telomeres are, by definition, the structures that maintain gene integrity throughout cell divisions and impede chromosome end-joining (Blackburn, 2000). In fact, experiments in mice (Chin *et al.*, 1999) and, more recently, in humans (Lantuejoul *et al.*, 2010) strongly suggest that the initial phase of carcinogenesis is marked by severe telomere shortening and concomitant genomic instability. Pre-neoplastic cells capable of enduring this scenario, namely through ablation of the Tp53 genome guardian function (Levine, 1997), may then experience a reactivation of telomerase, earning carte blanche for unchecked growth, division and accumulation of mutations (Hanahan & Weinberg, 2011).

Rather surprisingly, it was recently discovered that senescence, considered a paramount barrier to early tumorigenesis, can actually be driven by oncogene activation, rebating the common thinking that cell proliferation would increase indefinitely upon oncogene activation. In contrast with replicative senescence, oncogene-induced premature senescence cannot be attributed neither to high division rates nor to telomere erosion, as it is absent in normal epithelial proliferation, inflammatory responses and in cells expressing telomerase (Bartkova *et al.*, 2006). Whereas some oncogenes, such as RAS, induce senescence through *p16^{INK4a}* up-regulation (Serrano *et al.*, 1997), breakthrough studies indicated that others act by eliciting DNA repair: in particular, differential labeling with the thymidine analogues IdU and CldU has shown that induction of *Cyclin E* oncogene expression led to premature termination of most DNA replication forks, and that the resulting senescence could be suppressed by blocking DNA repair response (Bartkova *et al.*, 2006). Importantly, DNA damage repair prompts the expression of NKG2D ligands, which are recognized by the immune system natural killer (NK) cells, triggering the recognition and eradication of incipient tumor cells (Gasser *et al.*, 2005). As Tp53 is an upstream inducer of DNA damage repair (Levine, 1997), one may envisage here another mechanism to select for *Tp53*-deficient cells in early neoplastic lesions.

It turns out that most cancer cell types undergo a genetic reprogramming to bypass senescence, initially through inactivation of cell cycle guardians and later by telomerase activation. But how do they escape cell death that usually results from severe genomic damage? Remarkably, cancer cells evade apoptosis by short-circuiting this process at several stages. Mutations in *FAS*, whose gene product is a receptor that induces apoptosis upon interaction with the *FAS* ligand, have been associated with human lymphomas (Gronbaek *et al.*, 1998), whereas mutations that compromise *TP53* function, thus intracellular stress-induced apoptosis, are documented for a wide range of human cancers (Hollstein *et al.*, 1991). Moreover, gene expression may also be altered in order to further the expression of protector proteins, like *BCL-xL* (Foreman *et al.*, 1996) or to decrease levels of pro-apoptotic proteins, such as the tumor suppressors *BAX* and *BAK* (Degenhardt *et al.*, 2002). Similarly, the constitutive activity of *MAPK* cascade drives the inhibitory phosphorylation of the apoptosis agonists *BAD* (Zha *et al.*, 1996) and *BIM* (Ley *et al.*, 2003). In addition, a protein capable of associating with processed caspase-9, blocking the activation of downstream effector caspases, has been observed in non-small cell lung cancer (Yang *et al.*, 2003).

3.4 The onset of a tumor: Neovascularization, invasion and metastasis

A solid tumor comprises a heterogeneous assembly of cell subpopulations whose progression is dependent on the successful summoning of new vessels in order to replenish the tumor mass with oxygen and carbon sources (Folkman, 2003). Actually, the term cancer was probably coined to reflect similarities between the thick neovasculature that develops in later stages of tumorigenesis and the claws of a crab (Feinberg *et al.*, 2006).

In mammalian cells, *in situ* hypoxia stabilizes hypoxia-inducible factor (*HIF*)-1 α . *HIF*-1 α then dimerizes with *HIF*-1 β , forming a potent transcription factor, which is abnormally active in cancers (Dang *et al.*, 2008). This increased activity can result both from normal, physiological stimuli, i.e., the diminished oxygen tension in peri-necrotic areas of tumors (Dachs *et al.*, 1997), and from mutations in growth-associated genes. Indeed, oxygen-independent activation of *HIF* or its downstream responsive genes by loss of tumor suppressor activity or gain of oncogene function is a common feature in neoplastic lesions: loss of *PTEN* (Zundel *et al.*, 2000) or *TP53* (Ravi *et al.*, 2000), or, on the other hand, overexpression of *H-RAS* (Chen *et al.*, 2001), *v-SRC* (Jiang *et al.*, 1998) or *c-MYC* (Shim *et al.*, 1997), have all been described to amplify *HIF* response, suggesting that *HIF* may be a keystone gene of malignant progression. In fact, the pleiotropic action of *HIF-1* is a key part of cancer strategy to thrive and vanquish surrounding tissues. First, its activity is responsible for the transcriptional activation of angiogenic factors (Ikeda *et al.*, 1995), essential in the recruitment of new blood vessels to drive tumor growth. Furthermore, it also triggers the degradation of extracellular matrix (ECM) by the action of metalloproteinases (Pouyssegur *et al.*, 2006). This activity, concomitant with a conspicuous decrease in pH achieved by the abnormal metabolism of tumors (Gatenby & Gillies, 2004; Ferreira, 2010), sets the stage for cancer invasion.

Until rather recently, the mechanisms by which neoplastic tissues acquire their abnormal morphology and plasticity remained essentially mysterious. Nowadays, there is a growing perception that an epithelial-to-mesenchymal transition (EMT) may undergird cancer cell biological properties. This complex genetic reprogramming, essential in normal development, is thought to be responsible for the presence of mesenchymal populations in malignant tumors (Thiery & Sleeman, 2006). Strikingly, a recent breakthrough has shown

that the malignant cells responsible for metastasis are exactly the ones that have undergone EMT in the solid tumor, presenting both self-renewal capacity and enhanced motility (Mani *et al.*, 2008). In the context of a solid tumor, HIF-1 may be the main responsible for orchestrating this concerted change of cell type through blockade of E-cadherin expression (Imai *et al.*, 2003).

Altogether, this strongly suggests that a new integrative approach to the study of genomics may provide valuable insights not only for cancer, but also for other relevant maladies and even to normal organismal homeostasis. We shall see throughout the text the key role of DNA damage and repair dynamics in the inception, maintenance and exacerbation of a malignant phenotype.

4. DNA repair and misrepair in cancer: When the remedy is worse than the disease

Life as we know it would not be possible without the existence of DNA repair mechanisms. If DNA lesions were to accumulate, it is probable that no specialized cellular functions would have evolved, due to high inconstancies in the proteins' composition. Thus, it comes as no surprise that all living organisms have developed mechanisms to detect DNA lesions, signal their presence and promote their repair. These mechanisms, collectively termed the DNA-damage response (DDR), must be sufficiently accurate and efficient to preserve genome integrity. Interestingly, some of the key enzymes involved in DNA repair are highly conserved from bacteria to man (Hoeijmakers, 2001; Mellon, 2005). A refined set of surveillance and regulatory mechanisms, termed cell cycle checkpoints, controlled by a highly organized signal transduction network, ensures that, during each cell cycle, DNA replication and chromosomal segregation are orderly completed and genome fidelity is maintained (Harbour & Dean, 2000; Levine, 1997). DNA repair plays also a crucial role in aging and in a variety of human diseases. For instance, defects in various DNA repair pathways in hereditary diseases have been linked to the predisposition to a number of cancers (Heinen *et al.*, 2002) (section 5).

The importance of DNA repair dynamics in cancer biology became evident as early as the 1970s, when it was observed that mice treated with 4-nitroquinoline 1-oxide (4NQO), a chemical mutagen, displayed increased mortality and reduced incidence of tumors if treated with caffeine, up to five days after treatment with 4NQO (Kondo, 1977). Caffeine is now known to inhibit ATM and ATR kinases and, hence, DDR (Sarkaria *et al.*, 1999). These results suggested that 4NQO-induced damage in template DNA would occasionally be perpetuated during DNA synthesis, thereby indicating that carcinogenesis relies on the inheritance of damaged DNA through cell divisions. On the contrary, the inhibition of ATM and ATR-mediated error-prone translesion repair by caffeine selectively killed premalignant cells. A myriad of DNA repair mechanisms may be subverted during carcinogenesis, fostering the achievement of a fully malignant phenotype. Thus, one may envisage a cellular reprogramming of genomic maintenance towards augmented DNA misrepair as a driving force in tumorigenesis, favoring the progressive arising of clonal populations of cancer cells with higher genomic instability. This emerging feature doubtlessly possesses a highly malignant potential (Hanahan & Weinberg, 2011). The resultant hyper mutational phenotype then undergirds the stepwise acquisition of malignant traits in a Darwinian fashion (Fearon & Vogelstein, 1990).

4.1 Nucleotide excision repair

Nucleotide excision repair (NER) is a guardian against topological distortions in DNA, such as those induced by the CPDs resultant from UV radiation (section 2) (de Laat *et al.*, 1999). It comprises several steps, including lesion recognition, opening of the double helix around the damage, excision of the DNA fragment carrying the adduct and polymerization of a new fragment, followed by its ligation (Mu *et al.*, 1996). At least 16 different proteins are involved, including the XP proteins (groups A to G), named after the syndrome that is caused by their deficiency, *Xeroderma pigmentosum* (XP). This syndrome is characterized by hypersensitivity to UV radiation and a predisposition to skin cancer (Evans *et al.*, 1997).

The NER pathway has been divided into two sub-circuits: global genome repair (GGR), which repairs DNA lesions independently of their location in the genome, and transcription-coupled repair (TCR), which acts upon lesions in regions involved in transcription. In both cases, the unfolding of the double DNA helix is assured by helicases XPD (3'-5' polarity) and XPB (5'-3' polarity) (Scharer, 2003). This process renders the lesion accessible to endonuclease XPG, which, in conjunction with helicase XPA, recognizes the lesion and cleaves the nucleotide at its 3' edge. Next, endonuclease XPF, in association with ERCC1 (excision repair cross-complementation group 1), removes the damaged nucleotide from the 5' edge of the damaged chain and liberates a fragment of 24–32 bases. Finally, a complex of DNA polymerases and ligases is recruited to restore normal nucleotide sequence in the damaged chain (Friedberg, 2001).

TCR, which is triggered by RNA polymerase arrest at sites of DNA distortion (Lainé & Egly, 2006), was discovered following early key observations that CPDs were more efficiently removed from actively transcribed genes (Bohr *et al.*, 1985). One could envisage TCR overactivity as a strategy for cancer cell propagation, as rapidly dividing cells are particularly exposed to transcriptional stress and damage of active genes is a cell cycle-independent potent inducer of Tp53 accumulation and subsequent apoptosis (Yamaizumi & Sugano, 1994). This hypothesis finds support in the fact that cisplatin, a common anticancer drug that forms cross-links with DNA capable of stalling RNA polymerase (Tornaletti *et al.*, 2003), is most effective in TCR-deficient carcinoma cell lines (Stubbert *et al.*, 2010). Studies using transgenic mice deficient in either TCR or GGR (Berg *et al.*, 2000) and mouse embryonic stem cells (de Waard *et al.*, 2008), both valuable models for key malignant cell subpopulations within tumors (Wong *et al.*, 2008), strongly suggest that hampering GGR has even greater carcinogenic potential than hampering TCR. As GGR does not depend on gene expression, these findings led some authors to put forward the hypothesis that the greater oncogenic potential of GGR loss over TCR loss resides in the fact that, albeit both losses facilitate mutations in essential growth-related genes, GGR ablation would awaken inactive proto-oncogenes with simultaneous mutational inactivation of actively transcribed tumor suppressor genes, while TCR ablation would silence active tumor suppressor genes, with a concomitant increase in Tp53-dependent apoptosis (Berg *et al.*, 2000). In fact, *Tp53* null human fibroblasts display a defalcation in GGR and reduction in their overall capacity of repairing CPDs, but normal TCR activity and even an improved resistance against UV cytotoxicity (Ford & Hanawalt, 1995).

Inter-individual variation in lung cancer susceptibility may be modulated in part by single-nucleotide polymorphisms (SNPs) in NER genes. For instance, SNPs in *XPC* and *XPD* increased lung cancer risk in Northern Spain and Chinese populations (Lopez-Cima *et al.*, 2007; Xing *et al.*, 2003). Additionally, interactions between *XPC/XRCC3* and *XPD/XRCC3* polymorphisms were observed, suggesting that coordination between NER and BER repair

pathways contribute to the individual susceptibility to develop cancer (Lopez-Cima *et al.*, 2007). Impressively, it was reported that SNPs in NER genes modified the relation between breast cancer and smoking among African Americans and Caucasians, albeit with some differences (Mechanic *et al.*, 2006). Some studies also suggest that polymorphisms on *ERCC1* and *ERCC2*, two NER genes, may influence risk of glioma (Wrensch *et al.*, 2005). In addition, some minor allele variants of *ERCC4* and *BRIP1* (BRCA1-interacting protein 1) have been reported to increase meningioma risk, while variants of *ERCC2* and *ERCC5* augmented acoustic neuroma risk (Rajaraman *et al.*, 2010). Finally, *XPB/ERCC2* SNP rs13181 variant carriers display higher cutaneous melanoma risk (Mocellin *et al.*, 2009).

4.2 Translesion synthesis

Distortions in the DNA double helix pose problems not only to transcription, but also to replication. However, during evolution, cells have acquired a considerable number of DNA polymerases able to bypass CPDs and other obstacles in a process named DNA translesion synthesis (TLS) (Goodman & Tiffin, 2000). TLS is essential for normal physiology, and alterations in genes coding TLS polymerases are associated with medical conditions such as XP (Masutani *et al.*, 1999). It also provides a paragon of a DNA repair pathway that may be used by cancer cells for their benefit: recent experiments where several cycles of tumor engraftment and treatment with an anticancer drug were carried out have clearly shown that TLS activity drives drug resistance in tumors *in vivo* (Xie *et al.*, 2010b).

4.3 Mismatch repair

MMR is a DNA repair system that recognizes a wide range of genetic lesions, such as insertions, deletions and base mismatches introduced during DNA replication (Larrea *et al.*, 2010). MMR proteins were proposed to act as direct sensors of DNA damage by helping to recruit ATR (ATM and Rad3-related), a phosphoinositide 3-kinase-related kinase (PIKK) implicated in responding to several DNA lesions and stalled replication forks, to sites of DNA damage, triggering the intra-S phase checkpoint (Abraham, 2004; Choi *et al.*, 2010; Yoshioka *et al.*, 2006). Mismatch recognition is carried out by the MutS α (MSH2/MSH6) and MutS β (MSH2/MSH3) heterodimers, which recognize all eight single nucleotide mismatches, as well as small insertion/deletion loop (IDL)-type structures. Following recognition by these heterodimers, MUTL homologue heterodimers (MLH1/PMS2) are recruited and a necessary single-strand scission (nick) is introduced at either the 3' or 5' side of the mismatch by a 5'-exonuclease and some MLH members. The minimal 5'→3' and 3'→5' excision reaction requires hMSH2/hMSH6 (or hMSH2/hMSH3), hMLH1/hPMS2, EXO1, RPA, PCNA, and RFC, while re-synthesis of the single-stranded gap is carried out by DNA polymerase δ (Pol δ) and DNA ligase I (Larrea *et al.*, 2010).

In the context of oncology, MMR is best known for increased frameshift mutation rates (commonly called microsatellite instability (MSI or MSI-H)) caused by its deficiency, characteristic of various human malignancies (Oda *et al.*, 2005). It thus comes as no surprise that proteins involved in this pathway display properties of tumor suppressors. For instance, MSH2 has been recently described to abrogate mutations adjuvant of oncogenic c-MYC activity in early lymphomagenesis (Nepal *et al.*, 2009). Strikingly, breakthrough findings of fifteen years ago have established that chromosomal transfer designed to correct MMR defects in human tumor cells can also bypass TCR deficiency, establishing an

unequivocal link between NER and MMR pathways (Mellon *et al.*, 1996). Nonetheless, little is known about this still hotly debated interaction (Kobayashi *et al.*, 2005).

In humans, defects in MMR genes confer a strong predisposition to hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome (LS), and associated endometrial cancer. Moreover, epigenetic silencing of some MMR genes may contribute to the development of 5 to 15% of sporadic cancers with a microsatellite instability-high (MSI-H) phenotype (Peltomaki, 2003). MMR-deficient colorectal cancers (CRCs) have distinct features from other CRC types, such as MSI, multi-focality, increased likelihood of right-sided colon cancer location, mucinous histology and the presence of a Crohn's-like lymphocytic infiltrate (Greenson *et al.*, 2003; Jenkins *et al.*, 2007). In addition, MSI-H tumors also display multiple defects in other genes containing microsatellite repeats, such as those governing growth signaling (Markowitz *et al.*, 1995), apoptosis (Rampino *et al.*, 1997) and transcriptional activation (Duval *et al.*, 1999).

Three distinct genetic mechanisms are proposed for the onset of MMR-deficient CRCs. First, monoallelic germline MMR mutations and somatic loss of the second MMR allele cause HNPCC. LS carrier tissues are MMR-proficient, but when the second MMR gene allele is lost in some somatic cells, they become MMR-deficient and give rise to either CRC, small bowel, urethra, renal, pelvis, biliary tract, brain, gastric or ovarian cancers. HNPCC is clinically heterogeneous, depending on which of the MMR genes is affected, being classically associated with heterozygous *MLH1*, *MSH2*, *MSH6* and *PMS2* loss-of-function mutations. Recently, variants of the *MSH3* gene were proposed as low risk alleles, contributing to colon cancer risk in LS families when associated with other low risk alleles. Interestingly, some surveys indicate that the spectrum of MMR mutations in HNPCC differs between China and Western countries (Jin *et al.*, 2008) and even between northern and western China (Sheng *et al.*, 2006), suggesting that MMR mutation patterns depend on ethnicity. The second mechanism is germline biallelic MMR gene mutations, where all body tissues are MMR-deficient. The second mutation is associated with early onset of central nervous system (CNS) tumors, hematological malignancies and gastrointestinal neoplasia, as well as autoimmune disorders (Barnetson *et al.*, 2006; Felton *et al.*, 2007; Lindor *et al.*, 2005). Finally, both MMR alleles can be mutated or epigenetically inactivated in some cells, causing *de novo* sporadic MMR-deficient CRCs, which account for ca. 12.5% of CRCs (Barnetson *et al.*, 2006). *MLH1* promoter hypermethylation is the most common cause of sporadic MMR-deficient CRCs (Shen & Issa, 2002). In recent years, constitutional epimutations of *MLH1* and, more seldom, of *MSH2* have been identified in various mutation-negative HNPCC cases. In contrast to genetic mutations, *MLH1* epimutations are reversible between generations and thus display non-Mendelian inheritance, suggesting that these epimutations can be reversed in the gametes and re-established in the somatic cells in successive generations. Still, the molecular profile of tumors from individuals with constitutional *MLH1* epimutations is similar to those of individuals with conventional sequence mutations of *MLH1*, i.e. tumors with MSI and loss of the MLH1 protein (Hitchins & Ward, 2009).

4.4 Base excision repair

ROS are simultaneously a conspicuous byproduct of our metabolism, key molecules in signaling pathways and a major source of DNA damage (section 2). Albeit their function drastically depends upon their concentration and the cell type and environment where they occur (Hussain *et al.*, 2003), cancers in general display constitutive oxidative stress

(Toyokuni *et al.*, 1995). A direct consequence of oxidative stress is the infliction of mutations in DNA (Cooke *et al.*, 2003), which, when coupled with altered DNA repair mechanisms, set the stage for stepwise malignization. The main cellular machinery involved in fixing ROS-induced oxidative DNA damage is the ubiquitous BER. Although some experiments suggest that the aforementioned TCR may also be involved in the repair of oxidative DNA lesions, this field is currently under intense debate, as assessed by the retraction of several articles (Mellon, 2005). Components of the BER pathway constitute a versatile line of defense against not only DNA oxidative damage, but also SSBs and other small, non-helix-distorting lesions. It is initiated by damage-specific DNA glycosylases, which create abasic sites by cleaving the N-glycosidic bond (Hitomi *et al.*, 2007). AP endonuclease then recognizes AP sites and cleaves the DNA phosphodiester backbone, leaving a 3'-hydroxyl group and a 5'-deoxyribose phosphate group flanking the nucleotide gap. Poly(ADP-ribose) polymerase 1 (PARP1), together with PARP2 and poly(ADP-ribose) glycohydrolase (PARG), recognizes the DNA strand interruption and facilitates the recruitment of specific BER proteins, including the BER scaffold protein XRCC1 and DNA polymerase β (Pol β) (Almeida & Sobol, 2007a; Sobol *et al.*, 2000). Subsequently, the repair proceeds by two sub-pathways initiated by Pol β : short-patch (SN) BER repairs one nucleotide, while long-patch (LP) BER repairs 2 to 15 nucleotides. Albeit different subsets of enzymes are used, there is cooperation between the two sub-pathways (Hitomi *et al.*, 2007).

If left alone, most ROS-induced lesions, such as oxidized bases and abasic sites (Cooke *et al.*, 2003), could be replicated by either normal replication or TLS (Goodman & Tiffin, 2000). Misreplication of oxidized bases and non-instructional AP sites would often give rise to point and, sometimes, more complex mutations; SNPs, which are frequently observed (1 in 300 bp) in mammalian genomes, likely result from such mutations. Point mutations in growth-related genes can drive carcinogenesis (Fearon & Vogelstein, 1990), and certain SNPs in DNA repair genes impart greater cancer susceptibility (Goode *et al.*, 2002).

Several reports have confirmed the relationship between SNPs in BER genes and cancer susceptibility (Hung *et al.*, 2005). Particularly, polymorphisms on *hOGG1* and *XRCC1* genes are associated with lung, esophagus, stomach and nasopharyngeal (NPC) cancer risk. Similarly to MMR, SNP occurrence in BER genes appears to be dependent on ethnicity: single nucleotide changes at codons 194, 280 and 399 of *XRCC1* were associated with risk of several types of gastrointestinal, bladder, breast and lung cancers in the Japanese population (Arizono *et al.*, 2008), while SNPs in *hOGG1* codon 326 correlated with increased NPC and gallbladder risk in Southern Chinese populations (Cao *et al.*, 2006; Jiao *et al.*, 2007). The *APE1* Asp148Glu polymorphism is highly predictive for lung cancer in Caucasians, and cumulative cigarette smoking modifies the associations between *XRCC1* Arg399Gln and *XPD* Lys751Gln polymorphisms and lung cancer risk in nonsmokers and light smokers (de Ruyck *et al.*, 2007). Relevant epimutations also occur in BER genes, such as aberrant methylation of *XRCC1*, which contributes to gastric carcinogenesis (Wang *et al.*, 2010).

4.5 Non-homologous end-joining and homologous recombination

Despite the existence of some very specific scenarios where cleavage of both strands of the DNA molecule is essential, such as in recombination of some immune system genes (Jeggo *et al.*, 1995), DSBs are particularly genotoxic. DSBs usually result from insults such as X- or gamma rays or topoisomerase poisons, or simply arise when a replication fork encounters damaged DNA (Hartlerode & Scully, 2009). DSB repair takes place *in vivo* within defined foci characterized by a distinctive histone phosphorylation (γ -H2AX), accumulation of auto-

phosphorylated DNA-PKcs and recruitment of repair and signaling proteins, including 53BP1, NFBP1/MDC1 and the chromatin-bound form of the MRE11/RAD50/NBS1 complex (Chan *et al.*, 2002a; Lou *et al.*, 2003; Mirzoeva & Petrini, 2001; Paull *et al.*, 2000; Schultz *et al.*, 2000; Shang *et al.*, 2003). Two mechanisms have evolved to remediate this type of damage: non-homologous end joining (NHEJ) and homologous recombination (HR) (Hartlerode & Scully, 2009).

Ku70, DNA-protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV and Ku86/XRCC5 are the major proteins involved in NHEJ. Curiously, some Ku86 truncated C-terminus variants (Ku86v) with decreased Ku-DNA end binding and DNA-PKcs activities have been reported in human myeloma cells and associated with augmented chemo- and radiotherapy sensitivity (Tai *et al.*, 2000). The higher sensitivity of Ku86V-portraying cells may result from compromised DNA repair ability, making this gene a novel therapeutic target in cancer (Tai *et al.*, 2000).

A statistically significant correlation between three *Ku86/XRCC5* polymorphisms, one *XRCC6* tSNP and a single-locus variant at the *DNA ligase IV* SNP2 and the risk to develop gliomas has recently been established (Liu *et al.*, 2007). Moreover, an haplotype analysis performed in samples from glioma patients also recognized genetic variants in *XRCC4* as risk predictors and identified a three-locus interaction involving *DNA ligase IV* SNP4 rs1805388:C>T, *XRCC4* SNP12 rs7734849:A>T and SNP15 rs1056503:G>T as a common feature of these tumors (Liu *et al.*, 2007). A significant association between *XRCC4* (rs1805377) and *DNA ligase IV* (rs1805388) genotypes was also observed among non-small cell lung cancer (NSCLC) patients, of which those who had a homozygous variant guanine/guanine genotype of the *XRCC4* gene were given a poorer prognosis (Tseng *et al.*, 2009).

NHEJ overactivity has been implicated in human myeloid leukemia pathogenesis (Gaymes *et al.*, 2002), a cancer type marked by chromosomal aberrations, namely the Philadelphia translocation (sections 1 and 2) (Nowell, 2007). More precisely, this DNA repair deregulation, which seems to be present in both the acute and chronic forms of the disease, leads to conspicuous misrepair of DSBs, resulting in deletions of up to 400 bp. Ku70/Ku86, a protein heterodimer that binds free ends at a DSB (Mimori & Hardin, 1986), was implicated in repair infidelity, suggesting that alterations in this complex drive genomic instability in myeloid leukemia (Gaymes *et al.*, 2002).

HR is a cell-cycle dependent DNA repair pathway, as it normally relies on sister chromatids to perform error-free repair of DSBs (Richardson *et al.*, 1998). Similarly to NHEJ, which is instrumental in immune cell development (Jeggo *et al.*, 1995), HR also performs an important role in cell physiology, as it provides an alternative strategy for telomere length maintenance (Dunham *et al.*, 2000), as already mentioned in section 3. HR uses the RAD50, BRCA1 and BRCA2 protein families, as well as XRCC3, a member of the RECA/RAD51-related protein family. It can be divided essentially in two sub-pathways: in homology-directed repair (HDR), a homologous sequence in a sister chromatid is used to fix the damaged sequence, whereas in single-strand annealing (SSA), single strands of the same helix undergo annealing. This can happen in repetitive regions of DNA, leading to loss of information and may be considered as especially mutagenic if one bears in mind that ca. 50% of our genome is constituted by such sequence repeats (International Human Genome Consortium, 2001).

At first glance, it is not clear-cut whether prompt HR activity is advantageous for cancer cells or not. Remarkably, transcriptional repression of *RAD51*, a key player in HR (Sung *et al.*, 2003), is known to occur in several tumor types in response to their hypoxic environment

(Bindra *et al.*, 2004). In addition, mutations in HR genes do correlate with notable chromosome rearrangements (Patel *et al.*, 1998). Nonetheless, recombinational activity may also enhance anticancer drug resistance (Hansen *et al.*, 2003) and prevent telomere erosion (Dunham *et al.*, 2000), which may be valuable in aggressively dividing malignant cells.

135G>C of *RAD51*, as well as Arg188His and Thr241Met of *XRCC3* have recently been pointed as potential polygenic causes of CRC occurrence, and their screening has been suggested (Krupa *et al.*, 2011a). As the *RAD51* polymorphism implicates a lower level of *RAD51* protein, and consequently of other proteins such as *XRCC2* and *XRCC3*, it may explain the lower DSB repair capacity observed in CRC. Curiously, the same genetic hits have been observed in endometrial cancer and considered as an additional marker of the disease (Krupa *et al.*, 2011b). Although germline mutations in *BRCA1* and *BRCA2* have been vastly referred to confer higher risk of breast cancer development, recent evidence suggests that this risk is modified by other genetic or environmental factors that cluster in families. A recent genome-wide association study showed that common alleles at SNPs in *FGFR2*, *TNRC9* and *MAP3K* are also associated with increased breast cancer risk in the general population and in *BRCA1* and *BRCA2* mutation carriers. The different effects of *FGFR2* and *MAP3K1* SNPs in *BRCA1* and *BRCA2* carriers result in breast cancer tumors with an additional distinct nature (Antoniou *et al.*, 2010).

HR can compete with NHEJ for DSB repair: recent studies in yeast have shown that phosphorylation of *SAE2*, known to be involved in processing meiotic and mitotic DSB, by a CDK, functions as a regulator of the relative activity of NHEJ and HR after DSB resection (Huertas *et al.*, 2008). This coordination is of utmost importance, as HR activity during G1 would lead to the use of an allele as a template to the damaged one and, thus, to loss of heterozygosity, potentially unveiling mutations in tumor suppressor genes during the oncogenic process. In addition, it has been observed that *RAD51* impairment imparts mutagenicity to recombinational repair (Stark *et al.*, 2004).

4.6 Fanconi Anemia/BRCA pathway

Fanconi anemia (FA) is a genetic disease characterized by genomic instability, checkpoint arrest and cancer predisposition. Affected patients can develop various congenital abnormalities, including short stature, bone marrow failure during childhood and particular predisposition to myelodysplasia, acute myeloid leukemia and head and neck cancers (de Winter & Joenje, 2009). A network of at least 13 genes, designated *FANCA* to *FANCN*, is critical for maintaining chromosomal integrity (Thompson, 2005). Indeed, FA can be caused by mutations in any one of these genes (Mathew, 2006).

Although their molecular function is not completely understood, all FA proteins contribute to processing ICLs. Consequently, ICL induction in the absence of FA proteins leads to reduced cell viability and an accumulation of cells with a 4N DNA content, representing cells in either late S or G2/M (Thompson, 2005). FA proteins interact in the FANC/BRCA pathway, in which a pivotal event is the monoubiquitination of *FANCD2* (de Winter & Joenje, 2009). This monoubiquitination requires the FA nuclear core complex, formed by several FA proteins. *BRCA2/FANCD1*, *PALB2/FANCN* and *FANCI* proteins are not required for this event and are considered downstream of *FANCD2* monoubiquitination. The FANC/BRCA pathway is interconnected with HR and NHEJ systems and its disruption provokes the clinical and cellular abnormalities common to all FA subtypes (Wang & D'andrea, 2004).

Remarkably, some genetic mutations associated with FA are also associated with hereditary breast cancer. For example, *FANCD1*, the gene defective in the FA-D1 patient complementation group, was found to be the hereditary breast cancer gene *BRCA2* (Howlett *et al.*, 2002). Likewise, *FANCF* (also called *BACH1/BRIP1*), which was identified as the gene defective in the *FANCF*-null (FA-J) patient complementation group (Levitus *et al.*, 2005), was initially linked to hereditary breast cancer (Cantor *et al.*, 2004). FA patients that carry mutations in *BRCA2* only (de Winter & Joenje, 2009) differ from others in presenting much more severe phenotypes, with early-onset and high rates of leukemia and some solid tumors (Howlett *et al.*, 2002).

5. DNA repair in anti-cancer therapy: A double-edged sword

Chemotherapeutic drugs used in cancer treatment frequently take advantage of the intrinsic instability of the genome to inflict damage in the DNA of both healthy and tumor cells. Depending on their mechanisms of action and pharmacological properties, these drugs may be more genotoxic towards tumor cells than towards healthy ones. These agents include nitrosureas (carmustine, lomustine, fotemustine, streptozokine), tetrazines (temozolamide, dacarbazine), aziridines (thiotepa, mitomycin C), bischloroethylamines (melphalan, chlorambucil), DNA topo-isomerases I and II inhibitors (camptothecins and epipodophyllotoxins) and, notably, platinum complexes (cisplatin, carboplatin, oxaliplatin) (Bignami *et al.*, 2000; Wang, 1996).

5.1 Chemotherapeutic drugs and DNA repair systems

The outcome of cancer patients following chemo- and radiotherapy is mostly determined by DNA damage responses to the treatments, by both malignant and normal cells. As mentioned before, some of the long succession of random mutations that typically give rise to human cancers occur in determinant genes of important repair and survival pathways. In parallel with epigenetic changes either in DNA and/or histones, these mutations drive tumorigenesis (Esteller, 2008; Santella *et al.*, 2005). As such, cancer treatments that target a specific DNA repair defect can be selectively toxic to cancer cells exhibiting that defect, while sparing normal, DNA repair-proficient cells. Extreme care must, however, be exerted, as genetic and epigenetic perturbations of MMR and BER pathways, following the use of alkylating/antimetabolite chemotherapeutics and/or ionizing radiation, were associated with the onset of new cancers (David *et al.*, 2007; Iyer *et al.*, 2006; Jiricny, 2006; Karran & Attard, 2008; O'Brien & Brown, 2006). An increased understanding of how MMR and/or BER DNA repair pathways influence the cytotoxicity of chemotherapeutic drugs and/or ionizing radiation treatments in both normal and malignant tissues led to an important therapeutic distinction between these two DNA repair pathways, i.e., whereas MMR processing is required for the cytotoxicity of drug treatments, BER processing sometimes leads to reduced drug-related cytotoxicity. A similar distinction can be made for ionizing radiation damage processing by MMR versus BER, even though NHEJ and HR repair systems are the dominant pathways to repair DSBs induced by ionizing radiation (Matsuoka *et al.*, 2007; Workman *et al.*, 2006).

5.1.1 Tp53, the central player in DNA repair systems

Tp53 is a master pleiotropic guardian of genome integrity, being a key node in several pathways of the DNA repair circuitry that must be subverted during carcinogenesis.

Radiotherapy and most chemotherapeutic agents directly target DNA and, as a consequence, activate DNA repair processes and/or cell cycle arrest. Tp53 coordinates such functions, as it participates in the main DNA repair systems. Mutations within the tumor suppressor *Tp53* gene are highly frequent in human tumors and commonly associated with a resistance phenotype.

In cells with wild type Tp53, cisplatin resistance can be overridden by several mechanisms involving this protein, namely its direct interaction with proteins involved in cisplatin resistance and their targeting for proteasomal degradation, this way increasing cellular sensitivity to the drug (Abedini *et al.*, 2008). Other mechanism can involve up-regulation of *Tp53* in response to *PTEN* overexpression (Yan *et al.*, 2006) and enhanced Tp53-dependent apoptosis due to *XIAP* expression silencing (Fraser *et al.*, 2003). In contrast, decreased Tp53 phosphorylation, in the presence of active AKT, drives a decrease in cisplatin-induced cell death (Fraser *et al.*, 2008).

It has been shown that a combination of Tp53 inactivation and MMR deficiency results in cisplatin resistance (Lin & Howell, 2006). Paradoxically, loss of *Tp53* and/or MMR is a common event in cancers following treatment with cisplatin and its analogues (Fink *et al.*, 1998; Lin & Howell, 2006).

5.1.2 Overcoming Tp53 deficiency

The absence of wild type Tp53 in some tumor cells can be exploited therapeutically through the use of antagonistic drugs (Blagosklonny, 2002). An example is taxol, a β -tubulin target agent which simultaneously kills and/or blocks Tp53 deficient cancer cells during mitosis and arrests wild type cells in G1 or G2 phases (Demidenko *et al.*, 2008). Taking advantage of taxol specificity, its use in association with other mitotic and genotoxic drugs is frequent (Blagosklonny *et al.*, 2000). However, the usefulness of mitotic chemotherapeutics is limited by their ability to activate Tp53-independent checkpoint mechanism in cancer cells with mutant *Tp53* (Blagosklonny, 2002).

Recently, the MDM2 antagonist Nutlin-3 was reported to selectively activate Tp53 pathway, inducing cell cycle arrest of Tp53 wild type non-small cell lung cancer (NSCLC) cells, while taxol selectively killed *Tp53* deficient pharyngeal squamous-cell carcinoma cells (Tokalov & Abolmaali, 2010). Moreover, modulators of Nutlin-3 might also offer a new therapeutic option for patients with tumors expressing wild type Tp53, either in mono (Vassilev, 2005) or combined therapy (Kojima *et al.*, 2005). Inhibition of the Tp53-MDM2 interaction has been documented following nutlins administration in multiple types of cultured cells, with a high degree of specificity. The consequences are, generally, Tp53 stabilization induction, p21 induction, cell cycle arrest in G1 and G2 phases, apoptosis and growth inhibition of proliferating cancer cells (Vassilev *et al.*, 2004). It is interesting that nude mice treatment with Nutlin-3 inhibits tumor growth without overt toxicity, suggesting that normal tissues may have higher tolerance to Tp53 activation (Vassilev, 2007). Thus, depending on the genetic status of the tumor, Nutlin-3 might be administrated in monotherapy, for the activation of the Tp53 pathway, or in combination with taxol, for the protection of the surrounding normal tissue. The study of Nutlin-3 biodistribution in the body may be critical for the understanding of its pharmacodynamics and therapeutic effects *in vivo*.

Other strategies to overcome Tp53 mutant-dependent cancer cell resistance to certain anticancer drugs have been proposed. Reactivation of mutant Tp53 using small molecular therapeutic agents that change mutant Tp53 conformation or depletion of mutant Tp53 with Hsp90-active agents (e.g., geldanamycin) has been attempted (Fojo, 2002; Selivanova, 2001).

Yet, although depletion of mutant Tp53 *per se* cannot restore Tp53 functions, it may abolish dominant-positive effects (Wang *et al.*, 2003). A final strategy includes the use of the histone deacetylase inhibitor FR901228 (FK228) (Sandor *et al.*, 2002), a depsipeptide expected to be predominantly cytotoxic to Tp53 proficient cells, as it inhibits the trans-activating functions of wild type Tp53 (Juan *et al.*, 2000). However, recent studies unexpectedly revealed that FK228 was less active in Tp53 proficient cells (Blagosklonny *et al.*, 2002; Kitazono *et al.*, 2002) as its association with trichostatin A induced Tp53-regulated transcription in cells with mutant Tp53, resulting in complete depletion of the Tp53 protein. Restoring or mimicking Tp53 trans-functions appears to be highly cytotoxic to cells with mutant Tp53. Yet, care must be taken, since histone deacetylase inhibitors are cytotoxic to both normal and tumor cells with both wild type and mutant Tp53 (Blagosklonny *et al.*, 2005).

5.2 Combined therapies to overcome chemo- and radio-resistance

MMR is now accepted to be fundamental for the processing of the DNA damage induced by several classes of chemotherapeutic drugs (Seifert & Reichrath, 2006). The exact mechanisms that drive MMR activity are still not clear and two models – the futile cycle model and the direct signaling model – have been proposed. Nevertheless, prolonged G2 checkpoint arrest is triggered in both models following MMR activation, leading to an activation of some apoptotic pathways (Iyer *et al.*, 2006; Jiricny, 2006; O'Brien & Brown, 2006).

According to the futile cycle model, MMR has a single function with the MUTS α /MUTL α /EXO-1 complex ultimately creating persistent SSBs in the vicinity of chemically induced mismatches, as happens in the treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and thioguanine (6-TG). Briefly, when 6-methyl thioguanine (me6-TG) or O6-methyl guanine (O6meG) are formed following 6-TG and MNNG treatment, respectively, and are in the template during replication, the incorporation of a C or T into the newly synthesized strand creates an additional mismatch, as the modified base remains in the template strand after MMR-mediated excision (Yoshioka *et al.*, 2006). Repair synthesis proceeds with regeneration of O6meG-C or -T mismatches in a repetitive (futile) cycle, culminating with a G2 checkpoint arrest, probably mediated, in an initial phase, by the Tp53-ATR-CHK1 pathway and, later, by the ATM-CHK2 pathway (Adamson *et al.*, 2005; Jiricny, 2006; Yan *et al.*, 2003; Yan *et al.*, 2004).

Further MMR signaling pathways consistent with futile cycling have been reported after administration of other drugs. For example, cisplatin treatment of MMR-proficient cells results in the generation of ICLs, which can lead to MMR-related activation of c-JUN and c-ABL kinases (Gong *et al.*, 1999; Nehme *et al.*, 1999). Additionally, ionizing radiation-induced damage also activates MMR, resulting in modest cytotoxicity following acute and high-dose exposures. More significant cytotoxicity is only observed with prolonged exposures to low doses of radiation (Yan *et al.*, 2001). These observations may be a consequence of activation of Tp53-p21 pathways due to prolonged G2 delay, which is eventually followed by apoptosis or autophagy.

In the direct signaling model, MMR has two separate functions: repairing DNA damage and transducing the resulting signal (Fishel, 1999; Yoshioka *et al.*, 2006). After MUTS α /MUTL α complex recognition of the chemically induced mispair, it acts as a direct sensor, straightly activating the Tp53-ATR-CHK1 pathway (Iyer *et al.*, 2006; Jiricny, 2006). DNA mismatch processing through the downstream sub-processes of excision and re-synthesis can then follow independently of the damage-induced G2 cell cycle delay.

The absence of a functional MMR system confers cells a faulty ability to recognize chemotherapy-induced DNA damage and a damage-tolerant phenotype. As a consequence, these cells are spared by conventional therapies and accumulate more mutations that boost their malignancy (Karran, 2001; Seifert & Reichrath, 2006). For instance, cells with lower levels of MSH2 or MLH1 proteins are competent for recognizing damage, but fail to trigger checkpoint activation or apoptosis (Cejka *et al.*, 2003; Claij & Te Riele, 2004). In contrast, mutations in or near the nucleotide binding site of *MSH2* and *MSH6* disable MMR, but leave intact the apoptotic response to DNA damaging agents (Lin *et al.*, 2004; Yang *et al.*, 2004).

The importance of MMR proteins on chemotherapy outcome is well evidenced by the role of the MSH2 protein in recognizing base pairs involved in modified or damaged bases and, consequently, in triggering further MMR-mediated processing at the damaged sites (Friedman *et al.*, 2007; Yamada *et al.*, 1997). However, the activation of MSH2 is context dependent, as in non-transformed breast cell lines TGF- β activates *MSH2* promoter in a Tp53-dependent manner, while in Tp53-deficient breast cancer cells, TGF- β down-regulates *MSH2* expression through a miRNA-mediated mechanism, and confers resistance to DNA-damaging anticancer agents (Yu *et al.*, 2010).

The multiplicity of MMR functions in the DNA damage response has been recently uncovered through separation-of-function mutations (O'Brien & Brown, 2006). Interestingly, newly found deletions in the MLH1 C-terminus (703-725), which is important for maintaining the stability of the PMS2 MMR protein, also disrupted the FANCI/MLH1 interaction (Mohd *et al.*, 2006), delaying MMR signaling and apoptotic responses. This delay provides time for the O6-methylguanine-DNA methyltransferase (MGMT) enzyme to reverse DNA methylation and, thus, to confer resistance to agents that induce O6-meG lesions. In essence, FANCI deficiency alters the competition between two pathways: MGMT-pro-survival versus MMR-pro-death. A link between FANCI and HNPCC has also been established, providing insight towards directed therapies, as loss of the FANCI/MLH1 interaction sensitizes cells to DNA cross-linking agents (Xie *et al.*, 2010a).

5.2.1 Targeting simultaneously DNA, MMR, NER and Tp53

Cisplatin is one of the most widely used chemotherapeutic agents (Siddik, 2003). However, its clinical use is conditioned by the development of resistance, which can result from reduced intracellular accumulation, increased drug inactivation, increased repair of damaged DNA, increased activation of pro-survival pathways or inhibition of pathways that promote cell death (Siddik, 2003). An intermingling of these factors has also been suggested, justifying the difficulty to overcome platinum resistance (Dempke *et al.*, 2000). Defects in Tp53 and MMR pathways have been reported to underline cisplatin resistance, both *in vitro* and in the clinic. In agreement, loss of action of the MMR system can result in increased resistance to cisplatin, as cells are allowed to replicate damaged DNA instead of entering an apoptotic program (Martin *et al.*, 2008; Siddik, 2003; Vaisman *et al.*, 1998; Watanabe *et al.*, 2001). An additional correlation between MMR deficiency (due to a *BRAF* gene mutation), MSI and cisplatin resistance was also unveiled using non-seminomatous germ cell tumors (Honecker *et al.*, 2009).

To overcome cisplatin resistance, combined therapies using gemcitabine (2'-deoxy-2',2'-difluorocytidine) and cisplatin have been used (Vilella *et al.*, 2004). Attempts to explain gemcitabine enhanced cytotoxicity in platinum-resistant endometrial cancer cell lines revealed that gemcitabine downregulates *MSH2*, *Tp53* and *ERCC1* (the NER protein involved in intracellular nucleotide repair) as compared to cisplatin alone (Smith *et al.*, 2006).

5.2.2 Targeting simultaneously DNA and MGMT

DNA alkylation-induced damage is one of the most efficacious anticancer therapeutic strategies directed to cancer cells with weakened DNA repair capacity. There is ample preclinical evidence that MMR-deficient cancer cells are resistant to both methylating agents and some antimetabolites, as 5-fluorouracil (5FU), whereas they are sensitive to oxaliplatin and, possibly, even more sensitive to irinotecan (a topoisomerase I inhibitor) (Barratt *et al.*, 2002; Damia & D'incalci, 2010; Kim *et al.*, 2007; Ribic *et al.*, 2003). In the case of SN1 DNA methylating drugs like MNNG, temozolomide (TMZ) or procarbazine, the prevalent DNA lesion (O6meG) is largely responsible for their cytotoxicity (Goldmacher *et al.*, 1986; Haracska *et al.*, 2000; Karran & Bignami, 1992) and cells deficient in MGMT and MMR activities are highly resistant to killing by these drugs, as O6meG is easily repaired by MGMT (Iyer *et al.*, 2006; Kaina *et al.*, 2007). Actually, many colon tumors become resistant to DNA-alkylating agents due to overexpression of MGMT or MMR-deficiency (Liu & Gerson, 2006). In contrast, G:C to G:T transition mutations occur in MGMT-deficient cells, as a result of their inability to process O6meG during DNA synthesis (Kawate *et al.*, 1998). In MMR-proficient cells, G:T mismatches are easily repaired (Branch *et al.*, 1993). However, if the O6meG is not repaired before the re-synthesis step, it is believed that the repetitive cycle of futile MMR will generate tertiary lesions, most likely DSBs, eliciting a cell death response (Branch *et al.*, 1993).

Whereas a significant percentage of gliomas lack expression of MGMT, due to hypermethylation of the MGMT promoter, whereas at least half of glioblastomas multiforme (GBM) express MGMT, its expression being associated with resistance to chemotherapy and poor prognosis (Hegi *et al.*, 2005; Pollack *et al.*, 2006). Strategies to target the MMR pathway and to improve the efficacy of TMZ to overcome resistance resulting from MGMT activity have been implemented. Ironically, somatic mutations-induced loss of function of MSH6 has also been associated with glioblastoma recurrence post irradiation and TMZ treatment (Cahill *et al.*, 2007).

In vitro, MGMT inhibitors such as O6-benzylguanine (O6-BG) can effectively overcome TMZ resistance in MMR-proficient cells, but revealed clinically ineffective (McMurry, 2007; Tentori *et al.*, 1995). A viable option has been to target the BER pathway, which repairs the N7-methylguanine and N3-methyladenine lesions induced by TMZ. Pharmacological inhibition of this pathway resulted in TMZ-induced cytotoxicity enhancement, independently of MGMT status (Adhikari *et al.*, 2008).

5.2.3 Targeting simultaneously DNA and BER

Tumors' TMZ resistance strategies have been ascribed to elevated levels of MGMT and/or reduced MMR. Yet, recent data on human gliomas attributed a minor role to MMR deficiency and suggested the existence of other mechanisms (Maxwell *et al.*, 2008). The involvement of BER seems very probable, since more than 80% of the DNA lesions induced by TMZ are recognized and processed by BER DNA glycosylases, independently of the MMR status (Liu & Gerson, 2004). BER glycosylases can lead to antimetabolite drug resistance by processing antimetabolite-DNA base damage (Cortellino *et al.*, 2003; Jurado *et al.*, 2004; Morgan *et al.*, 2007). In TMZ-induced base damage, the repair process starts with the recognition and removal of the damaged bases by N-methylpurine DNA glycosylase (MPG), also known as alkyladenine DNA glycosylase (AAG). The resultant AP is then hydrolyzed by apurinic/aprimidinic (AP) endonuclease 1 (APE1) (Almeida & Sobol, 2007b). Enhanced sensitivity to alkylating agents upon modulation of the BER pathway has

recently been observed in preclinical studies (Kinsella, 2009). BER proteins as potential targets for chemotherapy sensitization are actually a field of active research.

5.2.3.1 Targeting APE1

The tumor microenvironment is characterized by acute/chronic hypoxia, low extracellular pH and nutrient access, affecting genomic stability (Reynolds *et al.*, 1996), local progression, metastatic potential and response to radio- and chemotherapy (Overgaard, 2007). Multiple DNA repair systems are inhibited under hypoxic and/or low pH extracellular conditions, including MMR (Koshiji *et al.*, 2005), NER (Yuan *et al.*, 2000) and HR (Bindra *et al.*, 2004).

In stark contrast, APE1 was found to have greater activity in various types of tumors compared with normal tissues (Yoo *et al.*, 2008). Potent direct inhibitors of APE1 have been identified, such as arylstibonic acid derivatives (Seiple *et al.*, 2008), which are being developed to sensitize cancer cells against other DNA damaging agents; however, it was reported that the APE1 inhibitor 7-nitroindole-2-carboxylic acid increases DNA oxidative damage, DSBs and cell death in the acidic tumor microenvironment (Horton & Wilson, 2007). Methoxyamine (MX) is a small molecule being evaluated in conjunction with TMZ in phase I clinical trials that specifically binds to and modifies AP sites, making them refractory to APE1 and highly cytotoxic, preventing their processing by BER ensuing steps (Yan *et al.*, 2007). Therefore, MX potentiates the action of AP sites-producing agents, regardless of MMR, MGMT or Tp53 status (Liu *et al.*, 1999).

5.2.3.2 Small molecular weight inhibitors of SN-BER and LP-BER

An emerging concept in cancer therapy is the sensitization of cancer cells to DNA-damaging agents by inhibiting various proteins in the DNA repair pathways. Small molecular weight inhibitors (SMIs) have been used to target the BER pathway by inhibiting APE1 and Pol β activities. Several Pol β inhibitors have been reported in recent years (Horton & Wilson, 2007). Pamoic acid is the most active inhibitor of Pol β (Hu *et al.*, 2004), blocking just Pol β -directed SN-BER only at high concentrations. As LP-BER can also repair abasic DNA sites, new agents that specifically block both Pol β -directed SN- and LP-BER pathways are being tested, as there is a protein, APC, that interacts with Pol β and FEN1, blocking both SN- and LP-BER pathways (Jaiswal & Narayan, 2008; Kundu *et al.*, 2007). Recently, two other potent SMIs, NSC-666715 [4-chloro-N-(3-(4-chloroanilino)-1H-1,2,4-triazol-5-yl)-2-mercapto-5-methylbenzenesulfonamide] and NSC-124854 [5-(4-amino-6-iodo-2-oxo-5,6-dihydropyrimidin-1-yl)-3-hydroxy-oxolan-2-yl] methoxyphosphonic acid, that interact with Pol β , blocking simultaneously SN- and LP-BER activities, without blocking neither APE1, FEN1 nor DNA ligase IV activities, were reported to enhance TMZ efficiency both *in vitro* and *in vivo* (Jaiswal *et al.*, 2011; Jaiswal *et al.*, 2009).

5.2.3.3 Inhibitors of PARP I and PARG

PARP I is an abundant nuclear enzyme that senses both SSBs and DSBs and functions in both SN- and LP- BER. In BER, PARP I acts as a nick sensor, catalyzing the addition of ADP-ribose units to DNA, histones and other target proteins: negatively charged ADP-ribose polymers then create electrostatic repulsions between DNA and histones, opening the chromatin for DNA repair. PARP I also recruits BER proteins to sites of single-stranded DNA breaks, initiating DNA repair (Ratnam & Low, 2007). PARP I expression/activity increases significantly in human normal and cancer cell lines after exposure to monofunctional and bifunctional alkylating agents, topoisomerase I inhibitors such as irinotecan, antimetabolites, including gemcitabine, as well as ionizing radiation (Ratnam & Low, 2007). High PARP I levels were also found in a variety of human cancers (Ratnam &

Low, 2007), being commonly associated with drug resistance and overall ability to survive genotoxic stress (Shiobara *et al.*, 2001). As *PARP I* knockdown mice are hypersensitive to ionizing radiation and alkylating agents (de Murcia *et al.*, 1997) and overexpression of dominant-negative *PARP I* in nude mice results in tumor cell apoptosis (Hans *et al.*, 1999), *PARP I* inhibition offers the opportunity to enhance chemotherapeutic- and ionizing radiation-mediated cytotoxicity in human cancers. In fact, *PARP I* inhibitors, in preclinical and clinical development for several decades, overcome TMZ resistance (Liu *et al.*, 1999) and enhance oxaliplatin efficacy *in vitro* and *in vivo* (Melisi *et al.*, 2009), while protecting against side effects of some anticancer drugs, such as doxorubicin (Pacher *et al.*, 2002).

PARG is the main enzyme that targets poly ADP-ribose (PAR) for degradation via endo- and exoglycosidic cleavage (Hassa *et al.*, 2006). Albeit *PARG* null mutations are embryonic lethal (Cortes *et al.*, 2004), a *PARG* inhibitor, GPI 16552, has been shown to chemosensitize malignant melanoma to TMZ (Tentori *et al.*, 2005). The same effect has been achieved by shRNA-mediated *PARG* knockdown in glioma cells (Tang *et al.*, 2011).

5.2.4 Therapies targeting simultaneously DNA and HR/NHEJ

5.2.4.1 Targeting DNA-PKcs

DSBs are induced by radiation and anticancer drugs such as cyclophosphamide, cisplatin, doxorubicin or etoposide (Barcellos-Hoff *et al.*, 2005; Christmann *et al.*, 2003). NHEJ is involved in DSB repair and does not depend on the presence of homologous DNA sequences (section 4) and requires the DNA-PKcs and one of its targets, the XRCC4/DNA-ligase IV complex (Lieber, 1999). Importantly, the catalytic subunit of DNA-PK, DNA-PKcs, phosphorylates itself, other repair proteins and Tp53 (Smith & Jackson, 1999). An inverse correlation between the level of DNA-PKcs and radiation sensitivity exists in human tumors: it was demonstrated that mutant cells radiosensitivity could be rescued by introduction of functional, but not kinase activity-deficient, DNA-PKcs cDNA, showing that DNA-PKcs kinase activity is essential for DNA repair (Kurimasa *et al.*, 1999). The binding of DNA-PKcs to DNA ends (Lieber, 1999), together with the ability to phosphorylate a variety of nuclear targets (Smith & Jackson, 1999), may determine whether a break is repaired by NHEJ, redirected for repair by an alternative pathway or left unrepaired, potentially leading to irreversible growth arrest or cell death. Therefore, induction of DNA-PKcs complex arrestment at the DNA termini is potentially more effective in radiosensitizing tumor cells than reducing DNA-PKcs expression itself, as the presence of a non-functional repair complex may block access to proteins from other DSB repair systems, leading to chronically unrepaired damage.

The first approach to inhibit DNA-PKcs *in vitro* and *in vivo* used rather unspecific pharmacological inhibitors that also inhibited ATM and ATR, such as wortmannin (Sarkaria *et al.*, 1998). More recently, 4'-bromo-3'-nitropropionophenone (NS-123) was shown to be more specific, enhancing, *in vitro* and *in vivo*, the cytotoxicity of biologically relevant doses of ionizing radiation, without any measurable normal tissue toxicity (Lally *et al.*, 2007). The use of monoclonal antibodies, immunotoxins and radioimmunoconjugates to ameliorate chemo- and radiotherapy results has emerged as a promising strategy (Milas *et al.*, 2005). A well-tolerated and effective radioimmunotherapy option for patients with high-risk leukemia uses β -emitting nuclides and antibodies (Kotzerke *et al.*, 2005). Nevertheless, the use of α -particles can achieve higher biological effectiveness and more specific tumor cell killing with less damage to surrounding normal tissues, due to their short path length (50-80 μ m) and

high energy transfer of α -particles emitting radioisotopes compared with β -emitters and external radiation, being ideal to eliminate residual or micrometastatic disease (Zalutsky & Pozzi, 2004).

β - and γ -irradiation-induced DNA damage activate apoptotic pathways involving CD95 ligand receptor-driven and the mitochondrial pathways (Friesen *et al.*, 2003). In contrast, α -irradiation-induced DSBs activate the mitochondrial apoptotic pathway independently of CD95 receptor/ligand interactions in leukemia cells. However, NHEJ inhibits doxorubicin-, γ -irradiation and β -irradiation-induced apoptosis, shedding light on why defective apoptosis signaling or increased DNA repair ability are involved in cross-resistance between radio- and chemotherapy (Friesen *et al.*, 2003). In order to override cross-resistance against β -irradiation, γ -irradiation, doxorubicin and radioactive [^{213}Bi]anti-CD45 has been successfully used recently: in the case of doxorubicin, it overcame NHEJ, possibly by decreasing DNA-PKcs or DNA-ligase IV activities, leading to efficient caspase activation and concomitant apoptosis (Friesen *et al.*, 2008).

5.2.4.2 Targeting Ku70

Hypoxic regions exist in many human cancers, and hypoxia-induced radioresistance has been postulated as an obstacle in achieving local control in tumors with a sizable hypoxic fraction. Even so, radioresistant tumor cells could be sensitized by modulating the cellular level/activity of Ku/DNA-PKcs and adjuvant strategies can be developed for targeted radiotherapy. Accordingly, a DNKu70 construct, designed based on the analysis of the structure-function of Ku70 and on the crystal structure of Ku70/Ku80 heterodimer, induced a decrease in Ku-DNA end-binding activity, and the persistence of γ -H2AX foci increased the radiosensitivity of infected human glioma U-87 MG cells and human colorectal tumor HCT-8 cells, both under aerobic and hypoxic conditions (He *et al.*, 2007).

In solid tumors, the tolerance of surrounding tissues often limits the dose of radiation that can be delivered. In gliomas, for instance, a recent prospective clinical trial found that radiotherapy combined with TMZ significantly improves patient's survival (Stupp *et al.*, 2005). Unsuccessful attempts have been made at increasing the radiation dose, either with additional external beam radiotherapy, brachytherapy or stereotactic radiosurgery (Chan *et al.*, 2002b; Regine *et al.*, 2000; Tatter *et al.*, 2003).

5.2.4.3 Targeting RAD51

Given the role of DNA repair in the radioresponse of human tumor cells, particularly of the DNA-PK-dependent NHEJ and HR systems, several strategies address either DNA-PK or RAD51. In the particular case of high grade gliomas, tumors that are strongly resistant and characterized by RAD51-mediated DNA repair activity, the *in vitro* cytotoxicity to combined TMZ and radiotherapies was achieved by suppressing the expression of RAD51 (Short *et al.*, 2011). Alternatively, in NSCLC cell lines, radiosensitization was achieved using antisense oligodeoxynucleotide, specifically targeting RAD51 mRNA, or wortmannin, a well-known inhibitor of NHEJ that also inhibits RAD51 foci formation (Sak *et al.*, 2005).

5-Iodo-2'-deoxyuridine (IUdR), a halogenated thymidine analogue, has been long recognized as an *in vitro/in vivo* radiosensitizer (Kinsella, 1996). The levels of thymidine replacement by IUdR in DNA, enhanced by caffeine and caffeine-like drugs in *Tp53*-deficient xenografts, directly correlate with the extent of radiosensitization (Seo *et al.*, 2006). This radiosensitization is ascribed to ionizing radiation-induced DNA strand breaks, most likely due to the generation of reactive free radicals from the IUdR incorporated into DNA.

As caffeine abrogates S- and G2-phase cell cycle arrests (Eastman, 2004), it can eventually be used to reduce DSB repair through the disruption of both ATR-CHK1-RAD51 and ATR-CHK1-CDC25 pathways (Sorensen *et al.*, 2005). Regrettably, IUdR is also incorporated into the DNA of rapidly proliferating normal tissue, resulting in myelosuppression and gastrointestinal toxicity. To circumvent this, 5-iodo-2-pyrimidinone-2'-deoxyribose (IPdR), an IUdR derivative, has recently been developed. Its low systemic toxicity and rapid liver conversion into IUdR renders this pro-drug quite useful in the treatment of tumors surrounded by non-proliferating normal tissues. That is the case of primary and metastatic brain and liver tumors and high-grade sarcomas (Kinsella *et al.*, 2007).

5.2.4.4 Targeting XRCC4/ DNA ligase IV

Given the plethora of potential targets within the NHEJ group of proteins, it is worth mentioning some potential advantages and disadvantages of targeting XRCC4/DNA ligase IV. One of these advantages resides in the fact that DNA ligase IV is apparently distinct from other DNA ligases and its relationship with XRCC4 and DNA repair seems unique. This may unveil specificity or, conversely, decrease efficacy. It is also possible that, by virtue of its low abundance and potential rate-limiting nature, XRCC4/DNA ligase IV may be a better target for intervention than DNA-PKcs or Ku proteins. Furthermore, cells lacking DNA ligase IV show considerably slower rejoining kinetics following irradiation than cells lacking DNA-PKcs or Artemis (a protein with major functions in V(D)J recombination and NHEJ). In addition, DNA ligase IV seems to be involved in all classes of breaks, unlike the other proteins, which may be more selective (Lobrich & Jeggo, 2005). Recently, adenovirus-mediated expression of the XRCC4 fragment resulted in radiosensitization in breast cancer cells (Jones *et al.*, 2005). One major drawback in clinic, even with intensity-modulated radiotherapy delivery, is that normal tissues will also be affected and, as such, careful monitoring is required.

5.2.4.5 Targeting BMP1

Ionizing radiation represents the most effective therapy for glioblastoma (World Health Organization grade IV glioma), one of the most lethal human malignancies. Yet, radiotherapy remains only palliative because of radioresistance. CD133-expressing (CD133+) tumor cells present in glioma have stem-like properties and, as such, are named neuronal cancer stem cells (NCSCs). NCSCs are major contributors to glioma radioresistance, through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. The efficient DNA damage response/repair and radioresistance of glioblastoma multiforme (GBM) was recently ascribed to the stem cell factor BMP1, present in high levels in CD133+ GBM cells (Facchino *et al.*, 2010). BMP1 is a key component of multiprotein Polycomb repression complex 1, critical for the maintenance of chromosome integrity in both normal and transformed cells (Chagraouia *et al.*, 2011). Cancer cells, including glioma, are very sensitive to genotoxic agents following BMP1 depletion, possibly by an acquired dependency upon BMP1 anti-apoptotic activity through transcriptional (i.e., tumor suppressor genes repression) and non-transcriptional (i.e., HR and NHEJ proteins DNA-PK, PARP I) activities (Chagraouia *et al.*, 2011; Facchino *et al.*, 2010). As the radioresistance of CD133+ glioma stem cells can be reversed with a specific inhibitor of the CHK1 and CHK2 checkpoint kinases, it is possible that an approach combining the cell's preferential sensitivity to BMP1 depletion and inhibitors of checkpoint kinases could be exploited to specifically target NCSCs following radiotherapy (Bao *et al.*, 2006).

5.2.5 Therapies targeting simultaneously DNA and NER repair system

Activation of DNA repair mechanisms circumvents chemotherapeutic drug-induced DNA damage (Edwards *et al.*, 2008), yet defects in those systems may also contribute to tumor drug resistance. In particular, impaired NER correlates with loss of susceptibility to cisplatin (Mountzios *et al.*, 2008) and trabectedin (Von Mehren, 2007).

Albeit enhancing NER protects against accumulation of DNA lesions and maintains genomic integrity, reducing the NER threshold may be beneficial for cancer patients undergoing chemotherapy to ensure the efficient action of DNA damage-inducing drugs (Liu *et al.*, 2010). The newly identified circadian oscillation of XPA expression, coupled with a short half-life of the protein, hints that an optimal time window for treatment with drugs whose provoked DNA lesions elicit BER, such as cisplatin and other base damaging drugs, may be found. Actually, transient suppression of NER through chronochemotherapy manipulation of core NER factors or regulatory pathways is anticipated to synergize with DNA damaging agents to optimize the chemotherapeutic outcome (Kang & Sancar, 2009)

5.2.6 Therapies addressed to MSI tumors

The epigenetic silencing that occurs during tumor development deeply affects the response of tumors to chemotherapy (Teodoridis *et al.*, 2005), as illustrated by the epigenetic inactivation of the MGMT gene and the consequent increase of gliomas' resistance to monofunctional alkylating agents (Hegi *et al.*, 2005). Another example is the relation between the epigenetic status of MLH1 protein and the sensitivity to a wide range of important chemotherapeutic agents (Gifford *et al.*, 2004; Sargent *et al.*, 2010).

Patients with MSI stage II CRCs receiving 5FU treatment require adjuvant chemotherapy with oxaliplatin to override 5FU negative effects and, consequently, increase their survival (Kim *et al.*, 2010). Other 5FU adjuvant therapies containing FU, irinotecan and leucovorin (folinic acid vitamin) were tested unsuccessfully (Van Cutsem *et al.*, 2009). Another strategy based on the topoisomerase 1 (TOP1) inhibitor irinotecan evidenced that MSI tumors respond better to this drug than tumors with intact MMR (Jacob *et al.*, 2001). The mechanism underlying this response is still not clear, but irinotecan, apparently, inhibits the catalytic function of TOP1 by stabilizing covalent complexes formed between DNA, prevents DNA from unwinding (Hsiang *et al.*, 1989) and induces SSBs that are later converted into DSBs after replication fork collapse (Vamvakas *et al.*, 1997). However, possibly it is not the MMR defect itself, but rather the loss of one or more of the genes associated to MSI, that causes the observed chemosensitivity.

Ironically, epigenetic silencing can also be achieved during chemotherapy and may be an important driving force behind acquired drug resistance (Glasspool *et al.*, 2006). In ovarian and peritoneal cancer patients, cisplatin and carboplatin/paclitaxel or carboplatin/docetaxel treatments were reported to induce *MLH1* down-regulation or promoter hypermethylation and, consequently, MSI (Strathdee *et al.*, 1999). Therefore, there is considerable interest in the association of epigenetic therapies with existing chemotherapeutic agents, to improve initial tumor response and to overcome acquired drug resistance. The use of the DNA hypomethylating agent decitabine (2-deoxy-5-azacytidine) resulted in partial reversal of DNA methylation of *MLH1* and was reported to increase the sensitivity to cisplatin and carboplatin both *in vitro* and *in vivo* (Cameron *et al.*, 1999). However, the dose limiting toxicity of decitabine (i.e., myelosuppression) plus the limited demethylation in tumors, coupled with the eventual re-methylation of genes, may limit the clinical use of decitabine in monotherapy of solid tumors.

5-Fluoro-2'-deoxycytidine (FdCyd) is a DNA methyltransferase inhibitor and a hypomethylating agent when incorporated into the DNA of exposed cells (Kaysen *et al.*, 1986). Indeed, to prevent re-methylation and re-silencing of genes, long-term exposures to DNA methylation inhibitors are needed, as are new strategies to use them. Manipulation of FdCyd metabolism using the cytidine deaminase inhibitor tetrahydrouridine (THU) enhanced the re-expression of *MLH1*, converting resistant hypermethylated *MLH1*- colon or ovarian cancer cells to sensitive 5FU cells, because they re-expressed functional *MLH1* and therefore competent MMR (Beumer *et al.*, 2008). Full exploitation of DNA methyltransferase inhibition may well need long-term exposure to low concentrations of the inhibitor, as observed with 5-azacytidine and 5-aza-2'-deoxycytidine (Lyko & Brown, 2005).

Finally, the combination of a demethylating agent and, the histone deacetylase (HDAC) inhibitor, trichostatin A has recently been examined in clinical trials of hematological malignancies. By combining non-toxic doses of decitabine with the HDAC inhibitor belinostat, a marked increase in *MLH1* expression both *in vitro* and *in vivo* was observed, as was a boost of cisplatin sensitivity in tumor xenografts (Steele *et al.*, 2009).

5.3 Modulating cancer therapies using mathematical models

The overall complexity of DNA repair pathways is a major obstacle in designing and analyzing clinical trials using cancer-targeted treatment; probabilistic computational modeling is being used to better integrate chemotherapeutics and ionizing radiation-induced damage. Recently, a stochastic model for cell cycle progression in two synchronized isogenic MMR-deficient (MMR⁻) and MMR-proficient (MMR⁺) CRC cells, treated or not with iododeoxyuridine (IUdR), was built, followed by a second model to obtain correlations between the percentage of cells in different cell cycle states and the corresponding IUdR-DNA incorporation at particular time points. Combining both models could predict IUdR incorporation in DNA incorporation at any time in the cell cycle. Consequently, maximum benefit of the therapeutic action of IUdR treatment and radiation was achieved in xenografts MMR⁻ tumors versus MMR⁺ normal tissues, before passing to clinical trials (Gurkan *et al.*, 2007). This suggests that the systems biology approach holds promise for strategies using chemo- and radiotherapy synergistically and to better comprehend the effect of DNA repair systems on them.

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7. References

- Abedini, M. R., Muller, E. J., Brun, J., Bergeron, R., Gray, D. A. & Tsang, B. K. 2008. 'Cisplatin induces p53-dependent FLICE-like inhibitory protein ubiquitination in ovarian cancer cells.' *Cancer Res*, 68:12, 4511-7.
- Abraham, R. T. 2004. 'PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways.' *DNA Repair (Amst)*, 3:8-9, 883-7.

- Adams, J. M. & Cory, S. 2007. 'The Bcl-2 apoptotic switch in cancer development and therapy.' *Oncogene*, 26:9, 1324-37.
- Adamson, A. W., Beardsley, D. I., Kim, W. J., Gao, Y., Baskaran, R. & Brown, K. D. 2005. 'Methylator-induced, mismatch repair-dependent G2 arrest is activated through Chk1 and Chk2.' *Mol Biol Cell*, 16:3, 1513-26.
- Adamson, E. D. 1987. 'Oncogenes in development.' *Development*, 99:4, 449-71.
- Adhikari, S., Choudhury, S., Mitra, P. S., Dubash, J. J., Sajankila, S. P. & Roy, R. 2008. 'Targeting base excision repair for chemosensitization.' *Anti-cancer Agents Med Chem*, 8:4, 351-7.
- Aktas, H., Cai, H. & Cooper, G. M. 1997. 'Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1.' *Mol Cell Biol*, 17:7, 3850-57.
- Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. & Harley, C. B. 1992. 'Telomere length predicts replicative capacity of human fibroblasts.' *Proc Natl Acad Sci USA*, 89, 10114-18.
- Almeida, K. H. & Sobol, R. W. 2007a. 'A unified view of base excision repair: lesion dependent protein complexes regulated by post-translational modification.' *DNA Repair (Amst)*, 6:6, 695-711.
- Almeida, K. H. & Sobol, R. W. 2007b. 'A unified view of base excision repair: lesion dependent protein complexes regulated by post-translational modification.' *DNA repair (Amst)*, 6:6, 695-711.
- American Cancer Society 2010. 'The history of cancer.' www.cancer.org/acs/groups/cid/documents/webcontent/002048-pdf.pdf (accessed March 2011).
- Antoniou, A. C., Beesley, J., McGuffog, L., Sinilnikova, O. M., Healey, S., Neuhausen, S. L., Ding, Y. C., Rebbeck, T. R., Weitzel, J. N., Lynch, H. T., Isaacs, C., Ganz, P. A., Tomlinson, G., Olopade, O. I., Couch, F. J., Wang, X., Lindor, N. M., Pankratz, V. S., Radice, P., Manoukian, S., Peissel, B., Zaffaroni, D., Barile, M., Viel, A., Allavena, A., Dall'Olio, V., Peterlongo, P., Szabo, C. I., Zikan, M., Claes, K., Poppe, B., Foretova, L., Mai, P. L., Greene, M. H., Rennert, G., Lejbkowitz, F., Glendon, G., Ozelik, H., Andrulis, I. L., Thomassen, M., Gerdes, A. M., Sunde, L., Cruger, D., Birk Jensen, U., Caligo, M., Friedman, E., Kaufman, B., Laitman, Y., Milgrom, R., Dubrovsky, M., Cohen, S., Borg, A., Jernstrom, H., Lindblom, A., Rantala, J., Stenmark-Askmal, M., Melin, B., Nathanson, K., Domchek, S., Jakubowska, A., Lubinski, J., Huzarski, T., Osorio, A., Lasa, A., Duran, M., Tejada, M. I., Godino, J., Benitez, J., Hamann, U., Kriege, M., Hoogerbrugge, N., van der Luijt, R. B., van Asperen, C. J., Devilee, P., Meijers-Heijboer, E. J., Blok, M. J., Aalfs, C. M., Hogervorst, F., Rookus, M., Cook, M., Oliver, C., Frost, D., Conroy, D., Evans, D. G., Lalloo, F., Pichert, G., Davidson, R., Cole, T., Cook, J., Paterson, J., Hodgson, S., Morrison, P. J., Porteous, M. E., Walker, L., Kennedy, M. J., Dorkins, H., Peock, S., Godwin, A. K., Stoppa-Lyonnet, D., de Pauw, A., et al. 2010. 'Common breast cancer susceptibility alleles and the risk of breast cancer for BRCA1 and BRCA2 mutation carriers: implications for risk prediction.' *Cancer Res*, 70:23, 9742-54.
- Arizono, K., Osada, Y. & Kuroda, Y. 2008. 'DNA repair gene hOGG1 codon 326 and XRCC1 codon 399 polymorphisms and bladder cancer risk in a Japanese population.' *Jpn J Clin Oncol*, 38:3, 186-91.

- Armelin, H. A., Armelin, M. C., Kelly, K., Stewart, T., Leder, P., Cochran, B. H. & Stiles, C. D. 1984. 'Functional role for c-myc in mitogenic response to platelet-derived growth factor.' *Nature*, 310:5979, 655-60.
- Aslakson, C. J. & Miller, F. R. 1992. 'Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor.' *Cancer Res*, 52:6, 1399-405.
- Bachelor, M. A. & Bowden, G. T. 2004. 'UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression.' *Semin Cancer Biol*, 14:2, 131-8.
- Bahar, R., Hartmann, C. H., Rodriguez, K. A., Denny, A. D., Busuttill, R. A., Dollé, M. E. T., Calder, R. B., Chisholm, G. B., Pollock, B. H., Klein, C. A. & Vijg, J. 2006. 'Increased cell-to-cell variation in gene expression in ageing mouse heart.' *Nature*, 441:7096, 1011-4.
- Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D. & Rich, J. N. 2006. 'Glioma stem cells promote radioresistance by preferential activation of the DNA damage response.' *Nature*, 444:7120, 756-60.
- Barcellos-Hoff, M. H., Park, C. & Wright, E. G. 2005. 'Radiation and the microenvironment - tumorigenesis and therapy.' *Nat Rev Cancer*, 5:11, 867-75.
- Barnes, D. E. & Lindahl, T. 2004. 'Repair and genetic consequences of endogenous DNA base damage in mammalian cells.' *Annu Rev Genet*, 38, 445-76.
- Barnetson, R. A., Tenesa, A., Farrington, S. M., Nicholl, I. D., Cetnarskyj, R., Porteous, M. E., Campbell, H. & Dunlop, M. G. 2006. 'Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer.' *N Engl J Med*, 354:26, 2751-63.
- Barratt, P. L., Seymour, M. T., Stenning, S. P., Georgiades, I., Walker, C., Birbeck, K. & Quirke, P. 2002. 'DNA markers predicting benefit from adjuvant fluorouracil in patients with colon cancer: a molecular study.' *Lancet*, 360:9343, 1381-91.
- Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C. L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T. D., Bartek, J. & Gorgoulis, V. G. 2006. 'Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints.' *Nature*, 444:7119, 633-7.
- Benson, K. R. 2001. 'T. H. Morgan's resistance to the chromosome theory.' *Nat Rev Genet*, 2:6, 469-74.
- Berenblum, I. & Haran, N. 1955. 'The significance of the sequence of initiating and promoting actions in the process of skin carcinogenesis in the mouse.' *Br J Cancer*, 9:2, 268-71.
- Berg, R. J., Rebel, H., van der Horst, G. T., van Kranen, H. J., Mullenders, L. H., van Vloten, W. A. & de Gruijl, F. R. 2000. 'Impact of global genome repair versus transcription coupled repair on ultraviolet carcinogenesis in hairless mice.' *Cancer Res*, 60:11, 2858-63.
- Beumer, J. H., Parise, R. A., Newman, E. M., Doroshow, J. H., Synold, T. W., Lenz, H. J. & Egorin, M. J. 2008. 'Concentrations of the DNA methyltransferase inhibitor 5-fluoro-2'-deoxycytidine (FdCyd) and its cytotoxic metabolites in plasma of patients treated with FdCyd and tetrahydrouridine (THU).' *Cancer Chemother Pharmacol*, 62:2, 363-8.

- Bignami, M., O'Driscoll, M., Aquilina, G. & Karran, P. 2000. 'Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents.' *Mutat Res*, 462:2-3, 71-82.
- Bignold, L. P., Coghlan, B. L. D. & Jersmann, H. P. A. 2006. *Cancer: Cell Structures, Carcinogens and Genomic Instability*. Basel: Birkhäuser Verlag.
- Bindra, R. S., Schaffer, P. J., Meng, A., Woo, J., Maseide, K., Roth, M. E., Lizardi, P., Hedley, D. W., Bristow, R. G. & Glazer, P. M. 2004. 'Down-regulation of Rad51 and decreased homologous recombination in hypoxic cancer cells.' *Mol Cell Biol*, 24:19, 8504-18.
- Blackburn, E. H. 2000. 'Telomere states and cell fates.' *Nature*, 408:6808, 53-6.
- Blagosklonny, M. V. 2002. 'Sequential activation and inactivation of G2 checkpoints for selective killing of p53-deficient cells by microtubule-active drugs.' *Oncogene*, 21:41, 6249-54.
- Blagosklonny, M. V., Robey, R., Bates, S. & Fojo, T. 2000. 'Pretreatment with DNA-damaging agents permits selective killing of checkpoint-deficient cells by microtubule-active drugs.' *J Clin Invest*, 105:4, 533-9.
- Blagosklonny, M. V., Robey, R., Sackett, D. L., Du, L., Traganos, F., Darzynkiewicz, Z., Fojo, T. & Bates, S. E. 2002. 'Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity.' *Mol Cancer Ther*, 1:11, 937-41.
- Blagosklonny, M. V., Trostel, S., Kayastha, G., Demidenko, Z. N., Vassilev, L. T., Romanova, L. Y., Bates, S. & Fojo, T. 2005. 'Depletion of mutant p53 and cytotoxicity of histone deacetylase inhibitors.' *Cancer Res*, 65:16, 7386-92.
- Boerner, J. L., Danielsen, A. & Maihle, N. J. 2003. 'Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm.' *Exp Cell Res*, 284:1,111-21.
- Bohr, V. A., Smith, C. A., Okumoto, D. S. & Hanawalt, P. C. 1985. 'DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall.' *Cell*, 40:2, 359-69.
- Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V. & Rosen, N. 1987. 'Activation of pp60c-src protein kinase activity in human colon carcinoma.' *Proc Natl Acad Sci U S A*, 84:8, 2251-5.
- Branch, P., Aquilina, G., Bignami, M. & Karran, P. 1993. 'Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage.' *Nature*, 362:6421, 652-4.
- Brash, D. E. 1997. 'Sunlight and the onset of skin cancer.' *Trends Genet*, 13:10, 410-4.
- Bruner, S. D., Norman, D. P. & Verdine, G. L. 2000. 'Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA.' *Nature*, 403:6772, 859-66.
- Cahill, D. P., Levine, K. K., Betensky, R. A., Codd, P. J., Romany, C. A., Reavie, L. B., Batchelor, T. T., Futreal, P. A., Stratton, M. R., Curry, W. T., Iafrate, A. J. & Louis, D. N. 2007. 'Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment.' *Clin Cancer Res*, 13:7, 2038-45.
- Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G. & Baylin, S. B. 1999. 'Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer.' *Nat Genet*, 21:1, 103-7.

- Campbell, P. J., Yachida, S., Mudie, L. J., Stephens, P. J., Pleasance, E. D., Stebbings, L. A., Morsberger, L. A., Latimer, C., McLaren, S., Lin, M.-L., McBride, D. J., Varela, I., Nik-Zainal, S. A., Leroy, C., Jia, M., Menzies, A., Butler, A. P., Teague, J. W., Griffin, C. A., Burton, J., Swerdlow, H., Quail, M. A., Stratton, M. R., Iacobuzio-Donahue, C. & Futreal, P. A. 2010. 'The patterns and dynamics of genomic instability in metastatic pancreatic cancer.' *Nature*, 467:7319, 1109-13.
- Cantor, S., Drapkin, R., Zhang, F., Lin, Y., Han, J., Pamidi, S. & Livingston, D. M. 2004. 'The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations.' *Proc Natl Acad Sci USA*, 101:8, 2357-62.
- Cao, Y., Miao, X. P., Huang, M. Y., Deng, L., Hu, L. F., Ernberg, I., Zeng, Y. X., Lin, D. X. & Shao, J. Y. 2006. 'Polymorphisms of XRCC1 genes and risk of nasopharyngeal carcinoma in the Cantonese population.' *BMC Cancer*, 6, 167.
- Carrel, A. & Ebeling, A. H. 1921. 'Age and multiplication of fibroblasts.' *J Exp Med*, 34:6, 599-623.
- Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., Murphree, A. L., Strong, L. C. & White, R. L. 1983. 'Expression of recessive alleles by chromosomal mechanisms in retinoblastoma.' *Nature*, 305:5937, 779-84.
- Cejka, P., Stojic, L., Mojas, N., Russell, A. M., Heinemann, K., Cannavo, E., di Pietro, M., Marra, G. & Jiricny, J. 2003. 'Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1.' *EMBO J*, 22:9, 2245-54.
- Chagraoui, J., Hébert, J., Girard, S. & Sauvageau, G. 2011. 'An anticlastogenic function for the Polycomb Group gene Bmi1.' *Proc Natl Acad Sci USA*, 108:13, 5284-9.
- Chakravarti, D., Mailander, P. C., Li, K. M., Higginbotham, S., Zhang, H. L., Gross, M. L., Meza, J. L., Cavalieri, E. L. & Rogan, E. G. 2001. 'Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene.' *Oncogene*, 20:55, 7945-53.
- Chan, D. W., Chen, B. P., Prithivirajasingh, S., Kurimasa, A., Story, M. D., Qin, J. & Chen, D. J. 2002a. 'Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks.' *Genes Dev*, 16:18, 2333-8.
- Chan, J. L., Lee, S. W., Fraass, B. A., Normolle, D. P., Greenberg, H. S., Junck, L. R., Gebarski, S. S. & Sandler, H. M. 2002b. 'Survival and failure patterns of high-grade gliomas after three-dimensional conformal radiotherapy.' *J Clin Oncol*, 20:6, 1635-42.
- Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F. & Maity, A. 2001. 'Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia'. *J Biol Chem*, 276:12, 9519-25.
- Chin, L., Artandi, S. E., Shen, Q., Tam, A., Lee, S. L., Gottlieb, G. J., Greider, C. W. & DePinho, R. A. 1999. 'p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis.' *Cell*, 97:4, 527-38.
- Choi, J. H., Lindsey-Boltz, L. A., Kemp, M., Mason, A. C., Wold, M. S. & Sancar, A. 2010. 'Reconstitution of RPA-covered single-stranded DNA-activated ATR-Chk1 signaling.' *Proc Natl Acad Sci USA*, 107:31, 13660-5.

- Christmann, M., Tomicic, M. T., Roos, W. P. & Kaina, B. 2003. 'Mechanisms of human DNA repair: an update.' *Toxicology*, 193:1-2, 3-34.
- Claij, N. & te Riele, H. 2004. 'Msh2 deficiency does not contribute to cisplatin resistance in mouse embryonic stem cells.' *Oncogene*, 23:1, 260-6.
- Cooke, M. S., Evans, M. D., Dizdaroglu, M. & Lunec, J. 2003. 'Oxidative DNA damage: mechanisms, mutation, and disease.' *FASEB J*, 17:10, 1195-214.
- Cortellino, S., Turner, D., Masciullo, V., Schepis, F., Albino, D., Daniel, R., Skalka, A. M., Meropol, N. J., Alberti, C., Larue, L. & Bellacosa, A. 2003. 'The base excision repair enzyme MED1 mediates DNA damage response to antitumor drugs and is associated with mismatch repair system integrity.' *Proc Natl Acad Sci USA*, 100:25, 15071-6.
- Cortes, U., Tong, W. M., Coyle, D. L., Meyer-Ficca, M. L., Meyer, R. G., Petrilli, V., Herceg, Z., Jacobson, E. L., Jacobson, M. K. & Wang, Z. Q. 2004. 'Depletion of the 110-kilodalton isoform of poly(ADP-ribose) glycohydrolase increases sensitivity to genotoxic and endotoxic stress in mice.' *Mol Cell Biol*, 24:16, 7163-78.
- Dachs, G. U., Patterson, A. V., Firth, J. D., Ratcliffe, P. J., Townsend, K. M., Stratford, I. J. & Harris, A. L. 1997. 'Targeting gene expression to hypoxic tumor cells.' *Nat Med*, 3:5, 515-20.
- Damia, G. & D'Incalci, M. 2010. 'Genetic instability influences drug response in cancer cells.' *Curr Drug Targets*, 11:10, 1317-24.
- Dang, C. V., Kim, J. W., Gao, P. & Yustein, J. 2008. 'The interplay between MYC and HIF in cancer.' *Nat Rev Cancer*, 8:1, 51-56.
- Daniel, N. N. & Korsmeyer, S. J. 2004. 'Cell death: critical control points.' *Cell*, 116:2, 205-19.
- David, S. S., O'Shea, V. L. & Kundu, S. 2007. 'Base-excision repair of oxidative DNA damage.' *Nature*, 447:7147, 941-50.
- Davies, M. A. & Samuels, Y. 2010. 'Analysis of the genome to personalize therapy for melanoma.' *Oncogene*, 29:41, 5545-55.
- de Laat, W. L., Jaspers, N. G. & Hoeijmakers, J. H. 1999. 'Molecular mechanism of nucleotide excision repair.' *Genes Dev*, 13:7, 768-85.
- de Larco, J. E. & Todaro, G. J. 1978. 'Growth factors from murine sarcoma virus-transformed cells.' *Proc Natl Acad Sci USA*, 75:8, 4001-5.
- de Murcia, J. M., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., Oliver, F. J., Masson, M., Dierich, A., LeMeur, M., Walztinger, C., Chambon, P. & de Murcia, G. 1997. 'Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells.' *Proc Natl Acad Sci USA*, 94:14, 7303-7.
- de Ruyck, K., Szaumkessel, M., De Rudder, I., Dehoorne, A., Vral, A., Claes, K., Velghe, A., Van Meerbeeck, J. & Thierens, H. 2007. 'Polymorphisms in base-excision repair and nucleotide-excision repair genes in relation to lung cancer risk.' *Mutat Res*, 631:2, 101-10.
- de Vries, H. 1910. "Intracellular Pangenesis". *Chicago The Open Court Publishing Co.*
- de Waard, H., Sonneveld, E., de Wit, J., Esveldt-van Lange, R., Hoeijmakers, J. H., Vrieling, H. & van der Horst, G. T. 2008. 'Cell-type-specific consequences of nucleotide excision repair deficiencies: Embryonic stem cells versus fibroblasts.' *DNA Repair (Amst)*, 7:10, 1659-69.

- de Winter, J. P. & Joenje, H. 2009. 'The genetic and molecular basis of Fanconi anemia.' *Mutat Res*, 668:1-2, 11-9.
- Degenhardt, K., Chen, G., Lindsten, T. & White, E. 2002. 'BAX and BAK mediate p53-independent suppression of tumorigenesis.' *Cancer Cell*, 2:3, 193-203.
- Demidenko, Z. N., Kalurupalle, S., Hanko, C., Lim, C. U., Broude, E. & Blagosklonny, M. V. 2008. 'Mechanism of G1-like arrest by low concentrations of paclitaxel: next cell cycle p53-dependent arrest with sub G1 DNA content mediated by prolonged mitosis.' *Oncogene*, 27:32, 4402-10.
- Dempke, W., Voigt, W., Grothey, A., Hill, B. T. & Schmoll, H. J. 2000. 'Cisplatin resistance and oncogenes--a review.' *Anti-cancer drugs*, 11:4, 225-36.
- Duesberg, P. H. & Vogt, P. K. 1970. 'Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses.' *Proc Natl Acad Sci USA*, 67:4, 1673-80.
- Dunham, M. A., Neumann, A. A., Fasching, C. L. & Reddel, R. R. 2000. 'Telomere maintenance by recombination in human cells.' *Nat Genet*, 26:4, 447-50.
- Duval, A., Gayet, J., Zhou, X. P., Iacopetta, B., Thomas, G. & Hamelin, R. 1999. 'Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability.' *Cancer Res*, 59:17, 4213-5.
- Eastman, A. 2004. 'Cell cycle checkpoints and their impact on anticancer therapeutic strategies.' *J Cell Biochem*, 91:2, 223-31.
- Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. 2003. 'Chromosomal instability and tumors promoted by DNA hypomethylation.' *Science*, 300:5618, 455.
- Edwards, S. L., Brough, R., Lord, C. J., Natrajan, R., Vatcheva, R., Levine, D. A., Boyd, J., Reis-Filho, J. S. & Ashworth, A. 2008. 'Resistance to therapy caused by intragenic deletion in BRCA2.' *Nature*, 451:7182, 1111-5.
- Esteller, M. 2008. 'Epigenetics in cancer.' *N Engl J Med*, 358:11, 1148-59.
- Evans, E., Moggs, J. G., Hwang, J. R., Egly, J. M. & Wood, R. D. 1997. 'Mechanism of open complex and dual incision formation by human nucleotide excision repair factors.' *EMBO J*, 16:21, 6559-73.
- Facchino, S., Abdouh, M., Chatoo, W. & Bernier, G. 2010. 'BMI1 confers radioresistance to normal and cancerous neural stem cells through recruitment of the DNA damage response machinery.' *J Neurosci*, 30:30, 10096-111.
- Fearon, E. R. & Vogelstein, B. 1990. 'A genetic model for colorectal tumorigenesis.' *Cell*, 61:5, 759-67.
- Feinberg, A. P., Ohlsson, R. & Henikoff, S. 2006. 'The epigenetic progenitor origin of human cancer.' *Nat Rev Genet*, 7:1, 21-33.
- Felton, K. E., Gilchrist, D. M. & Andrew, S. E. 2007. 'Constitutive deficiency in DNA mismatch repair: is it time for Lynch III?' *Clin Genet*, 71:6, 499-500.
- Fink, D., Nebel, S., Norris, P. S., Baergen, R. N., Wilczynski, S. P., Costa, M. J., Haas, M., Cannistra, S. A. & Howell, S. B. 1998. 'Enrichment for DNA mismatch repair deficient cells during treatment with cisplatin.' *Int J Cancer*, 77:5, 741-6.
- Finkel, T., Serrano, M. & Blasco, M. A. 2007. 'The common biology of cancer and ageing.' *Nature*, 448:7155, 767-74.
- Fishel, R. 1999. 'Signaling mismatch repair in cancer.' *Nat Med*, 5:11, 1239-41.
- Flemming, W. 1882. *Zellsubstanz, Kern und Zelltheilung*. Leipzig: F. C. W. Vogel.

- Fojo, T. 2002. 'p53 as a therapeutic target: unresolved issues on the road to cancer therapy targeting mutant p53.' *Drug Resist Updat*, 5:5, 209-16.
- Folkman, J. 2003. 'Fundamental concepts of the angiogenic process.' *Curr Mol Med*, 3:7, 643-51.
- Ford, J. M. & Hanawalt, P. C. 1995. 'Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription coupled repair and enhanced UV resistance.' *Proc Natl Acad Sci USA*, 92:19, 8876-80.
- Foreman, K. E., Wrone-Smith, T., Boise, L. H., Thompson, C. B., Polverini, P. J., Simonian, P. L., Nunez, G. & Nickoloff, B. J. 1996. 'Kaposi's sarcoma tumor cells preferentially express Bcl-xL.' *Am J Pathol*, 149:3, 795-803.
- Fraser, M., Bai, T. & Tsang, B. K. 2008. 'Akt promotes cisplatin resistance in human ovarian cancer cells through inhibition of p53 phosphorylation and nuclear function.' *Int J Cancer*, 122:3, 534-46.
- Fraser, M., Leung, B. M., Yan, X., Dan, H. C., Cheng, J. Q. & Tsang, B. K. 2003. 'p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells.' *Cancer Res*, 63:21, 7081-8.
- Friedberg, E. C. 2001. 'How nucleotide excision repair protects against cancer.' *Nat Rev Cancer*, 1:1, 22-33.
- Friedman, D. B., Wang, S. E., Whitwell, C. W., Caprioli, R. M. & Arteaga, C. L. 2007. 'Multivariable difference gel electrophoresis and mass spectrometry: a case study on transforming growth factor-beta and ERBB2 signaling.' *Mol Cell Proteomics*, 6:1,150-69.
- Friesen, C., Lubatschowski, A., Kotzerke, J., Buchmann, I., Reske, S. N. & Debatin, K. M. 2003. 'Beta-irradiation used for systemic radioimmunotherapy induces apoptosis and activates apoptosis pathways in leukaemia cells.' *Eur J Nucl Med Mol Imaging*, 30:9, 1251-61.
- Friesen, C., Uhl, M., Pannicke, U., Schwarz, K., Miltner, E. & Debatin, K. M. 2008. 'DNA ligase IV and DNA-protein kinase play a critical role in deficient caspases activation in apoptosis-resistant cancer cells by using doxorubicin.' *Mol Biol Cell*, 19:8, 3283-9.
- Gasser, S., Orsulic, S., Brown, E. J. & Raulet, D. H. 2005. 'The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor.' *Nature*, 436:7054, 1186-90.
- Gatenby, R. A. & Gillies, R. J. 2004. 'Why do cancers have high aerobic glycolysis?' *Nat Rev Cancer*, 4:11, 891-99.
- Gaymes, T. J., Mufti, G. J. & Rassool, F. V. 2002. 'Myeloid leukemias have increased activity of the nonhomologous end-joining pathway and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer.' *Cancer Res*, 62:10, 2791-97.
- Ghebranious, N. & Donehower, L. A. 1998. 'Mouse models in tumor suppression.' *Oncogene*, 17:25, 3385-400.
- Gifford, G., Paul, J., Vasey, P. A., Kaye, S. B. & Brown, R. 2004. 'The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients.' *Clin Cancer Res*, 10:13, 4420-6.
- Girard, F., Strausfeld, U., Fernandez, A. & Lamb, N. J. 1991. 'Cyclin A is required for the onset of DNA replication in mammalian fibroblasts.' *Cell*, 67:6, 1169-79.

- Glasspool, R. M., Teodoridis, J. M. & Brown, R. 2006. 'Epigenetics as a mechanism driving polygenic clinical drug resistance.' *Br J Cancer*, 94:8, 1087-92.
- Goldmacher, V. S., Cuzick, R. A., Jr. & Thilly, W. G. 1986. 'Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine.' *J Biol Chem*, 261:27, 12462-71.
- Gong, J. G., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G. Jr., Levrero, M. & Wang, J. Y. 1999. 'The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin induced DNA damage.' *Nature*, 399:6738, 806-9.
- Goode, E. L., Ulrich, C. M. & Potter, J. D. 2002. 'Polymorphisms in DNA repair genes and associations with cancer risk.' *Cancer Epidemiol Biomarkers Prev*, 11:12, 1513-30.
- Goodman, M. F. & Tiffin, B. 2000. 'Sloppier copier DNA polymerases involved in genome repair.' *Curr Opin Genet Dev*, 10:2, 162-8.
- Goodsell, D. S. 2001. 'The molecular perspective: ultraviolet light and pyrimidine dimers.' *Oncologist*, 6:3, 298-9.
- Gos, M., Miloszevska, J., Swoboda, P., Trembacz, H., Skierski, J. & Janik, P. 2005. 'Cellular quiescence induced by contact inhibition or serum withdrawal in C3H10T1/2 cells.' *Cell Prolif*, 38:2, 107-16.
- Greenon, J. K., Bonner, J. D., Ben-Yzhak, O., Cohen, H. I., Miselevich, I., Resnick, M. B., Trougouboff, P., Tomsho, L. D., Kim, E., Low, M., Almog, R., Rennert, G. & Gruber, S. B. 2003. 'Phenotype of microsatellite unstable colorectal carcinomas: Well differentiated and focally mucinous tumors and the absence of dirty necrosis correlate with microsatellite instability.' *Am J Surg Pathol*, 27:5, 563-70.
- Griffiths, A. J. F., Wessler, S. R., Lewontin, R. T., Gelbart, W. M., Suzuki, D. T. & Miller, J. H. 2004. *An introduction to genetic analysis*. New York: W.H. Freeman.
- Grivennikov, S. & Karin, M. 2008. 'Autocrine IL-6 signaling: a key event in tumorigenesis?' *Cancer Cell*, 13:1, 7-9.
- Groffen, J., Stephenson, J. R., Heisterkamp, N., de Klein, A., Bartram, C. R. & Grosveld, G. 1984. 'Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22.' *Cell*, 36:1, 93-9.
- Gronbaek, K., Straten, P. T., Ralfkiaer, E., Ahrenkiel, V., Andersen, M. K., Hansen, N. E., Zeuthen, J., Hou-Jensen, K. & Guldborg, P. 1998. 'Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity.' *Blood*, 92:9, 3018-24.
- Gurkan, E., Schupp, J. E., Aziz, M. A., Kinsella, T. J. & Loparo, K. A. 2007. 'Probabilistic modeling of DNA mismatch repair effects on cell cycle dynamics and iododeoxyuridine-DNA incorporation.' *Cancer Res*, 67:22, 10993-1000.
- Hahn, W. C. & Weinberg, R. A. 2002. 'Modelling the molecular circuitry of cancer.' *Nat Rev Cancer*, 2:5, 331-41.
- Hammond, E. M. & Giaccia, A. J. 2005. 'The role of p53 in hypoxia-induced apoptosis.' *Biochem Biophys Res Commun*, 331:3, 718-25.
- Hanahan, D. & Weinberg, R. A. 2000. 'The hallmarks of cancer.' *Cell*, 100:1, 57-70.
- Hanahan, D. & Weinberg, R. A. 2011. 'Hallmarks of cancer: the next generation.' *Cell*, 144:5, 646-74.

- Hans, M. A., Muller, M., Meyer-Ficca, M., Burkle, A. & Kupper, J. H. 1999. 'Overexpression of dominant negative PARP interferes with tumor formation of HeLa cells in nude mice: evidence for increased tumor cell apoptosis in vivo.' *Oncogene*, 18:50, 7010-5.
- Hansen, L. T., Lundin, C., Spang-Thomsen, M., Petersen, L. N. & Helleday, T. 2003. 'The role of RAD51 in etoposide (VP16) resistance in small cell lung cancer.' *Int J Cancer*, 105:4, 472-9.
- Haracska, L., Prakash, S. & Prakash, L. 2000. 'Replication past O(6)-methylguanine by yeast and human DNA polymerase eta.' *Mol Cell Biol*, 20:21, 8001-7.
- Harbour, J. W. & Dean, D. C. 2000. 'Rb function in cell-cycle regulation and apoptosis.' *Nat Cell Biol*, 2:4, E65-E67.
- Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdar, A. F., Minna, J. D. & Kaye, F. J. 1988. 'Abnormalities in structure and expression of the human retinoblastoma gene in SCLC.' *Science*, 241:4863, 353-7.
- Harris, H. 2008. 'Concerning the origin of malignant tumours by Theodor Boveri.' *J Cell Sci*, 121:Suppl 1, 1-84.
- Hartlerode, A. J. & Scully, R. 2009. 'Mechanisms of double-strand break repair in somatic mammalian cells.' *Biochem J*, 423:2, 157-68.
- Hassa, P. O., Haenni, S. S., Elser, M. & Hottiger, M. O. 2006. 'Nuclear adp-ribosylation reactions in mammalian cells: Where are we today and where are we going?' *Microbiol Mol Biol Rev*, 70:3, 789-829.
- Hayflick, L. 2000. 'The illusion of cell immortality.' *Br J Cancer*, 83:7, 841-6.
- Hayflick, L. & Moorhead, P. S. 1961. 'The serial cultivation of human diploid cell strains.' *Exp Cell Res*, 25, 585-621.
- Hayward, S. W., Wang, Y., Cao, M., Hom, Y. K., Zhang, B., Grossfeld, G. D., Sudilovsky, D. & Cunha, G. R. 2001. 'Malignant transformation in a nontumorigenic human prostatic epithelial cell line.' *Cancer Res*, 61:22, 8135-42.
- He, F., Li, L., Kim, D., Wen, B., Deng, X., Gutin, P. H., Ling, C. C. & Li, G. C. 2007. 'Adenovirus-mediated expression of a dominant negative Ku70 fragment radiosensitizes human tumor cells under aerobic and hypoxic conditions.' *Cancer Res*, 67:2, 634-42.
- He, S., Nakada, D. & Morrison, S. J. 2009. 'Mechanisms of stem cell self-renewal.' *Annu Rev Cell Dev Biol*, 25, 377-406.
- Hecht, J. L. & Aster, J. C. 2000. 'Molecular biology of Burkitt's lymphoma.' *J Clin Oncol*, 18:21, 3707-21.
- Hegi, M. E., Diserens, A. C., Gorlia, T., Hamou, M. F., de Tribolet, N., Weller, M., Kros, J. M., Hainfellner, J. A., Mason, W., Mariani, L., Bromberg, J. E., Hau, P., Mirimanoff, R. O., Cairncross, J. G., Janzer, R. C. & Stupp, R. 2005. 'MGMT gene silencing and benefit from temozolomide in glioblastoma.' *N Engl J Med*, 352:10, 997-1003.
- Heinen, C. D., Schmutte, C. & Fishel, R. 2002. 'DNA repair and tumorigenesis: lessons from hereditary cancer syndromes.' *Cancer Biol Ther*, 1:5, 477-85.
- Helleday, T. 2010. 'Homologous recombination in cancer development, treatment and development of drug resistance.' *Carcinogenesis*, 31:6, 955-60.
- Herman, J. G. & Baylin, S. B. 2003. 'Gene silencing in cancer in association with promoter hypermethylation.' *N Engl J Med*, 349:21, 2042-54.

- Hershko, A., Mamont, P., Shields, R. & Tomkins, G. M. 1971. 'Pleiotypic response'. *Nat New Biol*, 232:33, 206-11.
- Hitchins, M. P. & Ward, R. L. 2009. 'Constitutional (germline) MLH1 epimutation as an aetiological mechanism for hereditary non-polyposis colorectal cancer.' *J Med Genet*, 46:12, 793-802.
- Hitomi, K., Iwai, S. & Tainer, J. A. 2007. 'The intricate structural chemistry of base excision repair machinery: implications for DNA damage recognition, removal, and repair.' *DNA Repair (Amst)*, 6:4, 410-28.
- Hlavín, E. M., Smeaton, M. B., Miller, P. S. 2010. "Initiation of DNA Interstrand Cross-link Repair in Mammalian Cells". *Environ Mol Mutagen*, 51:6, 604-24.
- Hochedlinger, K., Blelloch, R., Brennan, C., Yamada, Y., Kim, M., Chin, L. & Jaenisch, R. 2004. 'Reprogramming of a melanoma genome by nuclear transplantation.' *Genes Dev*, 18:15, 1875-85.
- Hoeijmakers, J. H. 2001. 'Genome maintenance mechanisms for preventing cancer.' *Nature*, 411:6835, 366-74.
- Hollstein, M., Sidransky, B., Vogelstein, B. & Harris, C. C. 1991. 'p53 mutations in human cancers.' *Science*, 253:5015, 49-53.
- Honecker, F., Wermann, H., Mayer, F., Gillis, A. J., Stoop, H., van Gorp, R. J., Oechsle, K., Steyerberg, E., Hartmann, J. T., Dinjens, W. N., Oosterhuis, J. W., Bokemeyer, C. & Looijenga, L. H. 2009. 'Microsatellite instability, mismatch repair deficiency, and BRAF mutation in treatment-resistant germ cell tumors.' *J Clin Oncol*, 27:13, 2129-36.
- Horton, J. K. & Wilson, S. H. 2007. 'Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency.' *DNA Repair (Amst)*, 6:4, 530-43.
- Howard, A. & Pelc, S. 1953. 'Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage.' *Heredity*, 6 (Suppl.), 261-73.
- Howlett, N. G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E. A. & D'Andrea, A. D. 2002. 'Biallelic inactivation of BRCA2 in Fanconi anemia.' *Science*, 297:5581, 606-9.
- Hsiang, Y. H., Lihou, M. G. & Liu, L. F. 1989. 'Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin.' *Cancer Res*, 49:18, 5077-82.
- Hu, H. Y., Horton, J. K., Gryk, M. R., Prasad, R., Naron, J. M., Sun, D. A., Hecht, S. M., Wilson, S. H. & Mullen, G. P. 2004. 'Identification of small molecule synthetic inhibitors of DNA polymerase beta by NMR chemical shift mapping.' *J Biol Chem*, 279:38, 39736-44.
- Huebner, R. J. & Todaro, G. J. 1969. 'Oncogenes of RNA tumor viruses as determinants of cancer.' *Proc Natl Acad Sci USA*, 64:3, 1087-94.
- Huertas, P., Cortes-Ledesma, F., Sartori, A. A., Aguilera, A. & Jackson, S. P. 2008. 'CDK targets Sae2 to control DNA-end resection and homologous recombination.' *Nature*, 455:7213, 689-92.
- Hung, R. J., Hall, J., Brennan, P. & Boffetta, P. 2005. 'Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review.' *Am J Epidemiol*, 162:10, 925-42.

- Hussain, S. P., Hofseth, L. J. & Harris, C. C. 2003. 'Radical causes of cancer.' *Nat Rev Cancer*, 3:4, 276-85.
- Iba, H., Takeya, T., Cross, F. R., Hanafusa, T. & Hanafusa, H. 1984. 'Rous sarcoma virus variants that carry the cellular src gene instead of the viral src gene cannot transform chicken embryo fibroblasts.' *Proc Natl Acad Sci USA*, 81:14, 4424-8.
- Ikeda, E., Achen, M. G., Breier, G. & Risau, W. 1995. 'Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells.' *J Biol Chem*, 270:34, 19761-6.
- Imai, T., Horiuchi, A., Wang, C., Oka, K., Ohira, S., Nikaido, T. & Konishi, I. 2003. 'Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells.' *Am J Pathol*, 163:4, 1437-47.
- International Human Genome Consortium 2001. 'Initial sequencing and analysis of the human genome.' *Nature*, 409:6822, 860-921.
- Itahana, K., Dimri, G. P., Hara, E., Itahana, Y., Zou, Y., Desprez, P. Y. & Campisi, J. 2002. 'A role for p53 in maintaining and establishing the quiescence growth arrest in human cells.' *J Biol Chem*, 277:20, 18206-14.
- Iyer, R. R., Pluciennik, A., Burdett, V. & Modrich, P. L. 2006. 'DNA mismatch repair: functions and mechanisms.' *Chem Rev*, 106:2, 302-23.
- Jackson, S. P. & Bartek, J. 2009. 'The DNA-damage response in human biology and disease.' *Nature*, 461:7267, 1071-8.
- Jacob, S., Aguado, M., Fallik, D. & Praz, F. 2001. 'The role of the DNA mismatch repair system in the cytotoxicity of the topoisomerase inhibitors camptothecin and etoposide to human colorectal cancer cells.' *Cancer Res*, 61:17, 6555-62.
- Jacobs, C. & Rubsamén, H. 1983. 'Expression of pp60c-src protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammary carcinomas.' *Cancer Res*, 43:4, 1696-702.
- Jaiswal, A. S., Banerjee, S., Aneja, R., Sarkar, F. H., Ostrov, D. A. & Narayan, S. 2011. 'DNA polymerase beta as a novel target for chemotherapeutic intervention of colorectal cancer.' *PLoS One*, 6:2, e16691.
- Jaiswal, A. S. & Narayan, S. 2008. 'A novel function of adenomatous polyposis coli (APC) in regulating DNA repair.' *Cancer Lett*, 271:2, 272-80.
- Jaiswal, A. S., Banerjee, S., Panda, H., Bulkin, C. D., Izumi, T., Sarkar, F. H., Ostrov, D. A. & Narayan, S. 2009. 'A novel inhibitor of DNA polymerase beta enhances the ability of temozolomide to impair the growth of colon cancer cells.' *Mol Cancer Res*, 7:12, 1973-83.
- Jaskelioff, M., Muller, F. L., Paik, J. H., Thomas, E., Jiang, S., Adams, A. C., Sahin, E., Kost-Alimova, M., Protopopov, A., Cadinanos, J., Horner, J. W., Maratos-Flier, E. & Depinho, R. A. 2010. 'Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice.' *Nature*, 469:7328, 102-6.
- Jeggo, P. A., Taccioli, G. E. & Jackson, S. P. 1995. 'Menage a trois: double strand break repair, V(D)J recombination and DNA-PK.' *Bioessays*, 17:11, 949-57.
- Jenkins, M. A., Hayashi, S., O'Shea, A. M., Burgart, L. J., Smyrk, T. C., Shimizu, D., Waring, P. M., Ruszkiewicz, A. R., Pollett, A. F., Redston, M., Barker, M. A., Baron, J. A., Casey, G. R., Dowty, J. G., Giles, G. G., Limburg, P., Newcomb, P., Young, J. P., Walsh, M. D., Thibodeau, S. N., Lindor, N. M., Lemarchand, L., Gallinger, S., Haile, R. W., Potter, J. D., Hopper, J. L. & Jass, J. R. 2007. 'Pathology features in Bethesda

- guidelines predict colorectal cancer microsatellite instability: a population-based study.' *Gastroenterology*, 133:1, 48-56.
- Jiang, D., Yang, H., Willson, J. K., Liang, J., Humphrey, L. E., Zborowska, E., Wang, D., Foster, J., Fan, R. & Brattain, M. G. 1998. 'Autocrine transforming growth factor alpha provides a growth advantage to malignant cells by facilitating re-entry into the cell cycle from suboptimal growth states.' *J Biol Chem*, 273:47, 31471-9.
- Jiao, X., Huang, J., Wu, S., Lv, M., Hu, Y., Jianfu, Su, X., Luo, C. & Ce, B. 2007. 'hOGG1 Ser326Cys polymorphism and susceptibility to gallbladder cancer in a Chinese population.' *Int J Cancer*, 121:3, 501-5.
- Jin, H. Y., Liu, X., Li, V. K., Ding, Y., Yang, B., Geng, J., Lai, R., Ding, S., Ni, M. & Zhao, R. 2008. 'Detection of mismatch repair gene germline mutation carrier among Chinese population with colorectal cancer.' *BMC Cancer*, 8, 44.
- Jiricny, J. 2006. 'The multifaceted mismatch-repair system.' *Nat Rev Mol Cell Biol*, 7:5, 335-46.
- Johnson, A. & O'Donnell, M. 2005. 'Cellular DNA replicases: components and dynamics at the replication fork.' *Annu Rev Biochem*, 74, 283-315.
- Johnston, L. A. 2009. 'Competitive interactions between cells: death, growth, and geography.' *Science*, 324:5935, 1679-82.
- Jones, K. R., Gewirtz, D. A., Yannone, S. M., Zhou, S., Schatz, D. G., Valerie, K. & Povirk, L. F. 2005. 'Radiosensitization of MDA-MB-231 breast tumor cells by adenovirus mediated overexpression of a fragment of the XRCC4 protein.' *Mol Cancer Ther*, 4:10, 1541-7.
- Juan, L. J., Shia, W. J., Chen, M. H., Yang, W. M., Seto, E., Lin, Y. S. & Wu, C. W. 2000. 'Histone deacetylases specifically down-regulate p53-dependent gene activation.' *J Biol Chem*, 275:27, 20436-43.
- Jurado, J., Maciejewska, A., Krwawicz, J., Laval, J. & Saporbaev, M. K. 2004. 'Role of mismatch-specific uracil-DNA glycosylase in repair of 3,N4-ethenocytosine in vivo.' *DNA Repair (Amst)*, 3:12, 1579-90.
- Kaina, B., Christmann, M., Naumann, S. & Roos, W. P. 2007. 'MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents.' *DNA Repair (Amst)*, 6:8, 1079-99.
- Kang, T. H. & Sancar, A. 2009. 'Circadian regulation of DNA excision repair: implications for chrono-chemotherapy.' *Cell Cycle*, 8:11, 1665-7.
- Kapetanaki, M. G., Guerrero-Santoro, J., Bisi, D. C., Hsieh, C. L. & Rapic-Otrin, V. 2006. 'The DDB1-CUL4A DDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV- damaged DNA sites.' *Proc Natl Acad Sci USA*, 103:8, 2588-93.
- Karran, P. 2001. 'Mechanisms of tolerance to DNA damaging therapeutic drugs.' *Carcinogenesis*, 22:12, 1931-7.
- Karran, P. & Attard, N. 2008. 'Thiopurines in current medical practice: molecular mechanisms and contributions to therapy-related cancer.' *Nat Rev Cancer*, 8:1, 24-36.
- Karran, P. & Bignami, M. 1992. 'Self-destruction and tolerance in resistance of mammalian cells to alkylation damage.' *Nucleic Acids Res*, 20:12, 2933-40.

- Kawanishi, S., Hiraku, Y., Pinlaor, S. & Ma, N. 2006. 'Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation related carcinogenesis.' *Biol Chem*, 387:4, 365-72.
- Kawate, H., Sakumi, K., Tsuzuki, T., Nakatsuru, Y., Ishikawa, T., Takahashi, S., Takano, H., Noda, T. & Sekiguchi, M. 1998. 'Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes.' *Proc Natl Acad Sci USA*, 95:9, 5116-20.
- Kaysen, J., Spriggs, D. & Kufe, D. 1986. 'Incorporation of 5-fluorodeoxycytidine and metabolites into nucleic acids of human MCF-7 breast carcinoma cells.' *Cancer Res*, 46:9, 4534-8.
- Kerr, J. F., Wyllie, A. H. & Currie, A. R. 1972. 'Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.' *Br J Cancer*, 26:4, 239-57.
- Khanna, K. K. & Jackson, S. P. 2001. 'DNA double-strand breaks: signaling, repair and the cancer connection.' *Nat Genet*, 27:3, 247-54.
- Kim, G. P., Colangelo, L. H., Wieand, H. S., Paik, S., Kirsch, I. R., Wolmark, N. & Allegra, C. J. 2007. 'Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study.' *J Clin Oncol*, 25:7, 767-72.
- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L. & Shay, J. W. 1994. 'Specific association of human telomerase activity with immortal cells and cancer.' *Science*, 266:5193, 2011-5.
- Kim, S. T., Lee, J., Park, S. H., Park, J. O., Lim, H. Y., Kang, W. K., Kim, J. Y., Kim, Y. H., Chang, D. K., Rhee, P. L., Kim, D. S., Yun, H., Cho, Y. B., Kim, H. C., Yun, S. H., Lee, W. Y., Chun, H. K. & Park, Y. S. 2010. 'Clinical impact of microsatellite instability in colon cancer following adjuvant FOLFOX therapy.' *Cancer Chemother Pharmacol*, 66:4, 659-67.
- Kinsella, T. J. 1996. 'An approach to the radiosensitization of human tumors.' *Cancer J Sci Am*, 2:4, 184-93.
- Kinsella, T. J. 2009. 'Coordination of DNA mismatch repair and base excision repair processing of chemotherapy and radiation damage for targeting resistant cancers.' *Clin Cancer Res*, 15:6, 1853-9.
- Kinsella, T. J., Kinsella, M. T., Seo, Y. & Berk, G. 2007. '5-iodo-2-pyrimidinone-2'-deoxyribose-mediated cytotoxicity and radiosensitization in U87 human glioblastoma xenografts.' *Int J Radiat Oncol Biol Phys*, 69:4, 1254-61.
- Kinzler, K.W. & Vogelstein, B. 1996. 'Lessons from hereditary colorectal cancer.' *Cell*, 87:2, 159-70.
- Kitazono, M., Bates, S., Fok, P., Fojo, T. & Blagosklonny, M. V. 2002. 'The histone deacetylase inhibitor FR901228 (desipeptide) restores expression and function of pseudo-null p53.' *Cancer Biol Ther*, 1:6, 665-8.
- Knudson, A. G. Jr. 1971. 'Mutation and cancer: statistical study of retinoblastoma.' *Proc Natl Acad Sci USA*, 68:4, 820-3.
- Kobayashi, K., Karran, P., Oda, S. & Yanaga, K. 2005. 'Involvement of mismatch repair in transcription-coupled nucleotide excision repair.' *Hum Cell*, 18:3, 103-15.
- Kojima, K., Konopleva, M., Samudio, I. J., Shikami, M., Cabreira-Hansen, M., McQueen, T., Ruvolo, V., Tsao, T., Zeng, Z., Vassilev, L. T. & Andreeff, M. 2005. 'MDM2

- antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy.' *Blood*, 106:9, 3150-9.
- Kondo, S. 1977. 'A test for mutation theory of cancer: carcinogenesis by misrepair of DNA damaged by 4-Nitroquinoline 1-Oxide.' *Br J Cancer*, 35, 595-601.
- Koshiji, M., To, K. K., Hammer, S., Kumamoto, K., Harris, A. L., Modrich, P. & Huang, L. E. 2005. 'HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalph expression.' *Mol Cell*, 17:6, 793-803.
- Kotzerke, J., Bunjes, D. & Scheinberg, D. A. 2005. 'Radioimmunoconjugates in acute leukemia treatment: the future is radiant.' *Bone Marrow Transplant*, 36:12, 1021-6.
- Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. 2001. 'Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging.' *Proc Natl Acad Sci USA*, 98:21, 12072-7.
- Kruijer, W., Cooper, J. A., Hunter, T. & Verma, I. M. 1984. 'Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein.' *Nature*, 312:5996, 711-6.
- Krupa, R., Sliwinski, T., Wisniewska-Jarosinska, M., Chojnacki, J., Wasylecka, M., Dziki, L., Morawiec, J. & Blasiak, J. 2011a. 'Polymorphisms in RAD51, XRCC2 and XRCC3 genes of the homologous recombination repair in colorectal cancer-a case control study.' *Mol Biol Rep*, 38:4, 2849-54.
- Krupa, R., Sobczuk, A., Poplawski, T., Wozniak, K. & Blasiak, J. 2011b. 'DNA damage and repair in endometrial cancer in correlation with the hOGG1 and RAD51 genes polymorphism.' *Mol Biol Rep*, 38:2, 1163-70.
- Kumar-Sinha, C., Tomlins, S. A. & Chinnaiyan, A. M. 2008. 'Recurrent gene fusions in prostate cancer.' *Nat Rev Cancer*, 8:7, 497-511.
- Kundu, C. N., Balusu, R., Jaiswal, A. S. & Narayan, S. 2007. 'Adenomatous polyposis coli mediated hypersensitivity of mouse embryonic fibroblast cell lines to methylmethane sulfonate treatment: implication of base excision repair pathways.' *Carcinogenesis*, 28:10, 2089-95.
- Kurimasa, A., Kumano, S., Boubnov, N. V., Story, M. D., Tung, C. S., Peterson, S. R. & Chen, D. J. 1999. 'Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining.' *Mol Cell Biol*, 19:5, 3877-84.
- Kurzrock, R., Kantarjian, H. M., Druker, B. J. & Talpaz, M. 2003. 'Philadelphia chromosome positive leukemias: from basic mechanisms to molecular therapeutics.' *Ann Intern Med*, 138:10, 819-30.
- Lainé, J. P. & Egly, J. M. 2006. 'Initiation of DNA repair mediated by a stalled RNA polymerase II.' *EMBO J*, 25:2, 387-97.
- Lally, B. E., Geiger, G. A., Kridel, S., Arcury-Quandt, A. E., Robbins, M. E., Kock, N. D., Wheeler, K., Peddi, P., Georgakilas, A., Kao, G. D. & Koumenis, C. 2007. 'Identification and biological evaluation of a novel and potent small molecule radiation sensitizer via an unbiased screen of a chemical library.' *Cancer Res*, 67:18, 8791-9.
- Lantuejoul, S., Raynaud, C., Salameire, D., Gazzeri, S., Moro-Sibilot, D., Soria, J. C., Brambilla, C. & Brambilla, E. 2010. 'Telomere maintenance and DNA damage responses during lung carcinogenesis.' *Clin Cancer Res*, 16:11, 2979-88.

- Larrea, A. A., Lujan, S. A. & Kunkel, T. A. 2010. 'SnapShot: DNA mismatch repair.' *Cell*, 141:4, 730 e1.
- Lee, W. H., Bookstein, R., Hong, F., Young, L. J., Shew, J. Y. & Lee, E. Y. 1987. 'Human retinoblastoma susceptibility gene: cloning, identification, and sequence.' *Science*, 235:4794, 1394-9.
- Levine, A. J. 1997. 'p53, the cellular gatekeeper for growth and division.' *Cell*, 88:3, 323-31.
- Levitus, M., Waisfisz, Q., Godthelp, B. C., de Vries, Y., Hussain, S., Wiegant, W. W., Elghalbzouri-Maghrani, E., Steltenpool, J., Rooimans, M. A., Pals, G., Arwert, F., Mathew, C. G., Zdzienicka, M. Z., Hiom, K., de Winter, J. P. & Joenje, H. 2005. 'The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J.' *Nat Genet*, 37:9, 934-5.
- Ley, R., Balmanno, K., Hadfield, K., Weston, C. & Cook, S. J. 2003. 'Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim.' *J Biol Chem*, 278:21, 18811-6.
- Lieber, M. R. 1999. 'The biochemistry and biological significance of nonhomologous DNA end joining: an essential repair process in multicellular eukaryotes.' *Genes Cells*, 4:2, 77-85.
- Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M. & Lowe, S. W. 1998. 'Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling.' *Genes Dev*, 12:19, 3008-19.
- Lin, D. P., Wang, Y., Scherer, S. J., Clark, A. B., Yang, K., Avdievich, E., Jin, B., Werling, U., Parris, T., Kurihara, N., Umar, A., Kucherlapati, R., Lipkin, M., Kunkel, T. A. & Edelman, W. 2004. 'An Msh2 point mutation uncouples DNA mismatch repair and apoptosis.' *Cancer Res*, 64:2, 517-22.
- Lin, X. & Howell, S. B. 2006. 'DNA mismatch repair and p53 function are major determinants of the rate of development of cisplatin resistance.' *Mol Cancer Ther*, 5:5, 1239-47.
- Lindahl, T. 1993. 'Instability and decay of the primary structure of DNA.' *Nature*, 362:6422, 709-15.
- Lindahl, T. & Wood, R. D. 1999. 'Quality control by DNA repair.' *Science*, 286:5446, 1897-905.
- Lindor, N. M., Rabe, K., Petersen, G. M., Haile, R., Casey, G., Baron, J., Gallinger, S., Bapat, B., Aronson, M., Hopper, J., Jass, J., LeMarchand, L., Grove, J., Potter, J., Newcomb, P., Terdiman, J. P., Conrad, P., Moslein, G., Goldberg, R., Ziogas, A., Anton-Culver, H., de Andrade, M., Siegmund, K., Thibodeau, S. N., Boardman, L. A. & Seminara, D. 2005. 'Lower cancer incidence in Amsterdam-I criteria families without mismatch repair deficiency: familial colorectal cancer type X.' *JAMA*, 293:16, 1979-85.
- Lipinski, M. M. & Jacks, T. 1999. 'The retinoblastoma gene family in differentiation and development.' *Oncogene*, 18:55, 7873-82.
- Liu, L. & Gerson, S. L. 2004. 'Therapeutic impact of methoxyamine: blocking repair of abasic sites in the base excision repair pathway.' *Curr Opin Investig Drugs*, 5:6, 623-7.
- Liu, L. & Gerson, S. L. 2006. 'Targeted modulation of MGMT: clinical implications.' *Clin Cancer Res*, 12:2, 328-31.

- Liu, L., Lee, J. & Zhou, P. 2010. 'Navigating the nucleotide excision repair threshold.' *J Cell Physiol*, 224:3, 585-9.
- Liu, L., Taverna, P., Whitacre, C. M., Chatterjee, S. & Gerson, S. L. 1999. 'Pharmacologic disruption of base excision repair sensitizes mismatch repair-deficient and -proficient colon cancer cells to methylating agents.' *Clin Cancer Res*, 5:10, 2908-17.
- Liu, Y., Zhang, H., Zhou, K., Chen, L., Xu, Z., Zhong, Y., Liu, H., Li, R., Shugart, Y. Y., Wei, Q., Jin, L., Huang, F., Lu, D. & Zhou, L. 2007. 'Tagging SNPs in non-homologous end-joining pathway genes and risk of glioma.' *Carcinogenesis*, 28:9, 1906-13.
- Lobrich, M. & Jeggo, P. A. 2005. 'Harmonising the response to DSBs: a new string in the ATM bow.' *DNA Repair (Amst)*, 4:7, 749-59.
- Lopez-Cima, M. F., Gonzalez-Arriaga, P., Garcia-Castro, L., Pascual, T., Marron, M. G., Puente, X. S. & Tardon, A. 2007. 'Polymorphisms in XPC, XPD, XRCC1, and XRCC3 DNA repair genes and lung cancer risk in a population of northern Spain.' *BMC Cancer*, 7, 162.
- Lou, Z., Chini, C. C., Minter-Dykhouse, K. & Chen, J. 2003. 'Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control.' *J Biol Chem*, 278:16, 13599-602.
- Lugo, T. G., Pendergast, A. M., Muller, A. J. & Witte, O. N. 1990. 'Tyrosine kinase activity and transformation potency of bcr-abl oncogene products.' *Science*, 247:4946, 1079-82.
- Lyko, F. & Brown, R. 2005. 'DNA methyltransferase inhibitors and the development of epigenetic cancer therapies.' *J Nat Cancer Inst*, 97:20, 1498-506.
- Ma, W. Z., Sung, H. J., Park, J. Y., Matoba, S. & Hwang, P. M. 2007. 'A pivotal role for p53: balancing aerobic respiration and glycolysis.' *J Bioenerg Biomembr*, 39:3, 243-6.
- Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J. & Weinberg, R. A. 2008. 'The epithelial-mesenchymal transition generates cells with properties of stem cells.' *Cell*, 133:4, 704-15.
- Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B. & et al. 1995. 'Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability.' *Science*, 268:5215, 1336-8.
- Marquardt, H., Hunkapiller, M. W., Hood, L. E. & Todaro, G. J. 1984. 'Rat transforming growth factor type 1: structure and relation to epidermal growth factor.' *Science*, 223:4640, 1079-82.
- Martin, G. S. 1970. 'Rous sarcoma virus: a function required for the maintenance of the transformed state.' *Nature*, 227, 1021-23.
- Martin, L. P., Hamilton, T. C. & Schilder, R. J. 2008. 'Platinum resistance: the role of DNA repair pathways.' *Clin Cancer Res*, 14:5, 1291-5.
- Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. & Hanaoka, F. 1999. 'The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta.' *Nature*, 399:6737, 700-4.
- Mathew, C. G. 2006. 'Fanconi anaemia genes and susceptibility to cancer.' *Oncogene*, 25:43, 5875-84.

- Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R. 3rd, Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P. & Elledge, S. J. 2007. 'ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.' *Science*, 316:5828, 1160-6.
- Maxwell, J. A., Johnson, S. P., McLendon, R. E., Lister, D. W., Horne, K. S., Rasheed, A., Quinn, J. A., Ali-Osman, F., Friedman, A. H., Modrich, P. L., Bigner, D. D. & Friedman, H. S. 2008. 'Mismatch repair deficiency does not mediate clinical resistance to temozolomide in malignant glioma.' *Clin Cancer Res*, 14:15, 4859-68.
- McClellan, K. A., Ruzhynsky, V. A., Douda, D. N., Vanderluit, J. L., Ferguson, K. L., Chen, D., Bremner, R., Park, D. S., Leone, G. & Slack, R. S. 2007. 'Unique requirement for Rb/E2F3 in neuronal migration: evidence for cell cycle-independent functions.' *Mol Cell Biol*, 27:13, 4825-43.
- McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M. & Franklin, R. A. 2007. 'Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance.' *Biochim Biophys Acta*, 1773:8, 1263-84.
- McMurry, T. B. 2007. 'MGMT inhibitors--The Trinity College-Paterson Institute experience, a chemist's perception.' *DNA Repair (Amst)*, 6:8, 1161-9.
- Mechanic, L. E., Millikan, R. C., Player, J., de Cotret, A. R., Winkel, S., Worley, K., Heard, K., Tse, C. K. & Keku, T. 2006. 'Polymorphisms in nucleotide excision repair genes, smoking and breast cancer in African Americans and whites: a population-based case-control study.' *Carcinogenesis*, 27:7, 1377-85.
- Melisi, D., Ossovskaya, V., Zhu, C., Rosa, R., Ling, J., Dougherty, P. M., Sherman, B. M., Abbruzzese, J. L. & Chiao, P. J. 2009. 'Oral poly(ADP-ribose) polymerase-1 inhibitor BSI-401 has antitumor activity and synergizes with oxaliplatin against pancreatic cancer, preventing acute neurotoxicity.' *Clin Cancer Res*, 15:20, 6367-77.
- Mellon, I. 2005. 'Transcription-coupled repair: a complex affair.' *Mutat Res*, 577:1-2, 155-61.
- Mellon, I., Rajpal, D. K., Koi, M., Boland, C. R. & Champe, G. N. 1996. 'Transcription coupled repair deficiency and mutations in human mismatch repair genes.' *Science*, 272:5261, 557-60.
- Mendel, G. 1866. 'Versuche uber Pflanzenhybriden.' *Verh. Naturforsch. Ver. Brün*, 4, 3-47.
- Milas, L., Raju, U., Liao, Z. & Ajani, J. 2005. 'Targeting molecular determinants of tumor chemo-radioresistance.' *Semin Oncol*, 32:6 Suppl 9, S78-81.
- Mimori, T. & Hardin, J. A. 1986. 'Mechanism of interaction between Ku protein and DNA.' *J Biol Chem*, 261:22, 10375-9.
- Mirzoeva, O. K. & Petrini, J. H. 2001. 'DNA damage-dependent nuclear dynamics of the Mre11 complex.' *Mol Cell Biol*, 21:1, 281-8.
- Mizejewski, G. J. 1999. 'Role of integrins in cancer: survey of expression patterns.' *Proc Soc Exp Biol Med*, 222:2, 124-38.
- Mocellin, S., Verdi, D. & Nitti, D. 2009. 'DNA repair gene polymorphisms and risk of cutaneous melanoma: a systematic review and meta-analysis.' *Carcinogenesis*, 30:10, 1735-43.
- Mohd, A. B., Palama, B., Nelson, S. E., Tomer, G., Nguyen, M., Huo, X. & Buermeier, A. B. 2006. 'Truncation of the C-terminus of human MLH1 blocks intracellular

- stabilization of PMS2 and disrupts DNA mismatch repair.' *DNA Repair (Amst)*, 5:3, 347-61.
- Moreno-Bueno, G., Rodriguez-Perales, S., Sanchez-Estevez, C., Hardisson, D., Sarrío, D., Prat, J., Cigudosa, J. C., Matias-Guiu, X. & Palacios, J. 2003. 'Cyclin D1 gene (CCND1) mutations in endometrial cancer.' *Oncogene*, 22:38, 6115-8.
- Morgan, D. O. 2007. *The cell cycle: principles of control*. London: New Science Press Ltd.
- Morgan, M. T., Bennett, M. T. & Drohat, A. C. 2007. 'Excision of 5-halogenated uracils by human thymine DNA glycosylase. Robust activity for DNA contexts other than CpG.' *J Biol Chem*, 282:38, 27578-86.
- Mountzios, G., Dimopoulos, M. A. & Papadimitriou, C. 2008. "Excision repair cross-44 complementation group 1 enzyme as a molecular determinant of responsiveness to platinum-based chemotherapy for non small-cell lung cancer.' *Biomark Insights*, 3, 219-26.
- Mu, D., Hsu, D. S. & Sancar, A. 1996. 'Reaction mechanism of human DNA repair excision nuclease.' *J Biol Chem*, 271:14, 8285-94.
- Muller, H. J. 1927. 'Artificial transmutation of the gene.' *Science*, 66:1699, 84-7.
- Muntoni, A. & Reddel, R. R. 2005. 'The first molecular details of ALT in human tumor cells.' *Hum Mol Genet*, 14 Spec No. 2, R191-R96.
- Nambiar, M., Kari, V. & Raghavan, S. C. 2008. 'Chromosomal translocations in cancer.' *Biochim Biophys Acta*, 1786:2, 139-52.
- Nambiar, M. & Raghavan, S. C. 2011. 'How does DNA break during chromosomal translocations?' *Nucleic Acids Res*, 39:5813-25.
- Nehme, A., Baskaran, R., Nebel, S., Fink, D., Howell, S. B., Wang, J. Y. & Christen, R. D. 1999. 'Induction of JNK and c-Abl signalling by cisplatin and oxaliplatin in mismatch repair-proficient and -deficient cells.' *Br J Cancer*, 79:7-8, 1104-10.
- Nepal, R. M., Tong, L., Kolaj, B., Edelman, W. & Martin, A. 2009. 'Msh2-dependent DNA repair mitigates a unique susceptibility of B cell progenitors to c-Myc-induced lymphomas.' *Proc Natl Acad Sci USA*, 106:44, 18698-703.
- Nilausen, K. & Green, H. 1965. 'Reversible arrest of growth in G1 of an established fibroblast line (3T3).' *Exp Cell Res*, 40:1, 166-8.
- Nishigaki, M., Aoyagi, K., Danjoh, I., Fukaya, M., Yanagihara, K., Sakamoto, H., Yoshida, T. & Sasaki, H. 2005. 'Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays.' *Cancer Res*, 65:6, 2115-24.
- Nordling, C. O. 1953. 'A new theory on cancer-inducing mechanism.' *Br J Cancer*, 7:1, 68-72.
- Nowell, P. C. 2007. 'Discovery of the Philadelphia chromosome: a personal perspective.' *J Clin Invest*, 117:8, 2033-35.
- Nowell, P. C. & Hungerford, D. A. 1960. 'A minute chromosome in human chronic granulocytic leukemia.' *Science*, 132:3438, 1488-501.
- Nussenzweig, A. & Nussenzweig, M. C. 2010. 'Origin of chromosomal translocations in lymphoid cancer.' *Cell*, 141:1, 27-38.
- O'Brien, V. & Brown, R. 2006. 'Signalling cell cycle arrest and cell death through the MMR System.' *Carcinogenesis*, 27:4, 682-92.

- Oda, S., Maehara, Y., Ikeda, Y., Oki, E., Egashira, A., Okamura, Y., Takahashi, I., Kakeji, Y., Sumiyoshi, Y., Miyashita, K., Yamada, Y., Zhao, Y., Hattori, H., Taguchi, K., Ikeuchi, T., Tsuzuki, T., Sekiguchi, M., Karran, P. & Yoshida, M. A. 2005. 'Two modes of microsatellite instability in human cancer: differential connection of defective DNA mismatch repair to dinucleotide repeat instability.' *Nucleic Acids Res*, 33:5, 1628-36.
- Olovnikov, A. M. 1973. 'A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon.' *J Theor Biol*, 41:1, 181-90.
- Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D. & Cunha, G. R. 1999. 'Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium.' *Cancer Res*, 59:19, 5002-11.
- Overgaard, J. 2007. 'Hypoxic radiosensitization: adored and ignored.' *J Clin Oncol*, 25:26, 4066-74.
- Pacher, P., Liaudet, L., Bai, P., Virag, L., Mabley, J. G., Hasko, G. & Szabo, C. 2002. 'Activation of poly(ADP-ribose) polymerase contributes to development of doxorubicin-induced heart failure.' *J Pharmacol Exp Ther*, 300:3, 862-7.
- Painter, T. S. 1921. 'The Y-Chromosome in Mammals.' *Science*, 53:1378, 503-4.
- Pandey, V., Perry, J. K., Mohankumar, K. M., Kong, X. J., Liu, S. M., Wu, Z. S., Mitchell, M. D., Zhu, T. & Lobie, P. E. 2008. 'Autocrine human growth hormone stimulates oncogenicity of endometrial carcinoma cells.' *Endocrinology*, 149:8, 3909-19.
- Pardee, A. B. 1974. 'A restriction point for control of normal animal cell proliferation.' *Proc Natl Acad Sci USA*, 71:4, 1286-90.
- Park, J. I., Venteicher, A. S., Hong, J. Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., McLaughlin, M., Veenstra, T. D., Nusse, E., McCrea, P. D. & Artandi, S. E. 2009. 'Telomerase modulates Wnt signalling by association with target gene chromatin.' *Nature*, 460:7251, 66-72.
- Parker, J. B. & Stivers, J. T. 'Dynamics of uracil and 5-fluorouracil in DNA.' *Biochem*, 50:5, 612-7.
- Parker, R. C., Varmus, H. E. & Bishop, J. M. 1984. 'Expression of v-src and chicken c-src in rat cells demonstrates qualitative differences between pp60v-src and pp60c-src.' *Cell*, 37:1, 131-9.
- Parsons, R., Li, G. M., Longley, M. J., Fang, W. H., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K. W., Vogelstein, B. & Modrich, P. 1993. 'Hypermutability and mismatch repair deficiency in RER⁺ tumor cells.' *Cell*, 75:6, 1227-36.
- Patel, K. J., Yu, V. P., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A. & Venkitaraman, A. R. 1998. 'Involvement of Brca2 in DNA repair.' *Mol Cell*, 1:3, 347-57.
- Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M. & Bonner, W. M. 2000. 'A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.' *Curr Biol*, 10:15, 886-95.
- Peltomaki, P. 2003. 'Role of DNA mismatch repair defects in the pathogenesis of human cancer.' *J Clin Oncol*, 21:6, 1174-9.
- Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P. & Dedhar, S. 2001. 'Regulation of protein kinase B/Akt-serine 473

- phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343.' *J Biol Chem*, 276:29, 27462-9.
- Persad, S. & Dedhar, S. 2003. 'The role of integrin-linked kinase (ILK) in cancer progression.' *Cancer Metastasis Rev*, 22:4, 375-84.
- Pietenpol, J. A., Holt, J. T., Stein, R. W. & Moses, H. L. 1990. 'Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation.' *Proc Natl Acad Sci USA*, 87:10, 3758-62.
- Pollack, I. F., Hamilton, R. L., Sobol, R. W., Burnham, J., Yates, A. J., Holmes, E. J., Zhou, T. & Finlay, J. L. 2006. 'O6-methylguanine-DNA methyltransferase expression strongly correlates with outcome in childhood malignant gliomas: results from the CCG-945 Cohort.' *J Clin Oncol*, 24:21, 3431-7.
- Pouyssegur, J., Dayan, F. & Mazure, N. M. 2006. 'Hypoxia signalling in cancer and approaches to enforce tumour regression.' *Nature*, 441:7092, 437-43.
- Quelle, D. E., Cheng, M., Ashmun, R. A. & Sherr, C. J. 1997. 'Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF.' *Proc Natl Acad Sci USA*, 94:2, 669-73.
- Quelle, D. E., Zindy, F., Ashmun, R. A. & Sherr, C. J. 1995. 'Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest.' *Cell*, 83:6, 993-1000.
- Rajaraman, P., Hutchinson, A., Wichner, S., Black, P. M., Fine, H. A., Loeffler, J. S., Selker, R. G., Shapiro, W. R., Rothman, N., Linet, M. S. & Inskip, P. D. 2010. 'DNA repair gene polymorphisms and risk of adult meningioma, glioma, and acoustic neuroma.' *Neuro Oncol*, 12:1, 37-48.
- Rajewsky, M. F. & Muller, R. 2002. *DNA repair and the cell cycle as targets in cancer therapy*. New York: John Wiley and Sons, Ltd.
- Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J. C. & Perucho, M. 1997. 'Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype.' *Science*, 275:5302, 967-9.
- Ratnam, K. & Low, J. A. 2007. 'Current development of clinical inhibitors of poly(ADP17 ribose) polymerase in oncology.' *Clin Cancer Res*, 13:5, 1383-8.
- Regine, W. F., Patchell, R. A., Strottmann, J. M., Meigooni, A., Sanders, M. & Young, A. B. 2000. 'Preliminary report of a phase I study of combined fractionated stereotactic radiosurgery and conventional external beam radiation therapy for unfavorable gliomas.' *Int J Radiat Oncol Biol Phys*, 48:2, 421-6.
- Reya, T. & Clevers, H. 2005. 'Wnt signalling in stem cells and cancer.' *Nature*, 434:7035, 843-50.
- Reynolds, T. Y., Rockwell, S. & Glazer, P. M. 1996. 'Genetic instability induced by the tumor microenvironment.' *Cancer Res*, 56:24, 5754-7.
- Ribic, C. M., Sargent, D. J., Moore, M. J., Thibodeau, S. N., French, A. J., Goldberg, R. M., Hamilton, S. R., Laurent-Puig, P., Gryfe, R., Shepherd, L. E., Tu, D., Redston, M. & Gallinger, S. 2003. 'Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer.' *N Engl J Med*, 349:3, 247-57.
- Richardson, C., Moynahan, M. E. & Jasin, M. 1998. 'Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations.' *Genes Dev*, 12:24, 3831-42.

- Robinson, D. R., Wu, Y. M. & Lin, S. F. 2000. 'The protein tyrosine kinase family of the human genome.' *Oncogene*, 19:49, 5548-57.
- Rodriguez Fernandez, J. L., Geiger, B., Salomon, D., Sabanay, I., Zoller, M. & Ben-Ze'ev, A. 1992. 'Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA.' *J Cell Biol*, 119:2, 427-38.
- Rodriguez Fernandez, J. L., Geiger, B., Salomon, D. & Ben-Ze'ev, A. 1993. 'Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells.' *J Cell Biol*, 122:6, 1285-94.
- Rous, P. 1910. 'A transmissible avian neoplasm (sarcoma of the common fowl).' *J Exp Med*, 12:5, 696-705.
- Rous, P. 1911. 'A sarcoma of the fowl transmissible by an agent separable from the tumor cells.' *J Exp Med*, 13:4, 397-411.
- Rowley, J. D. 1973. 'A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining.' *Nature*, 243:5405, 290-3.
- Rowley, J. D. & Potter, D. 1976. 'Chromosomal banding patterns in acute nonlymphocytic leukemia.' *Blood*, 47:5, 705-21.
- Sage, E., Lamolet, B., Brulay, E., Moustacchi, E., Chteuneuf, A. & Drobetsky, E. A. 1996. 'Mutagenic specificity of solar UV light in nucleotide excision repair-deficient rodent cells.' *Proc Natl Acad Sci USA*, 93:1, 176-80.
- Sak, A., Stueben, G., Groneberg, M., Bocker, W. & Stuschke, M. 2005. 'Targeting of Rad51-dependent homologous recombination: implications for the radiation sensitivity of human lung cancer cell lines.' *Br J Cancer*, 92:6, 1089-97.
- Sandor, V., Bakke, S., Robey, R. W., Kang, M. H., Blagosklonny, M. V., Bender, J., Brooks, R., Piekarz, R. L., Tucker, E., Figg, W. D., Chan, K. K., Goldspiel, B., Fojo, A. T., Balcerzak, S. P. & Bates, S. E. 2002. 'Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms.' *Clin Cancer Res*, 8:3, 718-28.
- Santella, R. M., Gammon, M., Terry, M., Senie, R., Shen, J., Kennedy, D., Agrawal, M., Faraglia, B. & Zhang, F. 2005. 'DNA adducts, DNA repair genotype/phenotype and cancer risk.' *Mutat Res*, 592:1-2, 29-35.
- Sargent, D. J., Marsoni, S., Monges, G., Thibodeau, S. N., Labianca, R., Hamilton, S. R., French, A. J., Kabat, B., Foster, N. R., Torri, V., Ribic, C., Grothey, A., Moore, M., Zaniboni, A., Seitz, J. F., Sinicrope, F. & Gallinger, S. 2010. 'Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer.' *J Clin Oncol*, 28:20, 3219-26.
- Sarkaria, J. N., Tibbetts, R. S., Busby, E. C., Kennedy, A. P., Hill, D. E. & Abraham, R. T. 1998. 'Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin.' *Cancer Res*, 58:19, 4375-82.
- Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M. & Abraham, R. T. 1999. 'Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine.' *Cancer Res*, 59:17, 4375-82.
- Scharer, O. D. 2003. 'Chemistry and biology of DNA repair.' *Angew Chem Int Ed Engl*, 42:26, 2946-74.

- Schultz, L. B., Chehab, N. H., Malikzay, A. & Halazonetis, T. D. 2000. 'p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks.' *J Cell Biol*, 151:7, 1381-90.
- Schulze, A., Zerfass-Thome, K., Berges, J., Middendorp, S., Jansen-Durr, P. & Henglein, B. 1996. 'Anchorage-dependent transcription of the cyclin A gene.' *Mol Cell Biol*, 16:9, 4632-8.
- Seifert, M. & Reichrath, J. 2006. 'The role of the human DNA mismatch repair gene hMSH2 in DNA repair, cell cycle control and apoptosis: implications for pathogenesis, progression and therapy of cancer.' *J Mol Histol*, 37:5-7, 301-7.
- Seiple, L. A., Cardellina, J. H. 2nd, Akee, R. & Stivers, J. T. 2008. 'Potent inhibition of human apurinic/aprimidinic endonuclease 1 by arylstibonic acids.' *Mol Pharmacol*, 73:3, 669-77.
- Selivanova, G. 2001. 'Mutant p53: the loaded gun.' *Curr Opin Investig Drugs*, 2:8, 1136-41.
- Sellers, W. R., Novitch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., Lassar, A. B. & Kaelin, W. G. Jr. 1998. 'Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth.' *Genes Dev*, 12:1, 95-106.
- Seo, Y., Yan, T., Schupp, J. E., Yamane, K., Radivoyevitch, T. & Kinsella, T. J. 2006. 'The interaction between two radiosensitizers: 5-iododeoxyuridine and caffeine.' *Cancer Res*, 66:1, 490-8.
- Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. 1997. 'Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.' *Cell*, 88:5, 593-602.
- Shang, Y. L., Boderio, A. J. & Chen, P. L. 2003. 'NFBD1, a novel nuclear protein with signature motifs of FHA and BRCT, and an internal 41-amino acid repeat sequence, is an early participant in DNA damage response.' *J Biol Chem*, 278:8, 6323-9.
- Shen, J. C., Rideout, W. M. 3rd & Jones, P. A. 1994. 'The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA.' *Nucleic Acids Res*, 22:6, 972-6.
- Shen, L. & Issa, J. P. 2002. 'Epigenetics in colorectal cancer.' *Curr Opin Gastroenterol*, 18:1, 68-73.
- Sheng, J. Q., Chan, T. L., Chan, Y. W., Huang, J. S., Chen, J. G., Zhang, M. Z., Guo, X. L., Mu, H., Chan, A. S., Li, S. R., Yuen, S. T. & Leung, S. Y. 2006. 'Microsatellite instability and novel mismatch repair gene mutations in northern Chinese population with hereditary non-polyposis colorectal cancer.' *Chin J Dig Dis*, 7:4, 197-205.
- Sherr, C. J. & Roberts, J. M. 1999. 'CDK inhibitors: positive and negative regulators of G1-phase progression.' *Genes Dev*, 13:12, 1501-12.
- Shin, S. I., Freedman, V. H., Risser, R. & Pollack, R. 1975. 'Tumorigenicity of virus transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro.' *Proc Natl Acad Sci USA*, 72:11, 4435-9.
- Shiobara, M., Miyazaki, M., Ito, H., Togawa, A., Nakajima, N., Nomura, F., Morinaga, N. & Noda, M. 2001. 'Enhanced polyadenosine diphosphate-ribosylation in cirrhotic liver and carcinoma tissues in patients with hepatocellular carcinoma.' *J Gastroenterol Hepatol*, 16:3, 338-44.

- Short, S. C., Giampieri, S., Worku, M., Alcaide-German, M., Sioftanos, G., Bourne, S., Lio, K. I., Shaked-Rabi, M. & Martindale, C. 2011. 'Rad51 inhibition is an effective means of targeting DNA repair in glioma models and CD133+ tumor derived cells.' *Neuro Oncol*, 13:5, 487-99.
- Shrivastav, Li, D. & Essigmann, J. M. 2010. 'Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation.' *Carcinogenesis*, 31:1, 59-70.
- Siddik, Z. H. 2003. 'Cisplatin: mode of cytotoxic action and molecular basis of resistance.' *Oncogene*, 22:47, 7265-79.
- Sisken, J. E. & Morasca, L. 1965. 'Intrapopulation kinetics of the mitotic cycle.' *J Cell Biol*, 25:2, 179-89.
- Smith, G. C. & Jackson, S. P. 1999. 'The DNA-dependent protein kinase.' *Genes Dev*, 13:8, 916-34.
- Smith, J. A., Gaikwad, 1 A., Ramondetta, L. M., Wolf, J. K. & Brown, J. 2006. 'Determination of the mechanism of gemcitabine modulation of cisplatin drug resistance in panel of human endometrial cancer cell lines.' *Gynecol Oncol*, 103:2, 518-22.
- Sobol, R. W., Prasad, R., Evenski, A., Baker, A., Yang, X. P., Horton, J. K. & Wilson, S. H. 2000. 'The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity.' *Nature*, 405:6788, 807-10.
- Sorensen, C. S., Hansen, L. T., Dziegielewska, J., Syljuasen, R. G., Lundin, C., Bartek, J. & Helleday, T. 2005. 'The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair.' *Nat Cell Biol*, 7:2, 195-201.
- Stadler, L. J. 1928a. 'Genetic Effects of X-Rays in Maize.' *Proc Natl Acad Sci USA*, 14:1, 69-75.
- Stadler, L. J. 1928b. 'Mutations in Barley Induced by X-Rays and Radium.' *Science*, 68:1756, 186-7.
- Stark, J. M., Pierce, A. J., Oh, J., Pastink, A. & Jasin, M. 2004. 'Genetic steps of mammalian homologous repair with distinct mutagenic consequences.' *Mol Cell Biol*, 24:21, 9305-16.
- Steele, N., Finn, P., Brown, R. & Plumb, J. A. 2009. 'Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo.' *Br J Cancer*, 100:5, 758-63.
- Stehelin, D., Guntaka, R. V., Varmus, H. E. & Bishop, J. M. 1976a. 'Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses.' *J Mol Biol*, 101:3, 349-65.
- Stehelin, D., Varmus, H. E., Bishop, J. M. & Vogt, P. K. 1976b. 'DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA.' *Nature*, 260:5547, 170-3.
- Steinert, S., Shay, J. W. & Wright, W. E. 2000. 'Transient expression of human telomerase extends the life span of normal human fibroblasts.' *Biochem Biophys Res Commun*, 273:3, 1095-8.
- Stover, D. G., Bierie, B. & Moses, H. L. 2007. 'A delicate balance: TGF-beta and the tumor microenvironment.' *J Cell Biochem*, 101:4, 851-61.
- Strathdee, G., MacKean, M. J., Illand, M. & Brown, R. 1999. 'A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer.' *Oncogene*, 18:14, 2335-41.

- Stubbert, L. J., Smith, J. M. & McKay, B. C. 2010. 'Decreased transcription-coupled nucleotide excision repair capacity is associated with increased p53- and MLH1-independent apoptosis in response to cisplatin.' *BMC Cancer*, 10, 207.
- Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E. & Mirimanoff, R. O. 2005. 'Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.' *N Engl J Med*, 352:10, 987-96.
- Subauste, M. C., Nalbant, P., Adamson, E. D. & Hahn, K. M. 2005. 'Vinculin controls PTEN protein level by maintaining the interaction of the adherens junction protein beta catenin with the scaffolding protein MAGI-2.' *J Biol Chem*, 280:7, 5676-81.
- Sung, P., Krejci, L., Van Komen, S. & Sehorn, M. G. 2003. 'Rad51 recombinase and recombination mediators.' *J Biol Chem*, 278:44, 42729-32.
- Sutton, W. S. 1903. 'The chromosomes in heredity.' *Biological Bulletin*, 4, 231-51.
- Swift, H. 1950. 'The constancy of desoxyribose nucleic acid in plant nuclei.' *Proc Natl Acad Sci USA*, 36:11, 643-54.
- Tai, Y. T., Teoh, G., Lin, B., Davies, F. E., Chauhan, D., Treon, S. P., Raje, N., Hideshima, T., Shima, Y., Podar, K. & Anderson, K. C. 2000. 'Ku86 variant expression and function in multiple myeloma cells is associated with increased sensitivity to DNA damage.' *J Immunol*, 165:11, 6347-55.
- Tang, J. B., Svilar, D., Trivedi, R. N., Wang, X. H., Goellner, E. M., Moore, B., Hamilton, R. L., Banze, L. A., Brown, A. R. & Sobol, R. W. 2011. 'N-methylpurine DNA glycosylase and DNA polymerase {beta} modulate BER inhibitor potentiation of glioma cells to temozolomide.' *Neuro Oncol*, 13:5, 471-86.
- Tatter, S. B., Shaw, E. G., Rosenblum, M. L., Karvelis, K. C., Kleinberg, L., Weingart, J., Olson, J. J., Crocker, I. R., Brem, S., Pearlman, J. L., Fisher, J. D., Carson, K. & Grossman, S. A. 2003. 'An inflatable balloon catheter and liquid 125I radiation source (GliaSite Radiation Therapy System) for treatment of recurrent malignant glioma: multicenter safety and feasibility trial.' *J Neurosurg*, 99:2, 297-303.
- Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. & Leder, P. 1982. 'Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells.' *Proc Natl Acad Sci USA*, 79:24, 7837-41.
- Tentori, L., Graziani, G., Gilberti, S., Lacal, P. M., Bonmassar, E. & D'Atri, S. 1995. 'Triazene compounds induce apoptosis in O6-alkylguanine-DNA alkyltransferase deficient leukemia cell lines.' *Leukemia*, 9:111, 888-95.
- Tentori, L., Leonetti, C., Scarsella, M., Muzi, A., Vergati, M., Forini, O., Lacal, P. M., Ruffini, F., Gold, B., Li, W., Zhang, J. & Graziani, G. 2005. 'Poly(ADP-ribose) glycohydrolase inhibitor as chemosensitiser of malignant melanoma for temozolomide.' *Eur J Cancer*, 41:18, 2948-57.
- Teodoridis, J. M., Hall, J., Marsh, S., Kannall, H. D., Smyth, C., Curto, J., Siddiqui, N., Gabra, H., McLeod, H. L., Strathdee, G. & Brown, R. 2005. 'CpG island methylation of DNA damage response genes in advanced ovarian cancer.' *Cancer Res*, 65:19, 8961-7.
- Thiery, J. P. & Sleeman, J. P. 2006. 'Complex networks orchestrate epithelial-mesenchymal transitions.' *Nat Rev Mol Cell Biol*, 7:2, 131-42.

- Thompson, L. H. 2005. 'Unraveling the Fanconi anemia-DNA repair connection.' *Nat Genet*, 37:9, 921-2.
- Tijo, K. H. & Levan, A. 1956. 'The chromosome number of man.' *Hereditas*, 42, 1-6.
- Tobey, R. A. & Ley, K. D. 1970. 'Regulation of initiation of DNA synthesis in Chinese hamster cells. I. Production of stable, reversible G1-arrested populations in suspension culture.' *J Cell Biol*, 46:1, 151-7.
- Tokalov, S. V. & Abolmaali, N. D. 2010. 'Protection of p53 wild type cells from taxol by nutlin-3 in the combined lung cancer treatment.' *BMC Cancer*, 10, 57.
- Tornaletti, S., Patrick, S. M., Turchi, J. J. & Hanawalt, P. C. 2003. 'Behavior of T7 RNA polymerase and mammalian RNA polymerase II at site-specific cisplatin adducts in the template DNA.' *J Biol Chem*, 278:37, 35791-7.
- Toyokuni, S., Okamoto, K., Yodoi, J. & Hiai, H. 1995. 'Persistent oxidative stress in cancer.' *FEBS Lett*, 358:1, 1-3.
- Triolo, V. A. 1965. 'Nineteenth century foundations of cancer research advances in tumor pathology, nomenclature, and theories of oncogenesis.' *Cancer Res*, 25, 75-106.
- Tseng, R. C., Hsieh, F. J., Shih, C. M., Hsu, H. S., Chen, C. Y. & Wang, Y. C. 2009. 'Lung cancer susceptibility and prognosis associated with polymorphisms in the nonhomologous end-joining pathway genes: a multiple genotype-phenotype study.' *Cancer*, 115:13, 2939-48.
- Vaisman, A., Varchenko, M., Umar, A., Kunkel, T. A., Risinger, J. I., Barrett, J. C., Hamilton, T. C. & Chaney, S. G. 1998. 'The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts.' *Cancer Res*, 58:16, 3579-85.
- Vamvakas, S., Vock, E. H. & Lutz, W. K. 1997. 'On the role of DNA double-strand breaks in toxicity and carcinogenesis.' *Crit Rev Toxicol*, 27:2, 155-74.
- Van Cutsem, E., Labianca, R., Bodoky, G., Barone, C., Aranda, E., Nordlinger, B., Topham, C., Taberero, J., Andre, T., Sobrero, A. F., Mini, E., Greil, R., Di Costanzo, F., Collette, L., Cisar, L., Zhang, X., Khayat, D., Bokemeyer, C., Roth, A. D. & Cunningham, D. 2009. 'Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3.' *J Clin Oncol*, 27:19, 3117-25.
- Vassilev, L. T. 2005. 'p53 Activation by small molecules: application in oncology.' *J Med Chem*, 48:14, 4491-9.
- Vassilev, L. T. 2007. 'MDM2 inhibitors for cancer therapy.' *Trends Mol Med*, 13:1, 23-31.
- Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N. & Liu, E. A. 2004. 'In vivo activation of the p53 pathway by small-molecule antagonists of MDM2.' *Science*, 303:5659, 844-8.
- Villella, J., Marchetti, D., Odunsi, K., Rodabaugh, K., Driscoll, D. L. & Lele, S. 2004. 'Response of combination platinum and gemcitabine chemotherapy for recurrent epithelial ovarian carcinoma.' *Gynecol Oncol*, 95:3, 539-45.
- von Mehren, M. 2007. 'Trabectedin--a targeted chemotherapy?' *Lancet Oncol*, 8:7, 565-7.
- Waldeyer, W. 1888. 'Ueber karyokinese und ihre beziehungen zu den befruchtungsvorgängen.' *Arch Mikr Anat*, 32, 1-122.
- Wang, J. C. 1996. 'DNA topoisomerases.' *Annu Rev Biochem*, 65, 635-92.

- Wang, P., Tang, J. T., Peng, Y. S., Chen, X. Y., Zhang, Y. J. & Fang, J. Y. 2010. 'XRCC1 downregulated through promoter hypermethylation is involved in human gastric carcinogenesis.' *J Dig Dis*, 11:6, 343-51.
- Wang, S. Q., Setlow, R., Berwick, M., Polsky, D., Marghoob, A. A., Kopf, A. W. & Bart, R. S. 2001. 'Ultraviolet A and melanoma: a review.' *J Am Acad Dermatol*, 44:5, 837-46.
- Wang, W., Rastinejad, F. & El-Deiry, W. S. 2003. 'Restoring p53-dependent tumor suppression.' *Cancer Biol Ther*, 2:4 Suppl 1, S55-63.
- Wang, X. & D'Andrea, A. D. 2004. 'The interplay of Fanconi anemia proteins in the DNA damage response.' *DNA Repair (Amst)*, 3:8-9, 1063-9.
- Watanabe, Y., Koi, M., Hemmi, H., Hoshai, H. & Noda, K. 2001. 'A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer.' *Br J Cancer*, 85:7, 1064-9.
- Watson, J. D. 1972. 'Origin of concatemeric T7 DNA.' *Nat New Biol*, 239:94, 197-201.
- Williams, A. C., Collard, T. J. & Paraskeva, C. 1999. 'An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis.' *Oncogene*, 18:21, 3199-204.
- Wise, S. S., Elmore, L. W., Holt, S. E., Little, J. E., Antonucci, P. G., Bryant, B. H. & Wise, J. P. Sr. 2004. 'Telomerase-mediated lifespan extension of human bronchial cells does not affect hexavalent chromium-induced cytotoxicity or genotoxicity.' *Mol Cell Biochem*, 255:1-2, 103-11.
- Witkowski, J. A. 1980. 'Dr. Carrel's immortal cells.' *Med Hist*, 24:2, 129-42.
- Wong, D. J., Liu, H., Ridky, T. W., Cassarino, D., Segal, E. & Chang, H. Y. 2008. 'Module map of stem cell genes guides creation of epithelial cancer stem cells.' *Cell Stem Cell*, 2:4, 333-44.
- Workman, C. T., Mak, H. C., McCuine, S., Tagne, J. B., Agarwal, M., Ozier, O., Begley, T. J., Samson, L. D. & Ideker, T. 2006. 'A systems approach to mapping DNA damage response pathways.' *Science*, 312:5776, 1054-9.
- Wrensch, M., Kelsey, K. T., Liu, M., Miike, R., Moghadassi, M., Sison, J. D., Aldape, K., McMillan, A., Wiemels, J. & Wiencke, J. K. 2005. 'ERCC1 and ERCC2 polymorphisms and adult glioma.' *Neuro Oncol*, 7:4, 495-507.
- Xie, J., Guillemette, S., Peng, M., Gilbert, C., Buermeyer, A. & Cantor, S. B. 2010a. 'An MLH1 mutation links BACH1/FANCI to colon cancer, signaling, and insight toward directed therapy.' *Cancer Prev Res*, 3:11, 1409-16.
- Xie, K., Doles, J., Hemann, M. T. & Walker, G. C. 2010b. 'Error-prone translesion synthesis mediates acquired chemoresistance.' *Proc Natl Acad Sci USA*, 107:48, 20792-7.
- Xie, Y., Yang, H., Cunanan, C., Okamoto, K., Shibata, D., Pan, J., Barnes, D. E., Lindahl, T., McIlhatton, M., Fishel, R. & Miller, J. H. 2004. 'Deficiencies in mouse Myh and Ogg1 result in tumor predisposition and G to T mutations in codon 12 of the K-ras oncogene in lung tumors.' *Cancer Res*, 64:9, 3096-102.
- Xing, D. Y., Qi, J., Tan, W., Miao, X. P., Liang, G., Yu, C. Y., Lu, W. F., Zhou, C. N., Wu, M. & Lin, D. X. 2003. 'Association of genetic polymorphisms in the DNA repair gene XPD with risk of lung and esophageal cancer in a Chinese population in Beijing.' *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*, 20:1, 35-8.
- Yamada, M., O'Regan, E., Brown, R. & Karran, P. 1997. 'Selective recognition of a cisplatin-DNA adduct by human mismatch repair proteins.' *Nucleic Acids Res*, 25:3, 491-6.

- Yamaizumi, M. & Sugano, T. 1994. 'U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle.' *Oncogene*, 9:10, 2775-84.
- Yan, L., Bulgar, A., Miao, Y., Mahajan, V., Donze, J. R., Gerson, S. L. & Liu, L. 2007. 'Combined treatment with temozolomide and methoxyamine: blocking 40 apurinic/pyrimidinic site repair coupled with targeting topoisomerase II alpha.' *Clin Cancer Res*, 13:5, 1532-9.
- Yan, T., Berry, S. E., Desai, A. B. & Kinsella, T. J. 2003. 'DNA mismatch repair (MMR) mediates 6-thioguanine genotoxicity by introducing single-strand breaks to signal a G2-M arrest in MMR-proficient RKO cells.' *Clin Cancer Res*, 9:6, 2327-34.
- Yan, T., Desai, A. B., Jacobberger, J. W., Sramkoski, R. M., Loh, T. & Kinsella, T. J. 2004. 'CHK1 and CHK2 are differentially involved in mismatch repair-mediated 6-thioguanine-induced cell cycle checkpoint responses.' *Mol Cancer Ther*, 3:9, 1147-57.
- Yan, T., Schupp, J. E., Hwang, H. S., Wagner, M. W., Berry, S. E., Strickfaden, S., Veigl, M. L., Sedwick, W. D., Boothman, D. A. & Kinsella, T. J. 2001. 'Loss of DNA mismatch repair imparts defective cdc2 signaling and G(2) arrest responses without altering survival after ionizing radiation.' *Cancer Res*, 61:22, 8290-7.
- Yan, X., Fraser, M., Qiu, Q. & Tsang, B. K. 2006. 'Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis in a p53-dependent manner.' *Gynecol Oncol*, 102:2, 348-55.
- Yang, L., Mashima, T., Sato, S., Mochizuki, M., Sakamoto, H., Yamori, T., Oh-Hara, T. & Tsuruo, T. 2003. 'Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide.' *Cancer Res*, 63:4, 831-7.
- Yang, Q., Zhang, R., Wang, X. W., Linke, S. P., Sengupta, S., Hickson, I. D., Pedrazzi, G., Ferrera, C., Stagljar, I., Littman, S. J., Modrich, P. & Harris, C. C. 2004. 'The mismatch DNA repair heterodimer, hMSH2/6, regulates BLM helicase.' *Oncogene*, 23:21, 3749-56.
- Yeatman, T. J. 2004. 'A renaissance for SRC.' *Nat Rev Cancer*, 4:6, 470-80.
- Yoo, D. G., Song, Y. J., Cho, E. J., Lee, S. K., Park, J. B., Yu, J. H., Lim, S. P., Kim, J. M. & Jeon, B. H. 2008. 'Alteration of APE1/ref-1 expression in non-small cell lung cancer: the implications of impaired extracellular superoxide dismutase and catalase antioxidant systems.' *Lung Cancer*, 60:2, 277-84.
- Yoshioka, K., Yoshioka, Y. & Hsieh, P. 2006. 'ATR kinase activation mediated by MutSalpha and MutLalpha in response to cytotoxic O6-methylguanine adducts.' *Mol Cell*, 22:4, 501-10.
- Yu, Y., Wang, Y., Ren, X., Tsuyada, A., Li, A., Liu, L. J. & Wang, S. E. 2010. 'Context dependent bidirectional regulation of the MutS homolog 2 by transforming growth factor beta contributes to chemoresistance in breast cancer cells.' *Mol Cancer Res*, 8:12, 1633-42.
- Yuan, J., Narayanan, L., Rockwell, S. & Glazer, P. M. 2000. 'Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH.' *Cancer Res*, 60:16, 4372-6.
- Yung, W. K., Zhang, X., Steck, P. A. & Hung, M. C. 1990. 'Differential amplification of the TGF-alpha gene in human gliomas.' *Cancer Commun*, 2:6, 201-5.

- Zalutsky, M. R. & Pozzi, O. R. 2004. 'Radioimmunotherapy with alpha-particle emitting radionuclides.' *Q J Nucl Med Mol Imaging*, 48:4, 289-96.
- Zetterberg, A. & Larsson, O. 1985. 'Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells.' *Proc Natl Acad Sci USA*, 82:16, 5365-9.
- Zha, J., Harada, H., Yang, E., Jockel, J. & Korsmeyer, S. J. 1996. 'Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L).' *Cell*, 87:4, 619-28.
- Zhu, J., Wang, H., Bishop, J. M. & Blackburn, E. H. 1999. 'Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening.' *Proc Natl Acad Sci USA*, 96:7, 3723-8.

DNA Repair, Cancer and Cancer Therapy

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1. Introduction

The success of an organism to survive from one generation to the next is largely dependent upon the fidelity of replication of its genetic material, deoxyribonucleic acid (DNA). Unfortunately, DNA in living cell is labile and subject to many chemical alterations, and these alterations, if not corrected, can lead to diseases such as cancer (Fig. 1) (Pallis & Karamouzis, 2010). All eukaryotic cells have evolved a multifaceted response to counteract the potentially deleterious effects of DNA damage (Fig. 2). Upon sensing DNA damage, cell cycle checkpoints are activated to arrest cell cycle progression to allow time for repair before the damage is passed on to the next generation of cells. Depending on the type of damage, other cellular mechanisms such as transcriptional program activation, DNA repair pathways, and apoptosis can also be induced. All of these processes are coordinated so the genetic material is faithfully maintained, duplicated, and segregated within the cell. Important goals of cancer research are to determine the molecular mechanisms that are involved in the formation of genetic changes in human genes as a consequence of DNA mutations and to explain how cancer cells withstand and counteract DNA damage by the use of different defense mechanisms ranging from free radical scavengers to sophisticated DNA repair mechanisms.

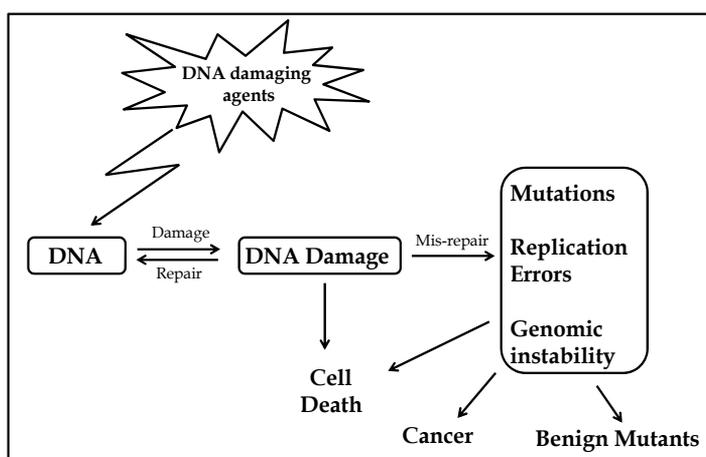


Fig. 1. General pathways linking DNA damage and cancer.

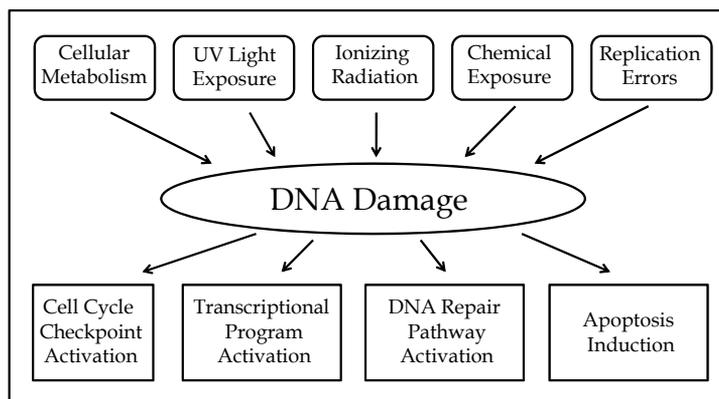


Fig. 2. DNA damage responses. When DNA is damaged by a variety of sources, the cellular response to damage involves activation of multiple processes in order to maintain genomic integrity.

Investigations into the regulation and the effects of DNA repair on tumor survival have expanded very rapidly in recent years. Research on targeting molecular pathways such as angiogenesis, DNA repair, and apoptosis is becoming one of the important areas in clinical oncology. Indeed, many pharmaceutical companies are developing inhibitors against DNA damage response pathways for cancer treatment.

2. Overview of DNA repair mechanisms

As a major defense system against DNA damage, DNA repair maintains genome fidelity that is essential to the health of the individual and to the reproductive success of a species. DNA repair is involved in many processes that minimize cell killing, mutations, replication errors, and genomic instability. Abnormalities in these processes have been implicated in cancer and other diseases (Preston et al., 2010). There are at least six different DNA repair pathways in mammalian systems, including base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end-joining (NHEJ), transcriptional-coupled repair (TCR), and mismatch repair (MMR). Disruption in these repair pathways can allow mutations to proliferate, leading to genomic instability. In fact, elevated levels of DNA repair proteins are often seen in drug-resistant tumor cells because a large number of conventional anti-cancer therapies are based on killing cancer cells through inducing DNA damage (Altaha et al., 2004; Drummond et al., 1996; Minn et al., 1995). Our understanding on the damage/errors repaired by each of these pathways has much improved from decades of intense biochemical and molecular genetic studies. Today we know that tumor cells respond differently to DNA damaging agent and their DNA repair activities vary. Thus, therapeutic targeting of specific components of the DNA repair pathways in cancer cells has become one of the major strategies in anti-cancer drug development.

2.1 Base excision repair (BER)

The BER pathway repairs base lesions and/or single-strand breaks (SSBs) induced by oxidative and alkylating agents in the DNA template. DNA glycosylases are responsible for initial recognition of the lesion. They flip the damaged base out of the double helix and cleave

the N-glycosidic bond of the damaged base, leaving an apurinic/aprimidinic (AP) site on damaged DNA strand. This site is identical to that generated by spontaneous depyrimidination or depurination. Six DNA glycosylases have been identified in humans to date - each excises an overlapping subset of either spontaneously formed (*e.g.*, hypoxanthine), oxidized (*e.g.*, 8-oxo-guanine), alkylated (*e.g.*, 3-methyladenine), or mismatched bases (Baute & Depicker, 2008). Then the AP endonucleases cleave an AP site to yield a 3'-hydroxyl adjacent to a 5'-deoxyribosephosphate. The resulting gap is subsequently filled by the 5'-deoxyribose-phosphodiesterase action of a DNA polymerase β and the strands are re-ligated by a DNA ligase. Defects in BER genes increase the mutation rate in a variety of organisms. For example, mutations in Pol β have been found in 30% of human cancers, and some of these mutations lead to transformation when expressed in mouse cells (Starcevic et al., 2004). Mutations in the DNA glycosylase MYH are also known to increase susceptibility to colon cancer (Kastrinos & Syngal, 2007).

2.2 Nucleotide excision repair (NER)

The NER is the predominant DNA repair pathway by which the cell maintains genomic integrity. It is responsible for removing a wide range of DNA damage, including UV-induced DNA cyclopurine dimers (CPDs), 6-4 photoproducts, and cisplatin induced DNA crosslinks (Ciccia & Elledge, 2010). There are 9 major proteins involved in NER in mammalian cells and their names come from the diseases associated with the deficiencies in those proteins. XPA, XPB, XPC, XPD, XPE, XPF, and XPG all derive from *Xeroderma pigmentosum* (XP) while CSA and CSB represent proteins linked to Cockayne syndrome (CS). In addition, other proteins are also found to participate in NER including ERCC1, RPA, PCNA, RAD23A, and RAD23B. There are four basic steps involved in NER: 1) Damage recognition, 2) Damage demarcation, 3) Incision, and 4) Repair patch synthesis and ligation. Two proteins, XPA and XPC-RAD23B, have been implicated in the damage recognition step, XPE has been shown to have a high affinity for damaged DNA, but whether it is required for the damage recognition step of NER remains unclear. CSA and CSB are mainly involved in the damage recognition step of the transcription-coupled repair. Once the DNA damage is recognized, XPB and XPD, which are subunits of transcription factor TF-IIH and have helicase activity, unwind the DNA at the sites of damages. XPF and ERCC1 form a protein complex which exhibits structure-specific endonuclease activity that is responsible for the 5' incision during the NER process. The 3' incision is made by the XPG protein and taken place prior to 5' incision. The resulting gap in DNA is then filled by DNA polymerases δ and ϵ . Proliferating cell nuclear antigen (PCNA) assists the DNA polymerases in the reaction, and replication protein A (RPA) protects the other DNA strand from degradation during NER. Finally DNA ligase seals the nicks to finish NER. One good example of targeting NER as an anti-cancer therapy is the use of cisplatin (Altaha et al., 2004; Balin-Gauthier et al., 2008; Prewett et al., 2007).

2.3 Homologous recombination (HR)

HR is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical DNA molecules. HR is most widely used by cells to repair potentially lethal double-strand breaks in DNA. HR produces new combinations of DNA sequences during meiosis and these new sequences represent genetic variation in offspring, which enables populations to adapt during the course of evolution. Although HR varies

among different organisms and cell types, most forms of HR share the same basic steps. After a double-strand break occurs, sections of DNA around the break on the 5'-end of the damaged chromosome are removed in a process called *resection*. In the *strand invasion* step that follows, an overhanging 3'-end of the damaged chromosome then "invades" an undamaged homologous chromosome. A mobile, cross-shaped intersection of four strands of DNA called a Holliday junction is formed between the two chromosomes after strand invasion. In the pathways of HR involved in DNA repair, a second Holliday junction forms. Depending on how the two junctions are resolved (*e.g.*, cut), the meiotic version of HR results in either chromosomal crossover or non-crossover. HR is also used in horizontal gene transfer to exchange genetic material between different strains and species of bacteria and viruses, and it has been targeted for cancer therapy (Helleday et al., 2005; Litman et al., 2005; Plo et al., 2008).

2.4 Non-homologous end-joining (NHEJ)

NHEJ is also a pathway that repairs double-strand breaks in DNA. NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template, in contrast to HR, which requires a homologous sequence to guide repair (Moore & Haber, 1996). NHEJ typically utilizes short homologous DNA sequences called microhomologies to guide repair. These microhomologies are often present in single-stranded overhangs on the ends of double-strand breaks. When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately (Boulton & Jackson, 1996; Budman & Chu, 2005; Moore & Haber, 1996; Wilson & Lieber, 1999). Imprecise repair leading to loss of nucleotides can also occur, but is much more common when the overhangs are not compatible. Inappropriate NHEJ can lead to translocations and telomere fusion, which are hallmarks of tumor cells (Espejel et al., 2002). NHEJ is evolutionarily conserved throughout all kingdoms of life and is the predominant double-strand break repair pathway in mammalian cells (Guirouilh-Barbat et al., 2004). In budding yeast (*Saccharomyces cerevisiae*), however, HR dominates when the organism is grown under common laboratory conditions.

When the NHEJ pathway is inactivated, double-strand breaks can be repaired by a more error-prone pathway called microhomology-mediated end joining (MMEJ). In this pathway, end resection reveals short microhomologies on either side of the break, which are then aligned to guide repair (McVey & Lee, 2008). This contrasts with classical NHEJ, which typically uses microhomologies already exposed in single-stranded overhangs on the double-strand breaks (DSBs) ends. Repair by MMEJ therefore leads to deletion of the DNA sequence between the microhomologies.

2.5 Transcriptional-coupled DNA repair (TCR)

The TCR pathway is an additional NER sub-pathway that allows for the preferential repair of transcription-blocking lesions on the transcribed strand of active genes (Tornaletti, 2009). It operates in tandem with transcription. DNA repair and transcription had long been considered as fully separable processes until recently when several discoveries showed that transcription could be coupled to the selective repair of the transcribed strand (Bohr et al., 1985; Hanawalt et al., 1994; Mellon & Hanawalt, 1989; Mellon et al., 1987). Failure of the TCR is the known cause of Cockayne syndrome (CS), an extreme form of accelerated aging that is fatal early in life (Sarker et al., 2005).

2.6 Mismatch repair (MMR)

MMR is primarily responsible for removing unpaired nucleotides. MMR discriminates between two strands so that the newly synthesized 'daughter' strand is repaired to match the 'parent' strand, rather than mutating the 'parent' strand to match the 'daughter' strand. MMR is a highly conserved process. First, MutS forms a dimer (MutS2) to recognize the mismatched base on the 'daughter' strand and binds the mutated DNA (Acharya et al., 2003). MutL then binds the MutS-DNA complex and recruit MutH to the damaged site. MutH subsequently binds and nicks the 'daughter' strand near the mismatched site and recruit an UvrD helicase (DNA helicase II) to separate the two strands with a specific 3' to 5' polarity. The entire MutSLH complex then slides along the DNA in the direction of the mismatch, liberating the strand to be excised as it goes. An exonuclease trails the complex and digests the ssDNA tail. The single-stranded gap created by the exonuclease is filled by DNA polymerase III using the other strand as a template. Finally the nicks are sealed by DNA ligase. Deficiencies in MMR are believed to account for almost all cases of hereditary nonpolyposis colon cancer (HNPCC) and many other cancers such as sporadic colorectal, endometrial, ovarian, gastric, and urothelial cancers, presumably due to the high rate of replication, which leads to the accumulation of DNA mismatches (Sancar, 1999). Mismatched nucleotides may arise from polymerase misincorporation errors, recombination between heteroallelic parental chromosomes, or chemical and physical damage to the DNA (Friedberg et al., 2006). MutS homologs (MSH) and MutL homologs (MLH/PMS) are highly conserved proteins that are essential for the mismatch repair (MMR) excision reaction (Kolodner et al., 2007). In human cells, hMSH2 and hMLH1 are the fundamental components of MMR. The hMSH2 protein forms a heterodimer with hMSH3 or hMSH6 and is required for mismatch/lesion recognition, whereas the hMLH1 protein forms a heterodimer with hMLH3 or hPMS2 and forms a ternary complex with MSH heterodimers to complete the excision repair reaction (Acharya et al., 2003; Kolodner et al., 2007). Human cells contain at least 10-fold more of the hMSH2-hMSH6/hMLH1-hPMS2 complex, which repairs single nucleotide and small insertion-deletion loop (IDL) mismatches, compared with the hMSH2-hMSH3/hMLH1-hMLH3 complex, which primarily repairs large IDL mismatches (Cannavo et al., 2005; Drummond et al., 1997; Raschle et al., 1999). In addition to MMR, the hMSH2-hMSH6/hMLH1-hPMS2 components have also been uniquely shown to recognize lesions in DNA and to signal cell cycle arrest and apoptosis (Fishel, 1999; Fishel, 2001).

3. Exploiting DNA damage response defects in cancer

In recent years, it has become evident that DNA damage responses are central for both the development and therapy of cancer. Defects in DNA damage response predispose to cancer by enhancing the accumulation of oncogenic mutations, and these mutations can provoke spontaneous DNA damage that suppresses the evolution of incipient cancer cells. Important goals of cancer research are to understand the molecular mechanisms by which cancers arise and to develop anti-cancer drugs that attack the Achilles' heel of cancer cells. Insight from understanding and targeting DNA damage response pathways has launched a new era in cancer therapy.

3.1 BRCA1/BRCA2 deficiencies

Breast cancer is the most common malignancy in women and an estimated 10% of the female population is affected by this disease (Alberg & Helzlsouer, 1997). About 5% of all breast

cancers are ascribed to hereditary predisposition. Extensive research efforts in the early 1990s have led to the identification of two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* (Futreal et al., 1994; Miki et al., 1994; Narod & Foulkes, 2004; Tavtigian et al., 1996; Wooster et al., 1995). Individuals carrying mutations in either one of the alleles will have a life-long high risk for either breast or ovarian cancers (Narod & Foulkes, 2004). Harmful *BRCA1* or *BRCA2* mutations may also increase a woman's risk of developing cervical, uterine, pancreatic, stomach, gallbladder, bile duct, melanoma, and colon cancers (Kadouri et al., 2007; Thompson & Easton, 2002). The likelihood that a breast and/or ovarian cancer is associated with a harmful mutation in *BRCA1* or *BRCA2* is highest in families with a history of multiple cases of breast cancer, cases of both breast and ovarian cancer, one or more family members with two primary cancers. However, it is important to note that most research related to *BRCA1* and *BRCA2* has been carried out on large families with many individuals affected by cancer. Thus, this risk estimate may not apply to general population. To date more than 600 mutations in the *BRCA1* and *BRCA2* genes are known. These mutations can be changes in one or a small number of DNA pairs or large rearrangements of DNA. Mutated BRCA proteins do not function properly. *BRCA1* is directly involved in the repair of damaged DNA by interacting with RAD51 to repair breaks in DNA. *BRCA2* has a similar function in repairing DNA. Defects in either or both proteins lead to unrepaired DNA damages in other genes. As these defects accumulate, they will allow cells to grow and divide in an uncontrollable manner and eventually form a tumor. Thus, direct or indirect targeting *BRCA1* or *BRCA2* and their interrelated pathways may have a significant clinical implication. For example, using a gene therapy to restore *BRCA1*'s tumor suppressor function in cancer cells in order to suppress tumor cell proliferation has been demonstrated (Tait et al., 1997). Preclinical and clinical findings indicated that restoration of normal function of *BRCA1* could have the therapeutic potential to inhibit tumor growth (Tait et al., 1999).

3.2 p53 mutations

p53 is a tumor suppressor protein encoded by the TP53 gene in humans (Isobe et al., 1986; Kern et al., 1991; Matlashewski et al., 1984; McBride et al., 1986). Mutations or inactivation of p53 is a universal feature of human cancers (Storey et al., 1998). As a transcription factor, p53 plays a critical role in apoptosis, genetic stability, and inhibition of angiogenesis (Farnebo et al., 2010; Gaiser et al., 2009; Strachan & Read, 1999). It is normally expressed at low levels so that it does not disrupt the cell cycle or induce the cell to undergo apoptosis. Thus its activity is mainly controlled by regulation of its protein expression levels mediated primarily by the ubiquitin ligase mouse double-minute 2 (MDM2), which targets p53 to the proteasome for degradation (Toledo & Wahl, 2006). It has been demonstrated that p53 becomes activated in response to a variety of stress types including DNA damage, oxidative stress, osmotic shock, ribonucleotide depletion, and deregulated oncogene expression (Han et al., 2008; Hollstein et al., 1991; Tyner et al., 2002). If the TP53 gene is mutated, tumor suppression will be severely reduced. High levels of mutant p53 protein are often observed in tumors (Bartek et al., 1991; Hassan et al., 2008; Iggo et al., 1990; Jonason et al., 1996; Lee et al., 2007; Rotter, 1983). Accumulation of mutant p53 has no correlation with tumor progression, however, it correlates well with increased metastasis (Morton et al., 2010). Previous studies suggest that ~50% of all human tumors overexpress a nonfunctional mutant p53 that accumulate to high concentrations in tumor cells (Brosh & Rotter, 2009; Brown et al., 2009; Nigro et al., 1989). Thus, targeting mutant p53 could be an extremely efficient strategy for selective killing of tumor cells (Mandinova & Lee, 2011).

3.3 BRCA-Fanconi Anemia (FA) pathway

Over the past few years, study of the rare inherited chromosome instability disorder, *Fanconi Anemia* (FA), has revealed a novel DNA damage response pathway, the BRCA-FA pathway. This pathway consists of *BRCA1*, *BRCA2*, and a network of at least 12 *FA* genes and is commonly inactivated in solid tumors (Thompson, 2005). Functional loss of the BRCA-FA pathway leads to increased cellular sensitivity to DNA damaging agents, defects in cell cycle checkpoints, and cancer predisposition (Litman et al., 2008). While the molecular function of the BRCA-FA protein complex remains unclear, evidence has suggested that the BRCA-FA protein complex is required to mediate the interstrand cross-link (ICL)-induced cellular response (Thompson, 2005). *FA* cells lacking any of the BRCA-FA proteins fail to respond to ICLs, which leads to cellular sensitivity and a prolonged accumulation of cells at the late S or G2/M checkpoint (Litman et al., 2008). Similarly, *BRCA1* mutant cells also fail to respond to ICLs by arresting DNA synthesis and are hypersensitive to ICLs, which causes profound genetic instability (Shen et al., 1998; Xu et al., 1999). Increased cancer risk has been observed in heterozygous carriers of *FA* gene mutations, in particular an increased susceptibility to breast and ovarian cancers (King et al., 2003). These observations suggest that the BRCA-FA pathway is important in the prevention of the female cancers and that unidentified mutations in *FA* genes may account for some familial breast/ovarian cancer pedigrees not accounted for by *BRCA1* or *BRCA2/FANCD1*. The association between abnormalities in the BRCA-FA pathway and cancer development may have important clinical implications as regards treatment. *FA* patients who are homozygous for mutation of a *FA* gene have a systemic DNA repair defect that results in a low tolerance for DNA-damaging chemotherapeutic agents. For this reason, chemotherapeutic agents are often given at low dosage or are avoided in favor of surgical approaches for these patients (Kutler et al., 2003). The situation, however, is different for cancer patients who carry a heterozygous mutation in a *FA* gene. In this scenario the tumor contains an abnormal *FA* pathway and would be predicted to be more DNA damage sensitive whereas the patient's other cells, such as those in the bone marrow, contain a functional pathway and would be relatively more DNA damage resistant. Consistent with this model *BRCA2/FANCD1* mutation carriers, with breast or ovarian cancer, demonstrate a high response to DNA-damaging chemotherapeutic agents (Cass et al., 2003; Chappuis et al., 2002). It remains to be seen if malignancies associated with heterozygosity for other *FA* gene mutations demonstrate the same level of chemo-sensitivity.

3.4 ATM/ATR

Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase activated by DNA double-strand breaks (DSBs) (Abraham, 2001). Its activity is increased 2-3 folds in response to DSBs. It phosphorylates several key proteins such as tumor suppressors p53, CHK2, and H2AX that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. ATM is recruited to DSBs by a trimeric complex of the three proteins MRE11/RAD50/NBS1. ATM directly interacts with the NBS1 subunit and phosphorylates the histone variant H2AX, generating binding sites for adaptor proteins with a BRCT domain. These adaptor proteins then recruit different factors including p53 and CHK2 to repair DSBs. ATM also phosphorylates MDM2 and p53, leading to stabilization and activation of p53 and subsequent transcription of numerous p53 target genes which eventually result in long-term cell-cycle arrest or apoptosis (Morgan, 2007). AT and most of other AT-like disorders are defective in ATM. Since one feature of the ATM

protein is its rapid increase in kinase activity immediately following DSBs, phosphorylating its substrates involved in DNA repair, apoptosis, G1/S, intra-S checkpoint, and G2/M checkpoints, gene regulation, translation initiation, and telomere maintenance (Kurz & Lees-Miller, 2004), a defect in ATM has severe consequences in repairing certain types of DNA damage, and cancer may result from improper DNA repair. For example, both leukemias and lymphomas are found to be associated with ATM defects (Chen, 2000). On the other hand, making ATM dysfunction can be an effective strategy for killing cancer cells. Many specific inhibitors of ATM have thus been developed for the treatment of cancer (Hickson et al., 2004).

The ATM- and Rad3-related (ATR) kinase is highly related to ATM (Abraham, 2001), and occupies a similar proximal position in checkpoint signaling cascades. ATM and ATR appear to phosphorylate many of the same substrates, though their functions are clearly distinct. ATR responds not only to DSBs, but also to damages caused by UV, cisplatin, hydroxyurea (HU), and stalled DNA synthesis (Abraham, 2001). Deficiency in ATR leads to a phenotype resembling mitotic catastrophe, suggesting an essential role for ATR in monitoring DNA replication (Hekmat-Nejad et al., 2000; Michael et al., 2000). ATR is constitutively bound to another protein, the ATR-interacting protein (ATRIP), which acts as a regulatory subunit for ATR. ATRIP cannot bind DNA without ATR and checkpoint activation requires both ATR and ATRIP, and possibly other proteins such as Rad9, Rad1, and Rad17/RFC complex (Abraham, 2001; Cortez et al., 2001). In addition, conditional deletion of ATR leads to G2 checkpoint defects and cell death (Cortez et al., 2001), indicating that ATR inhibition may be cytotoxic also to normal cells. However, short-term conditional expression of dominant negative ATR in human fibroblasts at a level that interfered with cell cycle checkpoint was not lethal (Cliby et al., 1998), raising the possibility that partial ATR inhibition may suppress checkpoints without causing cytotoxicity.

3.5 MMR deficiencies

The MMR pathway involves the removal of DNA base mismatches that arise during DNA replication or are caused by DNA damage. Mutations in seven known human MMR genes (*hMSH2*, *hMSH3*, *hMSH6*, *hMLH1*, *hMLH3*, *hPMS1*, and *hPMS2*) have been discovered, which lead to an inability to repair mismatches, causing an increased mutation rate and thus incidence of cancer. MMR deficiencies can be clearly observed in microsatellites - short tandem repetitive DNA sequences that are found throughout the genome (Laghi et al., 2008; Martin et al., 2010) and are predominantly linked to hereditary non-polyposis colorectal cancer (HNPCC), ovarian cancer, and leukemia (DeWeese et al., 1998; Pal et al., 2008; Whiteside et al., 2002). Cells mutated in either *hMSH2* or *hMLH1* have shown stronger mutator phenotypes and high microsatellite instability (MSI), which is often used as a marker for MMR deficiency (Jiricny & Nystrom-Lahti, 2000). A number of studies have also suggested a relationship between MMR deficiency and platinum-drug resistance (Aebi et al., 1996; Brown et al., 1997; Drummond et al., 1997; Lage & Dietel, 1999; Strathdee et al., 1999). However, the recent discovery that the MMR system plays an important role also in signaling the presence of DNA damage to the apoptotic machinery indicates that the function of MMR gene mutations may go beyond the mutator phenotype and MSI (D'Atri et al., 1998; Duckett et al., 1999; Hickman & Samson, 1999; Wu et al., 1999). Thus, MMR deficiency is likely to emerge as a frequent complication in the treatment of many types of cancers (Fleisher et al., 1999).

4. Targeting DNA repair pathways for cancer therapy

Radiation and genotoxic chemotherapies remain a mainstay of conventional cancer treatment and are likely to remain so for a foreseeable future. DNA damage responses are orchestrated by multiple signal transduction processes. Impaired DNA repair enables tumor cells to survive. Thus much current interest is focused on understanding how normal and tumor cells respond to DNA damage and determining whether DNA damage responses could be exploited or manipulated for therapeutic purposes.

DNA repair is a double-edged sword. First, deficiencies in DNA repair systems can lead to a higher incidence of cancer development; second, evidence also suggests that suppression of DNA repair capacity enhances the efficacy of conventional genotoxic anti-cancer agents, which has become an attractive strategy in anti-cancer therapeutics. Although promising, a full understanding of the biology and functions of the DNA repair pathways will be crucial for the future success of such approaches.

4.1 DNA damage checkpoint pathways

DNA damage checkpoints in the cell cycle serve as important barriers against cancer progression in human cells. Inhibition or inactivation of DNA damage checkpoint pathways can induce growth arrest, apoptosis and cellular senescence, and thus has been an attractive approach for cancer therapeutic interventions. The popular target proteins involved in these pathways are p53, ATM/ATR, and CHK1/CHK2. Efforts in targeting these proteins for therapeutic purposes are still in their infancy, and as understanding of the biological and molecular functions of these pathways becomes clearer, more effective and rational therapeutic strategies will likely emerge.

4.1.1 p53

Cancers have mutated *p53*. One approach to target *p53* pathway is to re-introduce wild-type *p53* via gene replacement. The desired outcome is a suppression of tumor growth and sensitization of the cancer cells against cytotoxic DNA damaging agents (Blagosklonny & El-Deiry, 1996; Meng & El-Deiry, 1998). Several adenovirus-based application of wild-type *p53* have moved into human clinical trials in combination with cisplatin or carboplatin, and the data from these clinical trials suggest that this gene-therapy approach may provide an effective strategy for selective killing of epithelial cancer cells (Seth et al., 1996). Another approach to target *p53* is the selective depletion of the mutant p53 protein. Geldanamycin (GA), a benzoquinone ansamycin, depletes mutant p53 in breast, prostate, and leukemia cell lines (An et al., 1997) and prevents nuclear translocation of mutant p53 (Dasgupta & Momand, 1997). 17AA-geldanamycin, a GA analog, has undergone a phase I clinical trial (Nowakowski et al., 2006). Additionally, efforts in restoring normal function of mutant *p53* using other approaches have been tested (Hietanen et al., 2000; Selivanova et al., 1999). A synthetic 22-mer peptide derived from the C-terminal domain of p53 has been shown to have the ability to restore the normal function of p53 in *p53*-mutant cell lines, leading to suppressed cell growth (Selivanova et al., 1999). Actinomycin D and leptomycin B also showed their activities in reactivating wt *p53* in cervical carcinoma cells (Hietanen et al., 2000). However, a potential problem with most of these approaches is to protect normal cells that harbor functioning p53. Often, intervention of one pathway can lead to the secondary inactivation of downstream components which may generate even more aggressive cancers as shown by Martins and co-workers (Martins et al., 2006).

4.1.2 ATM/ATR

The clinical use of ATM inhibitors is based on the rationale that ATM signaling is dysfunctional in tumor cells and inhibition of its activity would sensitize tumor cells to agents that cause DSBs. Two very specific ATM inhibitors, KU55933 and CP466722, have been shown to be able to effectively inhibit ATM function, reducing the phosphorylation of a wide range of ATM substrates such as p53, NBS1, H2AX, and SMC1 (Hickson et al., 2004) and rapidly sensitizing cancer cells to ionizing radiation (Rainey et al., 2008; White et al., 2008). The specificity and efficacy of both ATM inhibitors implies the potential of using these inhibitors as radiosensitizers in future cancer clinical trials.

The ATR kinase plays a role of monitoring the effect the damage has on DNA replication or transcription rather than sensing the damage directly (Derheimer et al., 2007; Jiang & Sancar, 2006). In this regard, inhibition of ATR could be cytotoxic to both tumor and normal cells, and the toxicity caused by inhibition of ATR to normal cells can be too severe to be used in clinical setting. Unlike ATM, there are no specific ATR inhibitors available. Considering that ATR may compensate partly for loss of ATM function, selective inhibition of ATR could preferentially sensitize ATM-deficient tumors (Zhou et al., 2003).

4.1.3 CHK1/CHK2

Both CHK1 and CHK2 are important members of protein kinases involved in DNA damage checkpoint control. Loss of CHK1 and/or CHK2 functions in combination with genotoxic therapeutic agents would allow the generation of lethal DNA lesions that could lead to apoptosis and cell death. CHK1 responds primarily to replication fork abnormalities through ATR-dependent phosphorylation, which activates an array of downstream events to elicit cell cycle arrest, preserve replication fork viability, activate DNA repair mechanisms, and terminate the activated checkpoint to resume cell division cycle. Numerous studies have revealed that CHK1 is overexpressed in various tumor cells and down-regulation of CHK1 leads to spontaneous cell death (Collis et al., 2007; Feng et al., 2008; Juvansuu et al., 2007; Leung-Pineda et al., 2009; Zhang et al., 2005; Zhang et al., 2009; Zhao & Piwnica-Worms, 2001). Thus, a strategy targeting the degradation of CHK1 in cancer cells would have a significant therapeutic implication in anti-cancer therapy.

CHK2 plays a similar role in DNA damage checkpoint pathways. Unlike CHK1, CHK2 is phosphorylated by ATM and is critical for DNA damage-induced apoptosis (Hirao et al., 2002; Takai et al., 2002). It regulates apoptosis in both ATM-dependent and ATM-independent manner (Hirao et al., 2002). Like p53, CHK2 is also a tumor suppressor and is highly expressed in both proliferating and differentiated normal tissues. Evidence has suggested that CHK2-p53 pathway is a determinant of the toxic side effects of anti-cancer treatment and CHK2 inhibitors may be very valuable for protecting tissues that are sensitive to DNA damage in patients with tumors that have a defective p53 pathway (Zhou et al., 2003). The therapeutic window of DNA-damaging therapies may be widened by CHK2 inhibitors via selective desensitization of normal cells.

To date, a number of CHK1 and CHK2 inhibitors have been developed (Collins & Garrett, 2005; Lin et al., 2006; Sorensen et al., 2003; Syljuasen et al., 2004; Wang et al., 2005). These compounds include G06976, isogranulutamide, SB-218078, urea, indolinones, XL844, and CEP-6367 (Collins & Garrett, 2005; Garber, 2005; Lin et al., 2006; Sorensen et al., 2003; Wang et al., 2005). However, the only known small-molecule inhibitor of CHK1 or CHK2 to enter clinical trial is XL844, which inhibits both CHK1 and CHK2 (Garber, 2005). While new checkpoint inhibitors are being developed, further understanding the functions of these

different tumor suppressors and checkpoint kinases in responding to DNA damage will better guide the use of selective checkpoint inhibitors in clinic.

4.2 Cell survival and proliferation pathways

Cell proliferation is governed by the cell cycle machinery which tightly controls cell cycle progression. Many kinases are involved in cell cycle regulation, including cyclin-dependent kinases (CDKs), PI-3 kinase, AKT, FOXO, EGFR, VGFR, and mTOR. The deregulation of many kinases is usually directly linked to cancer development. In solid tumors, changes in protein kinase expression levels and alterations in post-translational modifications can contribute to cancer and cancer progression. Thus, these kinases are often the targets for cancer therapeutic developments. In fact, protein kinase inhibitors are a major class of anti-cancer drugs.

4.2.1 BCL-2 family proteins

The B-cell lymphoma 2 (BCL-2) family proteins have been studied extensively for the past decade because their importance in apoptosis, tumorigenesis, and cellular responses to anti-cancer therapy (Adams & Cory, 1998). The interplay among BCL-2 family members integrates intracellular signals to maintain a balance between newly forming cells and old dying cells. When anti-apoptotic BCL-2 family members such as BCL-2 and BCL-XL are over-expressed, apoptotic cell death is prevented. In mammalian system, it has become evident that both BCL-2 and BCL-XL are over-expressed in many types of cancer cells (Chao & Korsmeyer, 1998; Motoyama et al., 1995; Veis et al., 1993). They inhibit apoptosis by interacting with Bax or Bak. Targeting the anti-apoptotic BCL-2 family of proteins has thus become a popular approach to improve apoptosis and overcome drug resistance to cancer chemotherapy (Del Poeta et al., 2003; Minn et al., 1995; Yoshino et al., 2006). The dysfunction of apoptosis can lead to disastrous consequences such as cancer cell proliferation. The initiator and effector caspases are the key players in apoptotic cascade (Motoyama et al., 1995; Veis et al., 1993). There are two major apoptotic pathways converge on the effector caspases: the intrinsic cell-death pathway (also known as the mitochondrial pathway) and the extrinsic cell-death pathway. The intrinsic pathway is activated by a wide range of signals including radiation, cytotoxic drugs, cellular stress, and growth factor withdrawal. The activation of Caspase-9 by mitochondria is a central checkpoint of apoptosis, which triggers a cascade of caspase activation (caspase-3, -6, and -7), resulting in the biochemical changes associated with apoptosis. In contrast, the extrinsic cell-death pathway functions independently of mitochondria and is activated by cell surface death receptors such as Fas and tumor necrosis factor related apoptosis inducing ligand (TRAIL) receptors (Wajant, 2002). To date, 25 members of the BCL-2 family of proteins have been identified and they all can be defined by the presence of conserved motifs known as BCL-2 homology domains (BH1 to BH4). Both BCL-2 and BCL-XL contain all four BH domains while other members may only contain BH1 and BH2. Heterodimerization of these domains is essential for the pro-apoptotic activity. Thus, disruption of the protein-protein interaction among these BCL-2 family members has been a focus of the development of BCL-2 inhibitors (Cao et al., 2001), even though some anti-sense drug has also been developed (*e.g.*, Oblimersen sodium) (Rai et al., 2008). At present, many agents have been designed to target the bcl-2 family members at the mRNA or protein level. Agents with high specificity may provide excellent opportunities for cancer treatment but unexpected systemic toxicities may also be a problem

if only one member of the bcl-2 family proteins is targeted (Kang & Reynolds, 2009). One approach to enhance therapeutic efficacy and reduce severe side effects is to inhibit multiple bcl-2 members using a combination of drugs.

4.2.2 EGFR

The epithelial growth factor receptor (EGFR) is a tyrosine kinase that participates in the regulation of cellular homeostasis. Following ligand binding, EGFR stimulates downstream signaling cascades such as the JAK/STAT pathway, the PI-3K/AKT pathway, the RAS/MAPK pathway, and the PKC pathway, influencing cell proliferation, apoptosis, migration, survival, and complex processes including angiogenesis and tumorigenesis (Nyati et al., 2006). EGFR is overexpressed in tumor cells, causing resistance to radiation and chemotherapeutic agents (Chakravarti et al., 2004; Liang et al., 2003; Milas et al., 2004). Thus, targeting EGFR for cancer treatment has been intensely pursued and a series of EGFR-targeting drugs has been developed and approved by FDA for clinical use, most noticeably Gefitinib, Panitumumab, Erlotinib, and Cetuximab (Ljunhman, 2009). Increased EGFR expression has been linked to poor clinical outcome in patients with breast, oropharyngeal HNSCC, and ovarian cancers (Lo et al., 2005; Psyrrri et al., 2005; Xia et al., 2009). Nuclear EGFR functions as a tyrosine kinase to phosphorylate and stabilize PCNA, and thus enhancing the proliferative potential of cancer cells (Wang et al., 2006). With its link to many different types of cancer, systematic laboratory and clinical research have facilitated the translation of EGFR inhibitors into common use in clinical oncology. For each new EGFR drug development, a complex series of preclinical and clinical tests have helped better understanding of the EGFR biology and advanced EGFR drug development in the both the laboratory and clinical settings.

4.2.3 CDKs

The Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases that regulate progression through each stage of the cell division cycle. In many cancers, CDKs are overactive or CDK-inhibiting proteins are not functional (Barriere et al., 2007; Malumbres & Barbacid, 2009). Thus, CDKs have been a major class of targets for deregulation in cancer cells to prevent unregulated proliferation of cancer cells. Two major cell cycle checkpoints are induced in response to DNA damage and take place before and after DNA synthesis during G1 and G2 phases. CHK1 and CHK2 are the two key transducers of these signaling pathways and they act indirectly on CDKs through their ability to inhibit members of the Cdc25 family of dual specificity phosphatases that dephosphorylate and activate CDKs (Bartek & Lukas, 2003). Roughly a dozen of CDK inhibitors have been developed to date (Collins & Garrett, 2005). Some are targeting multiple CDKs and others are targeting specific CDKs. However, the validity of these drug candidates should be carefully assessed because selectivity has been an issue. In addition, CDKs as effective anti-cancer targets may need to be re-evaluated, because genetic studies revealed that knockout of one specific type of CDK often does not affect proliferation of cells or has an effect only in specific tissue types (Malumbres & Barbacid, 2009). Furthermore, specific CDKs are only active in certain periods during the cell cycle. Therefore, the pharmacokinetics and dosing schedule of the candidate compound must be carefully evaluated to maintain active concentration of the drug throughout the entire cell cycle for cancer therapeutic purpose in clinical setting (Malumbres et al., 2008).

4.2.4 AMPK

AMP-activated protein kinase (AMPK) is an enzyme that plays a role in cellular energy homeostasis. AMPK is a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β -oxidation of fatty acids and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria (Bergeron et al., 1999; Durante et al., 2002; Ojuka, 2004; Thomson et al., 2007; Winder, 2001). Since its discovery, investigations into the regulation and the effects of AMPK have progressed very rapidly. Studies on the regulation of cellular proliferation by AMPK are becoming one of the critical areas in cancer research. Recent discoveries that three tumor-suppressors LKB1, p53, and TSC2 present either upstream (LKB1) or downstream (p53 and TSC2) have provided novel evidence that AMPK may function as a suppressor of cell proliferation. Thus inhibition of AMPK activity could lead to a suppressed cell proliferation. However, further studies are required for a full understanding of AMPK activation before it will emerge as an important target for the prevention and treatment of cancer.

4.2.5 PI-3K/AKT/mTOR

The PI-3K/AKT/mTOR pathway is frequently dysregulated in cancers (Cortot et al., 2006; LoPiccolo et al., 2008; Morgensztern & McLeod, 2005; Yap et al., 2008). PI-3 kinase activates AKT which subsequently activates mTOR. In many cancers, this pathway is overactive, reducing apoptosis and allowing proliferation. The phosphatase PTEN, which is often mutated or underexpressed in many cancer cells, negatively regulates this pathway via inhibiting PI-3K (Carnero et al., 2008). Importantly, hyperactivation of the PI-3K/AKT/mTOR pathway was found to be associated with resistance to radiation and chemotherapy (Jameel et al., 2004). Therefore it presents a promising therapeutic target for tumor sensitization. There has been a tremendous interest in developing novel drugs against this pathway. Many small-molecule inhibitors against PI-3K, AKT, and mTOR have been developed and tested in tumor cells (Carnero et al., 2008; Fasolo & Sessa, 2008; Franke, 2008; LoPiccolo et al., 2008; Marone et al., 2008; Nakamura et al., 2005; Steelman et al., 2008; Tokunaga et al., 2008; Yap et al., 2008). One unique feature of targeting this pathway is to target the apoptosis-protecting role of AKT without negating its HR-suppressing function (Plo et al., 2008). Such compounds could lead to a strong sensitization of cancer cells to treatments requiring HR such as IR, cisplatin, MMC, and PARP inhibitors.

4.2.6 VEGF

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells to stimulate vasculogenesis and angiogenesis. VEGF is a major regulator of blood vessel formation and function. It controls several processes in endothelial cells such as proliferation, survival, and migration. However, it is still unknown how these processes are coordinately regulated to result in more complex morphogenetic events such as tubular sprouting, fusion, and network formation. Over-expression of VEGF has been observed across a wide range of tumor types including colon, lung, breast, renal, glioblastoma, ovarian, and prostate cancers (Ferrara, 2004; Hicklin & Ellis, 2005; Margolin, 2002). Without blood vessels, the tumors cannot grow. For this reason, tumor angiogenesis has become a critical target for cancer therapy. Most common anti-VEGF strategies include ligand-binding with antibodies to prevent VEGF from binding to VEGF receptors (VEGFR1 and VEGFR2). Angiogenesis inhibitors targeting VEGF have shown antitumoral activity in preclinical and clinical trials. Currently available agents with established role include the anti-VEGF humanized mAb

bevacizumab, which is approved for the treatment of metastatic HER2/NEU-negative breast cancer (Miller et al., 2007). In many recent clinical trials, angiogenesis inhibitors were also being used in combination with conventional chemotherapy (Thanigaimani et al., 2010). One advantage of using angiogenesis inhibitors for cancer treatment is its low toxicity and less susceptibility to the induction of acquired drug resistance. However, like many other anti-cancer drugs, these inhibitors will need to be tested vigorously in the future clinical trials before they can be approved for use of cancer therapy alone because therapy with these inhibitors often does not prolong survival of cancer patients for more than months (Quesada et al., 2010).

4.2.7 HSP90

Heat shock protein 90 (HSP90) is a molecular chaperone involved in protein folding, cell signaling, and tumor repression. It is one of the most abundant proteins expressed in cells (Csermely et al., 1998). HSPs are a class of proteins that protect cells when stressed by elevated temperatures, dehydrating, or by other means. In this sense, HSPs seem to serve as biochemical buffers for the numerous genetic lesions that are characteristic of most human cancers. HSP90 is known to play a Janus-like role in the cell where it is essential for the creation, maintenance, and destruction of proteins. Its normal function is critical to maintaining the health of cells, whereas its dysregulation may lead to carcinogenesis. Cancerous cells over express a number of biologically critical proteins, including growth factor receptors, such as EGFR, or signal transduction proteins such as PI-3K and AKT (Lurje & Lenz, 2009). HSP90 stabilizes these proteins (Sawai et al., 2008), and loss of HSP90-mediated stabilization of these proteins selectively affects cancer cells (Mohsin et al., 2005; Stebbins et al., 1997). Another important role of HSP90 in cancer is the stabilization of mutant proteins such as v-Src, the fusion oncogene *Bcr/Abl*, and mutant forms of *p53* that appear during cell transformation (Calderwood et al., 2006). HSP90 is also required for induction of VEGF and nitric oxide synthase (NOS) (Fontana et al., 2002). Both are important for de novo angiogenesis that is required for tumor growth beyond the limit of diffusion distance of oxygen in tissues (Calderwood et al., 2006). HSP90 also promotes the invasion step of metastasis by assisting the matrix metalloproteinase MMP2 (Eustace et al., 2004). Together with its co-chaperones, HSP90 modulates tumor cell apoptosis mediated through effects on AKT (Sato et al., 2000), tumor necrosis factor receptors (TNFR) and nuclear factor- κ B (NF- κ B) function (Whitesell & Lindquist, 2005). Finally HSP90 participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis, and metastasis. Thus, as expected, the use of HSP90 inhibitors in cancer treatment has demonstrated its importance as a therapeutic target and shown promising effects in clinical trials. For example, the HSP90 inhibitor, geldanamycin has been used as an anti-tumor agent (Goetz et al., 2003). The drug was originally thought to function as a kinase inhibitor but was subsequently shown to be an HSP90 inhibitor where it uses a compact conformation to insert itself into the ATP binding site.

4.3 Accessory factor

Targeting DNA repair accessory factors as a therapeutic strategy has shown great promise for cancer treatment. The question remains as to whether these factors can be readily targeted, because many of them are multi-functional proteins involved in multiple pathways. For example, poly(ADP-ribose) polymerase (PARP) has recently emerged as one of the 'hot' anti-cancer targets. Inhibition of PARP impairs a tumor cell's DNA repair

activity, disabling its defense against DNA-damaging chemotherapy (Kling, 2009; Bryant & Helleday, 2004). Another DNA repair accessory factor, HMG-1, which is a specific marker of necrotic cell death, has also been suggested to facilitate protein-DNA and protein-protein interactions, enhancing effective binding of receptors such as progesterone receptor (PR) to its target DNA sequences and thus promoting cell survival (Onate et al., 1994). If inhibition of these factors can lead to cancer cell killing, it may provide clinically feasible opportunities for improved anti-cancer therapies. However, all the anti-cancer therapies targeting DNA repair pathways may also affect normal cells.

4.3.1 BRCA1/BRCA2

As stated earlier in this chapter, individuals with heterozygous, deleterious, germ line mutations in either *BRCA1* or *BRCA2* genes exhibit high life-time risks of developing breast, ovarian, and other types of cancer. A significant development in exploiting the DNA repair defect in *BRCA* mutant cells has been the use of synthetic lethality approaches. Cells lacking functional *BRCA1* or *BRCA2* have a deficiency in the repair of DSBs by HR. This deficiency results in the repair of these lesions by NHEJ or single-strand annealing (SSA) instead (Turner et al., 2005). Although it is still an area of intensive investigation, haplo-insufficiency phenotype remains a possibility for *BRCA1* and *BRCA2* mutant carriers (Santarosa & Ashworth, 2004). Agents that cause an increase in DSBs which are normally repaired by HR should selectively only affect *BRCA*-deficient cells, not normal cells (Tutt et al., 2006). This provides an ideal target for therapeutic intervention. Based on the concept that a lethal synthetic interaction between two genes occurs when mutation of either alone is compatible with viability, but mutation of both leads to cell death (Hartwell, 1997; Kaelin, 2005), a DNA repair protein, poly(ADP-ribose) polymerase (PARP), was identified as a synthetic lethal partner of *BRCA1* and *BRCA2* (Bryant et al., 2005; Farmer et al., 2005). Inhibition of this protein leads to severe and highly selective toxicity in *BRCA*-deficient cells. Similar results were obtained on xenografts and in animal models of spontaneous *BRCA2* loss of function (Bryant et al., 2005; Farmer et al., 2005; Hay et al., 2005). PARP inhibitors have been previously used as chemosensitizing and radiosensitizing agents. However, the use of these agents as therapeutic in the treatment of *BRCA*-deficient tumors is novel. There is still a great deal of research and development needed to be done before these PARP inhibitors can serve as medicine.

4.3.2 BRCA-FA

BRCA-FA pathway is essential for DNA damage response in cells. Loss of a functional *BRCA-FA* DNA damage response pathway, breast and ovarian tumors as well as leukemia can develop. *BRCA-FA* derived tumor cells must rely on alternative pathways for survival. Thus, to develop an effective therapeutic strategy, understanding specifically how these alternative pathways compensate for defects in the *BRCA-FA* pathway to promote survival is essential. The emerging role of *BRCA-FA* proteins in HR implies that tumor cells derived from mutations in these genes should have impaired HR. Several lines of evidence have supported this possibility, because *BRCA1*, *BRCA2*, *BRIP1/FANCI*, and *PALB2/FANCD1*-deficient cells all demonstrate defects in HR, consistent with a role for the *BRCA-FA* pathway in HR (Litman et al., 2005; Niedernhofer et al., 2005; Scully & Livingston, 2000; Venkitaraman, 2002; Xia et al., 2006). Thus there are opportunities to target the DNA repair defect in *BRCA-FA* tumors by increasing lesions repaired by HR. It was recently illustrated

that inhibiting BER increased the number of DSBs and also enhanced RAD51 foci formation, suggesting an increased activity in HR (Helleday et al., 2005). Based on this observation, it is reasonable to hypothesize that cells defective in HR would be sensitive to inhibition of BER. In fact, treatment of BRCA-FA-deficient cells with an inhibitor of the BER enzyme, PARP, leads to a dramatic reduction in cell survival, while BRCA-FA proficient cells were only mildly impacted by the PARP-inhibition (Helleday et al., 2005). Another important feature of targeting the BRCA-FA pathway is that cells defective in BRCA-FA are likely to repair DSBs by compensatory pathways such as NHEJ or SSA. For example, it was demonstrated that disruption of the NHEJ pathway cooperated with inactivation of the BRCA-FA pathway to enhance radiosensitivity in mouse embryonic fibroblasts (MEFs) (Houghtaling et al., 2005). Thus, by inactivating the BRCA-FA pathway, it may be possible to sensitize cancer cells that have become resistant to DNA cross-linking agents. The drug resistance and BRCA-FA pathway was also linked by a study in which cisplatin sensitive ovarian cancer cells developed cisplatin resistance by restoring expression of a previously silenced *FANCF* gene (Taniguchi et al., 2003). Similarly, restoring *BRCA2* gene expression in tumor cells leads to an acquired drug resistance to mytomycin C (MMC) (Chen et al., 2005). Thus, targeting BRCA-FA pathway may be effective in treating resistant tumors. However, directly targeting BRCA-FA pathway may be too toxic for most cells. It is conceivable that constitutive activation of the BRCA-FA pathway will be useful to dysregulate the pathway for therapeutic gain. In particular, deubiquitination of FANCD2 by USP1 could be targeted to reduce, but not to disable, the BRCA-FA pathway function. Clinically, it will be most beneficial if inhibitors of BRCA-FA pathway are used in combination with radiation and/or DNA cross-linkers.

4.3.3 Ribonucleotide reductase (RNR)

The ribonucleotide reductase (RNR, also known as ribonucleoside diphosphate reductase) is a ubiquitous radical-containing enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides, which are used in the synthesis of DNA (Elledge et al., 1992). The reaction catalyzed by RNR is strictly conserved in all living organisms (Torrents et al., 2002). Furthermore RNR plays a critical role in regulating the total rate of DNA synthesis so that DNA to cell mass is maintained at a constant ratio during cell division and DNA repair (Herrick & Sclavi, 2007). An unusual feature of the RNR enzyme is that it catalyzes a reaction that proceeds via a free radical mechanism of action (Eklund et al., 1997; Stubbe & Riggs-Gelasco, 1998). The levels and activity of RNR are highly regulated by the cell cycle and DNA checkpoints which maintain optimal dNTP pools required for genetic fidelity. The enzyme can be regulated by two factors: by transcription of the genes or by allosteric control of RNR by triphosphate effectors. When DNA damage occurs, a transcriptional induction of a new protein called p53R2, which is a p53-inducible RNR, is observed (Guittet et al., 2001; Shao et al., 2004). p53R2 has been shown to play an important role in supplying deoxyribonucleotides for DNA repair synthesis (Guittet et al., 2001; Tanaka et al., 2000; Yamaguchi et al., 2001) and the expression of p53R2 has been found to be up-regulated in various types of cancers (Devlin et al., 2008). Because inhibition of RNR has severe impact on DNA replication and repair, it makes an attractive target for cancer therapies. In fact, inhibition of p53R2 resulted in sensitization to both radiation and chemotherapeutic agents by attenuation of cell cycle checkpoints and enhanced apoptosis (Devlin et al., 2008; Wang et al., 2009; Yokomakura et al., 2007).

4.3.4 Thymidylate synthase

Thymidylate synthase (TS) is another important DNA repair accessory factor essential for DNA replication and repair. TS generates thymidine monophosphate (dTMP) which is subsequently phosphorylated to thymidine triphosphate (dTTP) for use in DNA synthesis and repair. TS is often found overexpressed in tumors and it has been suggested that TS overexpression promotes cell proliferation and resistance to radiation (Saga et al., 2002; Voeller et al., 2004). This makes TS an attractive cancer therapeutic target. Many TS inhibitors, such as fluorinated pyrimidine fluorouracil or certain folate analogues, have been developed and used in clinic for decades to treat advanced cancers (Clamp et al., 2008; Longley et al., 2003; Rustum, 2004; Showalter et al., 2008).

4.3.5 Proteasome

Proteasomes are very large protein complexes and act as a 'vacuum-cleaner' to degrade unneeded or damaged proteins by proteolysis. The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression, and responses to genotoxic stresses (Lodish et al., 2004). In response to cellular stresses such as infection, heat shock, or oxidative damage, heat shock proteins that identify misfolded or unfolded proteins and target them for proteasomal degradation are expressed. Both HSP27 and HSP90 have been implicated in increasing the activity of the ubiquitin-proteasome system, though they are not direct participants in the process (Garrido et al., 2006). HSP70, on the other hand, binds exposed hydrophobic patches on the surface of misfolded proteins and recruits E3 ubiquitin ligases such as CHIP to tag the proteins for proteasomal degradation (Park et al., 2007). Similar mechanisms exist to promote the degradation of oxidatively damaged proteins via the proteasome system. In particular, proteasomes localized to the nucleus are regulated by PARP and actively degrade inappropriately oxidized histones (Bader & Grune, 2006). Oxidized proteins, which often form large amorphous aggregates in the cell, can be degraded directly by the 20S core particle without the 19S regulatory cap and do not require ATP hydrolysis or tagging with ubiquitin (Shringarpure et al., 2003). However, high levels of oxidative damage increases the degree of cross-linking between protein fragments, rendering the aggregates resistant to proteolysis. Dysregulation of the ubiquitin proteasome system may contribute to several neural diseases. It may lead to brain tumors such as astrocytomas (Lehman, 2009). Proteasome inhibitors have effective anti-tumor activity in cell culture, inducing apoptosis by disrupting the regulated degradation of pro-growth cell cycle proteins (Adams et al., 1999). This approach of selectively inducing apoptosis in tumor cells has proven effective in animal models and human trials. Bortezomib, a molecule developed by Millennium Pharmaceuticals and marketed as Velcade, is the first proteasome inhibitor to reach clinical use as a chemotherapy agent. Bortezomib is used in the treatment of multiple myeloma (Fisher et al., 2006). Notably, multiple myeloma has been observed to result in increased proteasome levels in blood serum that decrease to normal levels in response to successful chemotherapy (Jakob et al., 2007). Studies in animals have indicated that bortezomib may also have clinically significant effects in pancreatic cancer (Nawrocki et al., 2004; Shah et al., 2001). Preclinical and early clinical studies have been started to examine bortezomib's effectiveness in treating other B-cell-related cancers (Schenkein, 2002), particularly some types of non-Hodgkin's lymphoma (O'Connor et al., 2005). The molecule ritonavir, marketed as Norvir, was developed as a protease inhibitor and used to target HIV infection. However, it has been shown to inhibit proteasomes as well as free proteases; to be specific,

the chymotrypsin-like activity of the proteasome is inhibited by ritonavir, while the trypsin-like activity is somewhat enhanced (O'Connor et al., 2005). Studies in animal models suggest that ritonavir may have inhibitory effects on the growth of glioma cells (Laurent et al., 2004). Proteasome inhibitors have also shown promise in treating autoimmune diseases in animal models. For example, studies in mice bearing human skin grafts found a reduction in the size of lesions from psoriasis after treatment with a proteasome inhibitor (Zollner, et al., 2002). Inhibitors also show positive effects in rodent models of asthma (Elliott et al., 1999). Labeling and inhibition of the proteasome is also of interest in laboratory settings for both *in vitro* and *in vivo* study of proteasomal activity in cells. The most commonly used laboratory inhibitors are lactacystin, a natural product synthesized by *Streptomyces* bacteria (Orlowski, 1999), and peptide MG132. Fluorescent inhibitors have also been developed to specifically label the active sites of the assembled proteasome (Verdoes et al., 2006).

4.3.6 MicroRNAs

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs of 18-25 nucleotides in length that regulate gene expression. Several miRNAs have been found to have links with some types of cancer (He et al., 2005; Mraz et al., 2009). MicroRNA-21 is one of the first microRNAs that was identified as an oncomiR. A study of mice altered to produce excess c-Myc – a protein with mutated forms implicated in several cancers – shows that miRNA has an effect on the development of cancer. Mice that were engineered to produce a surplus of types of miRNA found in lymphoma cells developed the disease within 50 days and died two weeks later. In contrast, mice without the surplus miRNA lived over 100 days (He et al., 2005). Leukemia can be caused by the insertion of a viral genome next to the 17-92 array of microRNAs leading to increased expression of this microRNA (Cui et al., 2007). Another study found that two types of miRNA inhibit the E2F1 protein, which regulates cell proliferation. miRNA appears to bind to messenger RNA before it can be translated to proteins that switch genes on and off (O'Donnell et al., 2005). By measuring activity among 217 genes encoding miRNA, patterns of gene activity that can distinguish types of cancers can be discerned. miRNA signatures may enable classification of cancer. This will allow doctors to determine the original tissue type which spawned a cancer and to be able to target a treatment course based on the original tissue type. miRNA profiling has already been able to determine whether patients with chronic lymphocytic leukemia had slow growing or aggressive forms of the cancer (Lu et al., 2005). Transgenic mice that over-express or lack specific miRNAs have provided insight into the role of small RNAs in various malignancies (Zanesi et al., 2010). A novel miRNA-profiling based screening assay for the detection of early-stage colorectal cancer has been developed and is currently in clinical trials. Early results showed that blood plasma samples collected from patients with early, resectable (Stage II) colorectal cancer could be distinguished from those of sex-and age-matched healthy volunteers. Sufficient selectivity and specificity could be achieved using small (less than 1 mL) samples of blood. The test has potential to be a cost-effective, non-invasive way to identify at-risk patients who should undergo colonoscopy (Nielsen et al., 2010).

5. Personalized medicine

Cancer is a multifaceted disease with many subtypes. Patients with identical clinical and pathological phenotypes often show different responses to the same therapy (Ely, 2009).

Today, numerous prescriptions written annually are ineffective in treating cancer patients (Phillips et al., 2001). This will increase the likelihood of overtreatment, the risk of adverse drug reactions (ADRs) in patients, and the costs of care for an individual patient (Ingelman-Sundberg, 2001). Thus, optimized treatments for individual patients will eventually lead to better clinical outcomes and patient satisfaction (Spears et al., 2001). Over the past decade, advances in genomics and proteomics have accelerated our understanding of individual differences in genetic makeup, allowing a more personalized approach to healthcare (Faratian et al., 2009; Marko-Varga et al., 2007; Phan et al., 2009; Yeatman et al., 2008). The prediction of treatment outcome based on an individual's biological information represents the future of oncology medicine – so called personalized medicine. Over the past ten years, personalized medicine has emerged as a rapidly advancing field in cancer patient care. Personalized medicine utilizes in-depth clinical, genomic, and proteomic information about an individual patient in order to determine which therapies will be safer and more effective for his/her care, matching the “right patients” to the “right drugs”. This new paradigm will no doubt improve health outcomes and patient satisfaction. However, decision making based on personalized biological information is far from simple. Understanding the probabilities, risk reduction, and short- and long-term consequences associated with each possible treatment based off the testing results is difficult. It requires not only accurate data collection and storage but also highly trained medical professionals to dissect the information and to use the complex data to make wise and effective treatment decisions. Even though many challenges are still ahead of us as clinical data continues to be generated and published. This new concept of personalized medicine will affect everyone in the cancer treatment community. How quickly the new integration of personal information into more effective health-care delivery occurs will largely depend on the development of predictive tools and the education of health-care providers. In addition to scientific and technological advances, personalized medicine also holds the promise of great cost-saving measures in health-care reform.

6. Future outlooks

As the DNA repair field continues to evolve, a better understanding of the DNA repair mechanisms and the players involved will certainly affect the development of anti-cancer therapies. Insights from understanding and targeting DNA damage response pathways have launched a new era in cancer therapy. As it appears, the weakness of tumor cells is that they either lack the ability to repair DNA damages or rely on other compensating DNA repair mechanisms for cell survival. Thus, new therapeutic development should focus on attacking these compensating pathways to compromise tumor cell viability, and this approach promises highly targeted therapies that potentially bypass the need for traditional radiation or chemical cancer therapies.

7. Conclusions

A rapid pace of discovery and development of anti-cancer therapy driven by new technologies makes cancer research into an exciting phase. Many previous studies have shed light on the complexity of tumor biology, showing that tumors rarely have similar sets of mutations in common. The fact that many tumors have defects in DNA repair pathways and/or cell cycle checkpoints presents unique opportunities for anti-cancer therapeutic

exploitation. With a better understanding of the mechanisms involved in DNA repair and DNA damage responses, tumor-specific therapies may be developed.

8. References

- Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases, *Genes & Development*, Vol. 15, No. 17, (September 2001), pp. 2177-2196, ISSN 0890-9369
- Acharya, S.; Foster, P. L.; Brooks, P. & Fishel, R. (2003). The coordinated functions of the E. coli MutS and MutL proteins in mismatch repair, *Molecular Cell*, Vol. 12, No. 1, (July 2003), pp. 233-246, ISSN 1097-2765
- Adams, J. M. & Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival, *Science*, Vol. 281, No. 5381, (August 1998), pp. 1322-1326, ISSN 0036-8075
- Adams, J.; Palombella, V. J.; Sausville, E. A.; Johnson, J.; Destree, A.; Lazarus, D. D.; Maas, J.; Pien, C. S.; Prakash, S. & Elliott, P. J. (1999). Proteasome inhibitors: a novel class of potent and effective antitumor agents, *Cancer Research*, Vol. 59, No. 11, (June 1999), pp. 2615-2622, ISSN 0008-5472
- Aebi, S.; Kurdi-Haidar, B.; Gordon, R.; Cenni, B.; Zheng, H.; Fink, D.; Christen, R. D.; Boland, C. R.; Koi, M.; Fishel, R. & Howell, S. B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin, *Cancer Research*, Vol. 56, No. 13, (July 1996), pp. 3087-3090, ISSN 0008-5472
- Alberg, A. J. & Helzlsouer, K. J. (1997). Epidemiology, prevention, and early detection of breast cancer, *Current Opinion in Oncology*, Vol. 9, No. 6, (November 1997), pp. 505-511, ISSN 1040-8746
- Altaha, R.; Liang, X.; Yu, J. J. & Reed, E. (2004). Excision repair cross complementing-group 1: gene expression and platinum resistance, *International Journal of Molecular Medicine*, Vol. 14, No. 6, (December 2004), pp. 959-970, ISSN 1107-3756
- An, W. G.; Schnur, R. C.; Neckers, L. M. & Blagosklonny, M. V. (1997). Depletion of p185erbB2, Raf-1 and mutant p53 proteins by geldanamycin derivatives correlates with antiproliferative activity, *Cancer Chemotherapy and Pharmacology*, Vol. 40, No. 1, (January 1997), pp. 60-64, ISSN 0344-5704
- Bader, N. & Grune, T. (2006). Protein oxidation and proteolysis, *Biological Chemistry*, Vol. 387, No. 10-11, (October-November 2006), pp. 1351-1355, ISSN 1431-6730
- Balin-Gauthier, D.; Delord, J. P.; Pillaire, M. J.; Rochaix, P.; Hoffman, J. S.; Bugat, R.; Cazaux, C.; Canal, P. & Allal, B. C. (2008). Cetuximab potentiates oxaliplatin cytotoxic effect through a defect in NER and DNA replication initiation, *British Journal of Cancer*, Vol. 98, No. 1, (January 2008), pp. 120-128, ISSN 0007-0920
- Barriere, C.; Santamaria, D.; Cerqueira, A.; Galan, J.; Martin, A.; Ortega, S.; Malumbres, M.; Dubus, P. & Barbacid, M. (2007). Mice thrive without Cdk4 and Cdk2, *Molecular Oncology*, Vol. 1, No. 1, (June 2007), pp. 72-83, ISSN 1574-7891
- Bartek, J.; Bartkova, J.; Vojtesek, B.; Staskova, Z.; Lukas, J.; Rejthar, A.; Kovarik, J.; Midgley, C. A.; Gannon, J. V. & Lane, D. P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies, *Oncogene*, Vol. 6, No. 9, (September 1991), pp. 1699-1703, ISSN 0950-9232
- Bartek, J. & Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer, *Cancer Cell*, Vol. 3, No. 5, (May 2003), pp. 421-429, ISSN 1535-6108

- Baute, J. & Depicker, A. (2008). Base excision repair and its role in maintaining genome stability, *Critical Reviews in Biochemistry and Molecular Biology*, Vol. 43, No. 4, (July-August 2008), pp. 239-276, ISSN 1040-9238
- Bergeron, R.; Russell, R. R.; Young, L. H.; Ren, J. M.; Marcucci, M.; Lee, A. & Shulman, G. I. (1999). Effect of AMPK activation on muscle glucose metabolism in conscious rats, *American Journal of Physiology*, Vol. 276, No. 5 Pt 1, (May 1999), pp. E938-944, ISSN 0002-9513
- Blagosklonny, M. V. & El-Deiry, W. S. (1996). In vitro evaluation of a p53-expressing adenovirus as an anti-cancer drug, *International Journal of Cancer*, Vol. 67, No. 3, (July 1996), pp. 386-392, ISSN 0020-7136
- Bohr, V. A.; Smith, C. A.; Okumoto, D. S. & Hanawalt, P. C. (1985). DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall, *Cell*, Vol. 40, No. 2, (February 1985), pp. 359-369, ISSN 0092-8674
- Boulton, S. J. & Jackson, S. P. (1996). Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways, *The EMBO Journal*, Vol. 15, No. 18, (September 1996), pp. 5093-5103, ISSN 0261-4189
- Brosh, R. & Rotter, V. (2009). When mutants gain new powers: News from the mutant p53 field, *Nature Reviews. Cancer*, Vol. 9, No. 10, (October 2009), pp. 701-713, ISSN 1474-175X
- Brown, C. J.; Lain, S.; Verma, C. S.; Fersht, A. R. & Lane, D. P. (2009). Awakening guardian angels: Drugging the p53 pathway, *Nature Reviews. Cancer*, Vol. 9, No. 12, (December 2009), pp. 862-873, ISSN 1474-175X
- Brown, R.; Hirst, G. L.; Gallagher, W. M.; McIlwrath, A. J.; Margison, G. P.; van der Zee, A. G. & Anthoney, D. A. (1997). hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents, *Oncogene*, Vol. 15, No. 1, (July 1997), pp. 45-52, ISSN 0950-9232
- Bryant, H. E. & Helleday, T. (2004). Poly(ADP-ribose) polymerase inhibitors as potential chemotherapeutic agents, *Biochemical Society Transactions*, Vol. 32, No. Pt. 6, (December 2004), pp. 959-961, ISSN 0300-5127
- Bryant, H. E.; Schultz, N.; Thomas, H. D.; Parker, K. M.; Flower, D.; Lopez, E.; Kyle, S.; Meuth, M.; Curtin, N. J. & Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase, *Nature*, Vol. 434, No. 7035, (April 2005), pp. 913-917, ISSN 0028-0836
- Budman, J. & Chu, G. (2005). Processing of DNA for nonhomologous end-joining by cell-free extract, *The EMBO Journal*, Vol. 24, No. 4, (February 2005), pp. 849-860, ISSN 0261-4189
- Calderwood, S. K.; Khaleque, M. A.; Sawyer, D. B. & Ciocca, D. R. (2006). Heat shock proteins in cancer: chaperones of tumorigenesis, *Trends in Biochemical Sciences*, Vol. 31, No. 3, (March 2006), pp. 164-172, ISSN 0968-0004
- Cannavo, E.; Marra, G.; Sabates-Bellver, J.; Menigatti, M.; Lipkin, S. M.; Fischer, F.; Cejka, P. & Jiricny, J. (2005). Expression of the MutL homologue hMLH3 in human cells and its role in DNA mismatch repair. *Cancer Research*, Vol. 65, No. 23, (December 2005), pp. 10759-10766, ISSN 0008-5472

- Cao, X. X.; Mohiuddin, I.; Ece, F.; McConkey, D. J. & Smythe, W. R. (2001). Histone deacetylase inhibitor downregulation of bcl-xl gene expression leads to apoptotic cell death in mesothelioma, *American Journal of Respiratory Cell and Molecular Biology*, Vol. 25, No. 5, (November 2001), pp. 562-568, ISSN 1044-1549
- Carnero, A.; Blanco-Aparicio, C.; Renner, O.; Link, W. & Leal, J. F. (2008). The PTEN/PI3K/AKT signaling pathway in cancer, therapeutic implications, *Current Cancer Drug Targets*, Vol. 8, No. 3, (May 2008), pp. 187-198, ISSN 1568-0096
- Cass, I.; Baldwin, R. L.; Varkey, T.; Moslehi, R.; Narod, S. A. & Karlan, B.Y. (2003). Improved survival in women with BRCA-associated ovarian carcinoma, *Cancer*, Vol. 97, No. 9, (May 2003), pp. 2187-2195, ISSN 1097-0142
- Chakravarti, A.; Dicker, A. & Mehta, M. (2004). The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data, *International Journal of Radiation Oncology, Biology, Physics*, Vol. 58, No. 3, (March 2004), pp. 927-931, ISSN 0360-3016
- Chao, D. T. & Korsmeyer, S. J. (1998). BCL-2 family: regulators of cell death, *Annual Review of Immunology*, Vol. 16, (1998), pp. 395-419, ISSN 0732-0582
- Chappuis, P. O.; Goffin, J.; Wong, N.; Perret, C.; Ghadirian, P.; Tonin, P. N. & Foulkes, W. D. (2002). A significant response to neoadjuvant chemotherapy in BRCA1/2 related breast cancer, *Journal of Medical Genetics*, Vol. 39, No. 8, (August 2002), pp. 608-610, ISSN 1468-6244
- Chen, J. (2000). Ataxia telangiectasia-related protein is involved in the phosphorylation of BRCA1 following deoxyribonucleic acid damage, *Cancer Research*, Vol. 60, No. 18, (September 2000), pp. 5037-5039. ISSN 0008-5472
- Chen, Q.; Van der Sluis, P. C.; Boulware, D.; Hazlehurst, L. A. & Dalton, W.S. (2005). The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells, *Blood*, Vol. 106, No. 2, (July 2005), pp. 698-705, ISSN 0006-4971
- Ciccia, A. & Elledge, S. J. (2010). The DNA damage response: making it safe to play with knives, *Molecular Cell*, Vol. 40, No. 2, (October 2010), pp. 179-204, ISSN 1097-2765
- Clamp, A. R.; Schoffski, P.; Valle, J. W.; Wilson, R. H.; Marreaud, S.; Govaerts, A. S.; Debois, M.; Lacombe, D.; Twelves, C.; Chick, J. & Jayson, G. C. (2008). A phase I and pharmacokinetic study of OSI-7904L, a liposomal thymidylate synthase inhibitor in combination with oxaliplatin in patients with advanced colorectal cancer, *Cancer Chemotherapy and Pharmacology*, Vol. 61, No. 4, (April 2008), pp. 579-585, ISSN 0344-5704
- Cliby, W. A.; Roberts, C. J.; Cimprich, K. A.; Stringer, C. M.; Lamb, J. R.; Schreiber, S. L. & Friend, S. H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints, *The EMBO Journal*, Vol. 17, No. 1, (January 1998), pp. 159-169, ISSN 0261-4189
- Collins, I. & Garrett, M. D. (2005). Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors, *Current Opinion in Pharmacology*, Vol. 5, No. 4, (August 2005), pp. 366-373, ISSN 1471-4892
- Collis, S. J.; Barber, L. J.; Clark, A. J.; Martin, J. S.; Ward, J. D. & Boulton, S. J. (2007). HCLK2 is essential for the mammalian S-phase checkpoint and impacts on Chk1 stability, *Nature Cell Biology*, Vol. 9, No. 4, (April 2007), pp. 391-401, ISSN 1465-7392

- Cortez, D.; Guntuku, S.; Qin, J. & Elledge, S. J. (2001). ATR and ATRIP: partners in checkpoint signaling, *Science*, Vol. 294, No. 5547, (November 2001), pp. 1713-1716, ISSN 0036-8075
- Cortot, A.; Armand, J. P. & Soria, J. C. (2006). PI3K-AKT-mTOR pathway inhibitors (in French), *Bulletin Du Cancer*, Vol. 93, No. 1, (January 2006), pp. 19-26, ISSN 0007-4551
- Csermely, P.; Schnaider, T.; Soti, C.; Prohászka, Z. & Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review, *Pharmacology & Therapeutics*, Vol. 79, No. 2, (August 1998), pp. 129-168, ISSN 0163-7258
- Cui, J. W.; Li, Y. J.; Sarkar, A.; Brown, J.; Tan, Y. H.; Premyslova, M.; Michaud, C.; Iscove, N.; Wang, G. J. & Ben-David, Y. (2007). Retroviral insertional activation of the Fli-3 locus in erythroleukemias encoding a cluster of microRNAs that convert Epo-induced differentiation to proliferation, *Blood*, Vol. 110, No. 7, (June 2007), pp. 2631-2640, ISSN 0006-4971
- Dasgupta, G. & Momand, J. (1997). Geldanamycin prevents nuclear translocation of mutant p53, *Experimental Cell Research*, Vol. 237, No. 1, (November 1997), pp. 29-37, ISSN 0014-4827
- D'Atri, S.; Tentori, L.; Lacal, P. M.; Graziani, G.; Pagani, E.; Benincasa, E.; Zambruno, G.; Bonmassar, E. & Jiricny, J. (1998). Involvement of the mismatch repair system in temozolomide-induced apoptosis, *Molecular Pharmacology*, Vol. 54, No. 2, (August 1998), pp. 334-341, ISSN 0026-895X
- Del Poeta, G.; Venditti, A.; Del Principe, M. I.; Maurillo, L.; Buccisano, F.; Tamburini, A.; Cox, M. C.; Franchi, A.; Bruno, A.; Mazzone, C.; Panetta, P.; Suppo, G.; Masi, M. & Amadori, S. (2003). Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML), *Blood*, Vol. 101, No. 6, (March 2003), pp. 2125-2131, ISSN 0006-4971
- Derheimer, F. A.; O'Hagan, H. M.; Krueger, H. M.; Hanasoge, S.; Paulsen, M. T. & Ljungman, M. (2007). RPA and ATR link transcription stress to p53, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 104, No. 31, (July 2007), pp. 12778-12783, ISSN 0027-8424
- Devlin, H. L.; Mack, P. C.; Burich, R. A.; Gumerlock, P. H.; Kung, H. J.; Mudryj, M. & deVere White, R. W. (2008). Impairment of the DNA repair and growth arrest pathways by p53R2 silencing enhances DNA damage-induced apoptosis in a p53-dependent manner in prostate cancer cells, *Molecular Cancer Research*, Vol. 6, No. 5, (May 2008), pp. 808-818, ISSN 1541-7786
- DeWeese, T. L.; Shipman, J. M.; Larrier, N. A.; Buckley, N. M.; Kidd, L. R.; Groopman, J. D.; Cutler, R. G.; te Riele, H. & Nelson, W. G. (1998). Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, No. 20, (September 1998), pp. 11915-11920, ISSN 0027-8424
- Drummond, J. T.; Anthoney, A.; Brown, R. & Modrich, P. (1996). Cisplatin and adriamycin resistance are associated with MutL α and mismatch repair deficiency in an ovarian tumor cell line, *Journal of Biological Chemistry*, Vol. 271, No. 33, (August 1996), pp. 19645-19648, ISSN 0021-9258

- Drummond, J. T.; Genschel, J.; Wolf, E. & Modrich, P. (1997). DHFR/MSH3 amplification in methotrexate-resistant cells alters the hMutSalpha/hMutSbeta ratio and reduces the efficiency of base-base mismatch repair. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 94, No. 19, (September 1997), pp. 10144-10149, ISSN 0027-8424
- Duckett, D. R.; Bronstein, S. M.; Taya, Y. & Modrich, P. (1999). hMutSalpha- and hMutLalpha-dependent phosphorylation of p53 in response to DNA methylator damage, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 96, No. 22, (October 1999), pp. 12384-12388, ISSN 0027-8424
- Durante, P. E.; Mustard, K. J.; Park, S. H.; Winder, W. W. & Hardie, D. G. (2002). Effects of endurance training on activity and expression of AMP-activated protein kinase isoforms in rat muscles, *American Journal of Physiology. Endocrinology and Metabolism*, Vol. 283, No. 1, (July 2002), pp. E178-186, ISSN 0193-1849
- Eklund, H.; Eriksson, M.; Uhlin, U.; Nordlund, P. & Logan, D. (1997). Ribonucleotide reductase--structural studies of a radical enzyme, *Biological Chemistry*, Vol. 378, No. 8, (August 1997), pp. 821-825, ISSN 1431-6730
- Elledge, S. J.; Zhou, Z. & Allen, J. B. (1992). Ribonucleotide reductase: regulation, regulation, regulation. *Trends in Biochemical Sciences*, Vol. 17, No. 3, (March 1992), pp. 119-123, ISSN 0968-0004
- Elliott, P. J.; Pien, C. S.; McCormack, T. A.; Chapman, I. D. & Adams, J. (1999). Proteasome inhibition: A novel mechanism to combat asthma, *Journal of Allergy and Clinical Immunology*, Vol. 104, No. 2 Pt 1, (August 1999), pp. 294-300, ISSN 0091-6749
- Ely, S. (2009). Personalized medicine: individualized care of cancer patients, *Translational Research*, Vol. 154, No. 6, (December 2009), pp. 303-308, ISSN 1931-5244
- Espejel, S.; Franco, S.; Rodriguez-Perales, S.; Bouffler, S. D.; Cigudosa, J. C. & Blasco, M. A. (2002). Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres, *The EMBO Journal*, Vol. 21, No. 9, (May 2002), pp. 2207-2219, ISSN 0261-4189
- Eustace, B. K.; Sakurai, T.; Stewart, J. K.; Yimlamai, D.; Unger, C.; Zehetmeier, C.; Lain, B.; Torella, C.; Henning, S. W.; Beste, G.; Scroggins, B. T.; Neckers, L.; Ilag, L. L. & Jay, D. G. (2004). Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness, *Nature Cell Biology*, Vol. 6, No. 6, (June 2004), pp. 507-514, ISSN 1465-7392
- Faratian, D.; Goltsov, A.; Lebedeva, G.; Sorokin, A.; Moodie, S.; Mullen, P.; Kay, C.; Um, I. H.; Langdon, S.; Goryanin, I. & Harrison, D. J. (2009). Systems biology reveals new strategies for personalized cancer medicine and confirms the role of PTEN in resistance to trastuzumab, *Cancer Research*, Vol. 69, No. 16, (August 2009), pp. 6713-6720, ISSN 0008-5472
- Farmer, H.; McCabe, N.; Lord, C. J.; Tutt, A. N.; Johnson, D. A.; Richardson, T. B.; Santarosa, M.; Dillon, K. J.; Hickson, I.; Knights, C.; Martin, N. M.; Jackson, S. P.; Smith, G. C. & Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy, *Nature*, Vol. 434, No. 7035, (April 2005), pp. 917-921, ISSN 0028-0836
- Farnebo, M.; Bykov, V. J. & Wiman, K. G. (2010). The p53 tumor suppressor: a master regulator of diverse cellular processes and therapeutic target in cancer, *Biochemical*

- and Biophysical Research Communications*, Vol. 396, No. 1, (May 2010), pp. 85-89, ISSN 0006-291X
- Fasolo, A. & Sessa, C. (2008). mTOR inhibitors in the treatment of cancer, *Expert Opinion in Investigational Drugs*, Vol. 17, No. 11, (November 2008), pp. 1717-1734, ISSN 1354-3784
- Feng, J. M.; Zhu, H.; Zhang, X. W.; Ding, J. & Miao, Z. H. (2008). Proteasome-dependent degradation of Chk1 kinase induced by the topoisomerase II inhibitor R16 contributes to its anticancer activity, *Cancer Biology & Therapy*, Vol. 7, No. 11, (November 2008), pp. 1726-1731, ISSN 1538-4047
- Ferrara, N. (2004). Vascular endothelial growth factor: basic science and clinical progress, *Endocrine Reviews*, Vol. 25, No. 4, (August 2004), pp. 581-611, ISSN 0163-769X
- Fishel, R. (1999). Signaling mismatch repair in cancer, *Nature Medicine*, Vol. 5, No. 11, (November 1999), pp. 1239-1241, ISSN 1078-8956
- Fishel, R. (2001). The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: Revising the mutator hypothesis, *Cancer Research*, Vol. 61, No. 20, (October 2001), pp. 7369-7374, ISSN 0008-5472
- Fisher, R. I.; Bernstein, S. H.; Kahl, B. S.; Djulbegovic, B.; Robertson, M. J.; de Vos, S.; Epner, E.; Krishnan, A.; Leonard, J. P.; Lonial, S.; Stadtmauer, E. A.; O'Connor, O. A.; Shi, H.; Boral, A. L. & Goy, A. (2006). Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma, *Journal of Clinical Oncology*, Vol. 24, No. 30, (October 2006), pp. 4867-4874, ISSN 0732-183X
- Fleisher, A. S.; Esteller, M.; Wang, S.; Tamura, G.; Suzuki, H.; Yin, J.; Zou, T. T.; Abraham, J. M.; Kong, D.; Smolinski, K. N.; Shi, Y. Q.; Rhyu, M. G.; Powell, S. M.; James, S. P.; Wilson, K. T.; Herman, J. G. & Meltzer, S. J. (1999). Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability, *Cancer Research*, Vol. 59, No. 5, (March 1999), pp. 1090-1095, ISSN 0008-5472
- Fontana, J.; Fulton, D.; Chen, Y.; Fairchild, T. A.; McCabe, T. J.; Fujita, N.; Tsuruo, T. & Sessa, W. C. (2002). Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release, *Circulation Research*, Vol. 90, No. 8, (May 2002), pp. 866-873, ISSN 0009-7330
- Franke, T. F. (2008). PI3K/Akt: getting it right matters, *Oncogene*, Vol. 27, No. 50, (October 2008), pp. 6473-6488, ISSN 0950-9232
- Friedberg, E. C.; Walker, G. C.; Siede, W.; Wood, R. D.; Schultz, R. A. & Ellenberger, T. (2006). *DNA Repair and Mutagenesis*, 2nd Ed, ASM Press, ISBN 1-55581-319-4, Washington, D. C., USA
- Futreal, P. A.; Liu, Q.; Shattuck-Eidens, D.; Cochran, C.; Harshman, K.; Tavtigian, S.; Bennett, L. M.; Haugen-Strano, A.; Swensen, J.; Miki, Y. & et al. (1994). BRCA1 mutations in primary breast and ovarian carcinomas, *Science*, Vol. 266, No. 5182, (October 1994), pp. 120-122, ISSN 0036-8075
- Gaiser, T.; Becker, M. R.; Meyer, J.; Habel, A. & Siegelin, M. D. (2009) p53-mediated inhibition of angiogenesis in diffuse low-grade astrocytomas, *Neurochemistry International*, Vol. 54, No. 7, (June 2009), pp. 458-463, ISSN 0197-0186
- Garber, K. (2005). New checkpoint blockers begin human trials, *Journal of the National Cancer Institute*, Vol. 97, No. 14, (July 2005), pp. 1026-1028, ISSN 0027-8874

- Garrido, C.; Brunet, M.; Didelot, C.; Zermati, Y.; Schmitt, E. & Kroemer, G. (2006). Heat Shock Proteins 27 and 70: Anti-Apoptotic Proteins with Tumorigenic Properties, *Cell Cycle*, Vol. 5, No. 22, (November 2006), pp. 2592-2601, ISSN 1538-4101
- Goetz, M. P.; Toft, D. O.; Ames, M. M. & Erlichman, C. (2003). The Hsp90 chaperone complex as a novel target for cancer therapy, *Annals of Oncology*, Vol. 14, No. 8, (August 2003), pp. 1169-1176, ISSN 0923-7534
- Guirouilh-Barbat, J.; Huck, S.; Bertrand, P.; Pirzio, L.; Desmaze, C.; Sabatier, L. & Lopez, B. S. (2004). Impact of the KU80 pathway on NHEJ-induced genome rearrangements in mammalian cells, *Molecular Cell*, Vol. 14, No. 5, (June 2004), pp. 611-623, ISSN 1097-2765
- Guittet, O.; Hakansson, P.; Voevodskaya, N.; Fridd, S.; Graslund, A.; Arakawa, H.; Nakamura, Y. & Thelander, L. (2001). Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells, *Journal of Biological Chemistry*, Vol. 276, No. 44, (November 2001), pp. 40647-40651, ISSN 0021-9258
- Han, E. S.; Muller, F. L.; Pérez, V. I.; Qi, W.; Liang, H.; Xi, L.; Fu, C.; Doyle, E.; Hickey, M.; Cornell, J.; Epstein, C. J.; Roberts, L. J.; Van Remmen, H. & Richardson, A. (2008). The in vivo gene expression signature of oxidative stress, *Physiological Genomics*, Vol. 34, No. 1, (June 2008), pp. 112-126, ISSN 1094-8341
- Hanawalt, P. C.; Donahue, B. A. & Sweder, K. S. (1994). Repair and transcription. Collision or collusion? *Current Biology*, Vol. 4, No. 6, (June 1994), pp. 518-521, ISSN 0960-9822
- Hartwell, L. (1997). Theoretical biology. A robust view of biochemical pathways, *Nature*, Vol. 387, No. 6636, (June 1997), pp. 855-857, ISSN 0028-0836
- Hassan, N. M.; Tada, M.; Hamada, J.; Kashiwazaki, H.; Kameyama, T.; Akhter, R.; Yamazaki, Y.; Yano, M.; Inoue, N. & Moriuchi, T. (2008). Presence of dominant negative mutation of TP53 is a risk of early recurrence in oral cancer, *Cancer Letters*, Vol. 270, No. 1, (October 2008), pp. 108-119, ISSN 0304-3835
- Hay, T.; Jenkins, H.; Sansom, O. J.; Martin, N. M.; Smith, G. C. & Clarke, A. R. (2005). Efficient deletion of normal Brca2-deficient intestinal epithelium by poly(ADP-ribose) polymerase inhibition models potential prophylactic therapy, *Cancer Research*, Vol. 65, No. 22, (November 2005), pp. 10145-10148, ISSN 0008-5472
- He, L.; Thomson, J. M.; Hemann, M. T.; Hernando-Monge, E.; Mu, D.; Goodson, S.; Powers, S.; Cordon-Cardo, C.; Lowe, S. W.; Hannon, G. J. & Hammond, S. M. (2005). A microRNA polycistron as a potential human oncogene, *Nature*, Vol. 435, No. 7043, (June 2005), pp. 828-833, ISSN 0028-0836
- Hekmat-Nejad, M.; You, Z.; Yee, M. C.; Newport, J. W. & Cimprich, K. A. (2000). Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint, *Current Biology*, Vol. 10, No. 24, (December 2000), pp. 1565-1573, ISSN 0960-9822
- Helleday, T.; Bryant, H. E. & Schultz, N. (2005). Poly(ADP-ribose) polymerase (PARP-1) in homologous recombination and as a target for cancer therapy, *Cell Cycle*, Vol. 4, No. 9, (September 2005), pp. 1176-1178, ISSN 1538-4101

- Herrick, J. & Sclavi, B. (2007). Ribonucleotide reductase and the regulation of DNA replication: an old story and an ancient heritage, *Molecular Microbiology*, Vol. 63, No. 1, (January 2007), pp. 22–34, ISSN 0950-382X
- Hicklin, D. J. & Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis, *Journal of Clinical Oncology*, Vol. 23, No. 5, (February 2005), pp. 1011-1027, ISSN 0732-183X
- Hickman, M. J. & Samson, L. D. (1999). Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 96, No. 19, (September 1999), pp. 10764-10769, ISSN 0027-8428
- Hickson, I.; Zhao, Y.; Richardson, C. J.; Green, S. J.; Martin, N. M.; Orr, A. I.; Reaper, P. M.; Jackson, S. P.; Curtin, N. J. & Smith G. C. (2004). Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM, *Cancer Research*, Vol. 64, No. 24, (December 2004), pp. 9152-9159, ISSN 0008-5472
- Hietanen, S.; Lain, S.; Krausz, E.; Blattner, C. & Lane, D. P. (2000). Activation of p53 in cervical carcinoma cells by small molecules, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, No. 15, (July 2000), pp. 8501-8506, ISSN 0027-8428
- Hirao, A.; Cheung, A.; Duncan, G.; Girard, P. M.; Elia, A. J.; Wakeham, A.; Okada, H.; Sarkissian, T.; Wong, J. A.; Sakai, T.; De Stanchina, E.; Bristow, R. G.; Suda, T.; Lowe, S. W.; Jeggo, P. A.; Elledge, S. J. & Mak, T. W. (2002). Chk2 is a tumor suppressor that regulates apoptosis in both an ATM-dependent and ATM-independent manner, *Molecular and Cellular Biology*, Vol. 22, No. 18, (September 2002), pp. 6521-6532, ISSN 0270-7306
- Hollstein, M.; Sidransky, D.; Vogelstein, B. & Harris, C. C. (1991). p53 mutations in human cancers, *Science*, Vol. 253, No. 5015 (July 1991), pp. 49–53, ISSN 0036-8075
- Houghtaling, S.; Newell, A.; Akkari, Y.; Taniguchi, T.; Olson, S. & Grompe, M. (2005). Fancd2 functions in a double strand break repair pathway that is distinct from non-homologous end joining, *Human Molecular Genetics*, Vol. 14, No. 20, (October 2005), pp. 3027-3033, ISSN 0964-6906
- Iggo, R.; Gatter, K.; Bartek, J.; Lane, D. & Harris, A. L. (1990). Increased expression of mutant forms of p53 oncogene in primary lung cancer, *The Lancet*, Vol. 335, No. 8691, (March 1990), pp. 675-679, ISSN 0140-6736
- Ingelman-Sundberg, M. (2001). Pharmacogenetics: an opportunity for a safer and more efficient pharmacotherapy, *Journal of Internal Medicine*, Vol. 250, No. 3, (September 2001), pp. 186-200, ISSN 0954-6820
- Isobe, M.; Emanuel, B. S.; Givol, D.; Oren, M. & Croce, C. M. (1986). Localization of gene for human p53 tumour antigen to band 17p13, *Nature*, Vol. 320, No. 6057, (March, 1986), pp. 84–85, ISSN 0028-0836
- Jakob, C.; Egerer, K.; Liebisch, P.; Türkmen, S.; Zavrski, I.; Kuckelkorn, U.; Heider, U.; Kaiser, M.; Fleissner, C.; Sterz, J.; Kleeberg, L.; Feist, E.; Burmester, G. R.; Kloetzel, P. M. & Sezer, O. (2007). Circulating proteasome levels are an independent prognostic factor for survival in multiple myeloma, *Blood*, Vol. 109, No. 5, (March 2007), pp. 2100–2105, ISSN 0006-4971

- Jameel, J. K.; Rao, V. S.; Cawkwell, L. & Drew, P. J. (2004). Radioresistance in carcinoma of the breast, *Breast*, Vol. 13, No. 6, (December 2004), pp. 452-460, ISSN 0960-9776
- Jiang, G. & Sancar, A. (2006). Recruitment of DNA damage checkpoint proteins to damage in transcribed and nontranscribed sequences, *Molecular and Cellular Biology*, Vol. 26, No. 1, (January 2006), pp. 39-49, ISSN 0270-7306
- Jiricny, J. & Nystrom-Lahti M. (2000). Mismatch repair defects in cancer, *Current Opinion in Genetics & Development*, Vol. 10, No. 2, (April 2000), pp. 157-161, ISSN 0959-437X
- Jonason, A. S.; Kunal, S.; Price, G. J.; Restifo, R. J.; Spinelli, H. M.; Persing, J. A.; Leffell, D. J.; Tarone, R. E. & Brash, D. E. (1996). Frequent clones of p53-mutated keratinocytes in normal human skin, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 93, No. 24, (November 1996), pp. 14025-14029, ISSN 0027-8424
- Jurvansuu, J.; Fragkos, M.; Ingemarsdotter, C. & Beard, P. (2007). Chk1 Instability Is Coupled to Mitotic Cell Death of p53-deficient Cells in Response to Virus-induced DNA Damage Signaling, *Journal of Molecular Biology*, Vol. 372, No. 2, (September 2007), pp. 397-406, ISSN 0022-2836
- Kadouri, L.; Hubert, A.; Rotenberg, Y.; Hamburger, T.; Sagi, M.; Nechushtan, C.; Abeliovich, D. & Peretz, T. (2007). Cancer risks in carriers of the BRCA1/2 Ashkenazi founder mutations, *Journal of Medical Genetics*, Vol. 44, No. 7, (July 2007), pp. 467-471, ISSN 1468-6244
- Kaelin, W. G. (2005). Proline hydroxylation and gene expression, *Annual Review of Biochemistry*, Vol. 74, pp. 115-128, ISSN 0066-4154
- Kang, M. H. & Reynolds, C. P. (2009). Bcl-2 inhibitors: Targeting mitochondrial apoptotic pathways in cancer therapy, *Clinical Cancer Research*, Vol. 15, No. 4, (February 2009), pp. 1126-1132, ISSN 1078-0432
- Kastrinos, F. & Syngal, S. (2007). Recently identified colon cancer predispositions: MYH and MSH6 mutations, *Seminars in Oncology*, Vol. 34, No. 5, (October 2007), pp. 418-424, ISSN 0093-7754
- Kern, S. E.; Kinzler, K. W.; Bruskin, A.; Jarosz, D.; Friedman, P.; Prives, C. & Vogelstein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein, *Science*, Vol. 252, No. 5013, (June 1991), pp. 1708-1711, ISSN 0036-8075
- King, M. C.; Marks, J. H. & Mandell, J. B. (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2, *Science*, Vol. 302, No. 5645, (October 2003), pp. 643-646, ISSN 0036-8075
- Kling, J. (2009). PARP inhibitors blaze a trail in difficult-to-treat cancers, *Nature Biotechnology*, Vol. 27, No. 9, (September 2009), pp. 784-786, ISSN 1087-0156
- Kolodner, R. D.; Mendillo, M. L. & Putnam, C. D. (2007). Coupling distant sites in DNA during DNA mismatch repair, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 104, No. 32, (August 2007), pp. 12953-12954, ISSN 0027-8424
- Kurz, E. U. & Lees-Miller, S. P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways, *DNA Repair*, Vol. 3, No. 8-9, (August-September 2004), pp. 889-900, ISSN 1568-7864
- Kutler, D. I.; Auerbach, A. D.; Satagopan, J.; Giampietro, P. F.; Batish, S. D.; Huvos, A. G.; Gubergh, A.; Shah, J. P. & Singh, B. (2003). High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia, *Archives of*

- Otolaryngology - Head & Neck Surgery*, Vol. 129, No. 1, (January 2003), pp. 106-112, ISSN 0886-4470
- Lage, H. & Dietel, M. (1999). Involvement of the DNA mismatch repair system in antineoplastic drug resistance, *Journal of Cancer Research and Clinical Oncology*, Vol. 125, No. 3-4, (April 1999), pp. 156-165, ISSN 0171-5216
- Laghi, L.; Bianchi, P. & Malesci, A. (2008). Differences and evolution of the methods for the assessment of microsatellite instability, *Oncogene*, Vol. 27, No. 49, (October 2008), pp. 6313-6321, ISSN 0950-9232
- Laurent, N.; de Boüard, S.; Guillamo, J. S.; Christov, C.; Zini, R.; Jouault, H.; Andre, P.; Lotteau, V. & Peschanski, M. (2004). Effects of the proteasome inhibitor ritonavir on glioma growth in vitro and in vivo, *Molecular Cancer Therapeutics*, Vol. 3, No. 2, (February 2004), pp. 129-136, ISSN 1535-7163
- Lee, Y.; Miron, A.; Drapkin, R.; Nucci, M. R.; Medeiros, F.; Saleemuddin, A; Garber, J.; Birch, C.; Mou, H.; Gordon, R. W., Cramer, D. W.; McKeon, F. D. & Crum, C. P. (2007). A candidate precursor to serous carcinoma that originates in the distal Fallopian tube, *Journal of Pathology*, Vol. 211, No. 1, (January 2007), pp. 26-35, ISSN 1096-9896
- Lehman, N. L. (2009). The ubiquitin proteasome system in neuropathology, *Acta Neuropathologica*, Vol. 118, No. 3, (September 2009), pp. 329-347, ISSN 0001-6322
- Leung-Pineda, V.; Huh, J. & Piwnica-Worms, H. (2009). DDB1 targets Chk1 to the Cul4 E3 ligase complex in normal cycling cells and in cells experiencing replication stress, *Cancer Research*, Vol. 69, No. 6, (March 2009), pp. 2630-2637, ISSN 0008-5472
- Liang, K.; Ang, K. K.; Milas, L.; Hunter, N. & Fan, Z. (2003). The epidermal growth factor receptor mediates radioresistance, *International Journal of Radiation Oncology, Biology, Physics*, Vol. 57, No. 1, (September 2003), pp. 246-254, ISSN 0360-3016
- Lin, N. H.; Xia, P.; Kovar, P.; Park, C.; Chen, Z.; Zhang, H.; Rosenberg, S. H. & Sham, H. L. (2006). Synthesis and biological evaluation of 3-ethylidene-1,3-dihydro-indol-2-ones as novel checkpoint 1 inhibitors, *Bioorganic & Medicinal Chemistry Letters*, Vol. 16, No. 2, (January 2006), pp. 421-426, ISSN 0960-894X
- Litman, R.; Peng, M.; Jin, Z.; Zhang, F.; Zhang, J.; Powell, S.; Andreassen, P. R. & Cantor, S. B. (2005). BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCF, *Cancer Cell*, Vol. 8, No. 3, (September 2005), pp. 255-265, ISSN 1535-6108
- Litman, R.; Gupta, R.; Brosh, R. M. & Cantor, S. B. (2008). BRCA-FA pathway as a target for anti-tumor drugs, *Anticancer Agents in Medicinal Chemistry*, Vol. 8, No. 4 (May 2008), pp. 426-430, ISSN 1871-5206
- Ljungman, M. (2009). Targeting the DNA damage response in cancer, *Chemical Reviews*, Vol. 109, No. 7, (July 2009), pp. 2929-2950, ISSN 0009-2665
- Lo, H. W.; Xia, W.; Wei, Y.; Ali-Seyed, M.; Huang, S. F. & Hung, M. C. (2005). Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer, *Cancer Research*, Vol. 65, No. 1, (January 2005), pp. 338-348, ISSN 0008-5472
- Lodish, H.; Berk, A.; Matsudaira, P.; Kaiser, C. A.; Krieger, M.; Scott, M. P.; Zipursky, S. L. & Darnell, J. (2004). *Molecular cell biology* (5th ed.), pp. 66-72, ISBN-10: 0-71674366-3, W.H. Freeman and CO, New York, USA
- Longley, D. B.; Harkin, D. P. & Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies, *Nature Reviews. Cancer*, Vol. 3, No. 5, (May 2003), pp. 330-338, ISSN 1474-175X

- LoPiccolo, J.; Blumenthal, G. M.; Bernstein, W. B. & Dennis, P. A. (2008). Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations, *Drug Resistance Updates*, Vol. 11, No. 1-2, (February-April 2008), pp. 32-50, ISSN 1368-7646
- Lu, J.; Getz, G.; Miska, E. A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B. L.; Mak, R. H.; Ferrando, A. A.; Downing, J. R.; Jacks, T.; Horvitz, H. R. & Golub, T. R. (2005). MicroRNA expression profiles classify human cancers, *Nature*, Vol. 435, No. 7043, (June 2005), pp. 834-838, ISSN 0028-0836
- Lurje, G. & Lenz, H. J. (2009). EGFR Signaling and Drug Discovery, *Oncology*, Vol. 77, No. 6, (May 2009), pp. 400-410, ISSN 0030-2414
- Malumbres, M.; Pevarello, P.; Barbacid, M. & Bischoff, J.R. (2008). CDK inhibitors in cancer therapy: what is next? *Trends in Pharmacological Sciences*, Vol. 29, No. 1, (January 2008), pp. 16-21, ISSN 0165-6147
- Malumbres, M. & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm, *Nature Reviews. Cancer*, Vol. 9, No. 3, (March 2009), pp. 153-166, ISSN 1474-175X
- Mandinova, A. & Lee, S. W. (2011). The p53 pathway as a target in cancer therapeutics: obstacles and promise, *Science Translational Medicine*, Vol. 3, No. 64, (January 2011), pp. 64rv1, ISSN 1946-6234
- Margolin, K. (2002). Inhibition of vascular endothelial growth factor in the treatment of solid tumors, *Current Oncology Reports*, Vol. 4, No. 1, (January 2002), pp. 20-28, ISSN 1523-3790
- Marko-Varga, G.; Ogiwara, A.; Nishimura, T.; et al. (2007). Personalized medicine and proteomics: lessons from non-small cell lung cancer, *Journal of Proteome Research*, Vol. 6, No. 8, (August 2007), pp. 2925-2935, ISSN 1535-3893
- Marone, R.; Cmiljanovic, V.; Giese, B. & Wymann, M. P. (2007). Targeting phosphoinositide 3-kinase: moving towards therapy, *Biochimica et Biophysica Acta*, Vol. 1784, No. 1, (January 2008), pp. 159-185, ISSN 0006-3002
- Martin, S. A.; Lord, C. J. & Ashworth, A. (2010). Therapeutic targeting of the DNA mismatch repair pathway, *Clinical Cancer Research*, Vol. 16, No. 21, (November 2010), pp. 5107-5113, ISSN 1078-0432
- Martins, C. P.; Brown-Swigart, L. & Evan, G. I. (2006). Modeling the therapeutic efficacy of p53 restoration in tumors, *Cell*, Vol. 127, No. 7, (December 2006), pp. 1323-1334, ISSN 0092-8674
- Matlashewski, G.; Lamb, P.; Pim, D.; Peacock, J.; Crawford, L. & Benchimol, S. (1984). Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene, *The EMBO Journal*, Vol. 3, No. 13, (December 1984), pp. 3257-3262, ISSN 0261-4189
- McBride, O. W.; Merry, D. & Givol, D. (1986). The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13), *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 83, No. 1, (January 1986), pp. 130-134, ISSN 0027-8424
- McVey, M. & Lee, S. E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings, *Trends in Genetics*, Vol. 24, No. 11, (November 2008), pp. 529-538, ISSN 0168-9525

- Mellon, I. & Hanawalt, P. C. (1989). Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand, *Nature*, Vol. 342, No. 6245, (November 1989), pp. 95-98, ISSN 0028-0836
- Mellon, I.; Spivak, G. & Hanawalt, P. C. (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene, *Cell*, Vol. 51, No. 2, (October 1987), pp. 241-249, ISSN 0092-8674
- Meng, R. D. & El-Deiry, W. S. (1998). Tumor suppressor gene therapy for cancer from the bench to the clinic, *Drug Resistance Updates*, Vol. 1, No. 3, (May 1998), pp. 205-210, ISSN 1368-7646
- Michael, W. M.; Ott, R.; Fanning, E. & Newport, J. (2000). Activation of the DNA replication checkpoint through RNA synthesis by primase, *Science*, Vol. 289, No. 5487, (September 2000), pp. 2133-2137, ISSN 0036-8075
- Miki, Y.; Swensen, J.; Shattuck-Eidens, D.; Futreal, P. A.; Harshman, K.; Tavtigian, S.; Liu, Q.; Cochran C.; Bennett, L. M.; Ding, W. & et al. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1, *Science*, Vol. 266, No. 5182, (October 1994), pp. 66-71, ISSN 0036-8075
- Milas, L.; Fan, Z.; Andratschke, N. H. & Ang, K. K. (2004). Epidermal growth factor receptor and tumor response to radiation: in vivo preclinical studies, *International Journal of Radiation Oncology, Biology, Physics*, Vol. 58, No. 3, (March 2004), pp. 966-971, ISSN 0360-3016
- Miller, K.; Wang, M.; Gralow, J.; Dickler, M.; Cobleigh, M.; Perez, E. A.; Shenkier, T.; Cella, D. & Davidson, N. E. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer, *New England Journal of Medicine*, Vol. 357, No. 26, (December 2007), pp. 2666-2676, ISSN 0028-4793
- Minn, A. J.; Rudin, C. M.; Boise, L. H.; & Thompson, C. B. (1995). Expression of Bcl-xL can confer a multidrug resistance phenotype, *Blood*, Vol. 86, No. 5, (September 1995), pp. 1903-1910, ISSN 0006-4971
- Mohsin, S. K.; Weiss, H. L.; Gutierrez, M. C.; Chamness, G. C.; Schiff, R.; Digiovanna, M. P.; Wang, C. X.; Hilsenbeck, S. G.; Osborne, C. K.; Allred, D. C.; Elledge, R. & Chang, J. C. (2005). Neoadjuvant trastuzumab induces apoptosis in primary breast cancers, *Journal of Clinical Oncology*, Vol. 23, No. 11, (April 2005), pp. 2460-2468, ISSN 0732-183X
- Moore, J. K. & Haber, J. E. (1996). Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*, *Molecular and Cellular Biology*, Vol. 16, No. 5, (May 1996), pp. 2164-2173, ISSN 0270-7306
- Morgan, D. O. (2007). *The cell cycle: Principles of control*, Oxford University Press, ISBN 0-19-920610-4, Oxford, United Kingdom
- Morgensztern, D. & McLeod, H. L. (2005). PI3K/Akt/mTOR pathway as a target for cancer therapy, *Anti-cancer Drugs*, Vol. 16, No. 8, (September 2005), pp. 797-803, ISSN 0959-4973
- Morton, J. P.; Timpson, P.; Karim, S. A.; Ridgway, R. A.; Athineos, D.; Doyle, B.; Jamieson, N. B.; Oien, K. A.; Lowy, A. M.; Brunton, V. G.; Frame, M. C.; Evans, T. R. & Sansom, O. J. (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer, *Proceedings of the National Academy of Sciences*

- Sciences of the United States of America*, Vol. 107, No. 1, (January 2010), pp. 246-251, ISSN 0027-8424
- Motoyama, N.; Wang, F.; Roth, K. A.; Sawa, H.; Nakayama, K.; Nakayama, K.; Negishi, I.; Senju, S.; Zhang, Q.; Fujii, S.; et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice, *Science*, Vol. 267, No. 5203, (March 1995), pp. 1506-1510, ISSN 0036-8075
- Mraz, M.; Pospisilova, S.; Malinova, K.; Slapak, I. & Mayer, J. (2009). MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes, *Leukemia and Lymphoma*, Vol. 50, No. 3, (March 2009), pp. 506-509, ISSN 1042-8194
- Nakamura, J. L.; Karlsson, A.; Arvold, N. D.; Gottschalk, A. R.; Pieper, R. O.; Stokoe, D. & Haas-Kogan, D. A. (2005). PKB/Akt mediates radiosensitization by the signaling inhibitor LY294002 in human malignant gliomas, *Journal of Neuro-Oncology*, Vol. 71, No. 3, (February 2005), pp. 215-222, ISSN 0167-594X
- Narod, S. A. & Foulkes, W. D. (2004). BRCA1 and BRCA2: 1994 and beyond, *Nature Reviews. Cancer*, Vol. 4, No. 9, (September 2004), pp. 665-676, ISSN 1474-175X
- Nawrocki, S. T.; Sweeney-Gotsch, B.; Takamori, R. & McConkey, D. J. (2004). The proteasome inhibitor bortezomib enhances the activity of docetaxel in orthotopic human pancreatic tumor xenografts, *Molecular Cancer Therapeutics*, Vol. 3, No. 1, (January 2004), pp. 59-70, ISSN 1535-7163
- Niedernhofer, L. J.; Lalai, A. S. & Hoeijmakers, J. H. (2005). Fanconi anemia (cross) linked to DNA repair, *Cell*, Vol. 123, No. 7, (December 2005), pp. 1191-1198, ISSN 0092-8674
- Nielsen, B. S.; Jørgensen, S.; Fog, J. U.; Søkilde, R.; Christensen, I. J.; Hansen, U.; Brüner, N.; Baker, A.; Møller, S. & Nielsen, H. J. (2010). High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. *Clinical and Experimental Metastasis*, Vol. 28, No. 1, (October 2010), pp. 27-38, ISSN 0262-0898
- Nigro, J. M.; Baker, S. J.; Preisinger, A. C.; Jessup, J. M.; Hostetter, R.; Cleary, K.; Bigner, S. H.; Davidson, N.; Baylin, S.; Davilee, P.; et al. (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature*, Vol. 342, No. 6250, (December 1989), pp. 705-708, ISSN 0028-0836
- Nowakowski, G. S.; McCollum, A. K.; Ames, M. M.; Mandrekar, S. J.; Reid, J. M.; Adjei, A. A.; Toft, D. O.; Safgren, S. L. & Erlichman, C. (2006). A phase I trial of twice-weekly 17-allylamino-demethoxy-geldanamycin in patients with advanced cancer, *Clinical Cancer Research*, Vol. 12, No. 20, (October 2006), pp. 6087-6093, ISSN 1078-0432
- Nyati, M. K.; Morgan, M. A.; Feng, F. Y. & Lawrence, T. S. (2006). Integration of EGFR inhibitors with radiochemotherapy, *Nature Reviews. Cancer*, Vol. 6, No. 11, (November 2006), pp. 876-885, ISSN 1474-175X
- O'Connor, O. A.; Wright, J.; Moskowitz, C.; Muzzy, J.; MacGregor-Cortelli, B.; Stubblefield, M.; Straus, D.; Portlock, C.; Hamlin, P.; Choi, E.; Dumetrescu, O.; Esseltine, D.; Trehu, E.; Adams, J.; Schenkein, D. & Zelenetz, A. D. (2005). Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma, *Journal of Clinical Oncology*, Vol. 23, No. 4, (February 2005), pp. 676-684, ISSN 0732-183X
- O'Donnell, K. A.; Wentzel, E. A.; Zeller, K. I.; Dang, C. V. & Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression, *Nature*, Vol. 435, No. 7043, (June 2005), pp. 839-843, ISSN 0028-0836

- Ojuka, E. O. (2004). Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle, *Proceedings of the Nutrition Society*, Vol. 63, No. 2, (May 2004), pp. 275-278, ISSN 0029-6651
- Onate, S. A.; Prendergast, P.; Wagner, J. P.; Nissen, M.; Reeves, R.; Pettijohn, D. E. & Edwards, D. P. (1994). The DNA-bending protein HMG-1 enhances progesterone receptor binding to its target DNA sequences, *Molecular and Cellular Biology*, Vol. 14, No. 5, (May 1994), pp. 3376-3391, ISSN 0270-7306
- Orlowski, R. Z. (1999). The role of the ubiquitin-proteasome pathway in apoptosis, *Cell Death and Differentiation*, Vol. 6, No. 4, (April 1999), pp. 303-313, ISSN 1350-9047
- Pal, T.; Permuth-Wey, J. & Sellers, T. A. (2008). A review of the clinical relevance of mismatch-repair deficiency in ovarian cancer, *Cancer*, Vol. 113, No. 4, (August 2008), pp. 733-742, ISSN 1097-0142
- Pallis, A. G. & Karamouzis, M. V. (2010). DNA repair pathways and their implication in cancer treatment, *Cancer and Metastasis Reviews*, Vol. 29, No. 4, (December 2010), pp. 677-685, ISSN 0167-7659
- Park, S. H.; Bolender, N.; Eisele, F.; Kostova, Z.; Takeuchi, J.; Coffino, P. & Wolfet, D. H. (2007). The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system, *Molecular Biology of the Cell*, Vol. 18, No. 1, (January 2007), pp. 153-165, ISSN 1939-4586
- Phan, J. H.; Moffitt, R. A.; Stokes, T. H.; Liu, J.; Young, A. N.; Nie, S. & Wang, M. D. (2009). Convergence of biomarkers, bioinformatics and nanotechnology for individualized cancer treatment, *Trends in Biotechnology*, Vol. 27, No. 6, (June 2009), pp. 350-358, ISSN 0167-7799
- Phillips, K. A.; Veenstra, D. L.; Oren, E.; Lee, J. K. & Sadee, W. (2001). Potential role of pharmacogenomics in reducing adverse drug reactions: a systematic review, *Journal of the American Medical Association*, Vol. 286, No. 18, (November 2001), pp. 2270-2279, ISSN 0002-9955
- Plo, I.; Laulier, C.; Gauthier, L.; Lebrun, F.; Calvo, F. & Lopez, B. S. (2008). AKT1 inhibits homologous recombination by inducing cytoplasmic retention of BRCA1 and RAD51, *Cancer Research*, Vol. 68, No. 22, (November 2008), pp. 9404-9412, ISSN 0008-5472
- Preston, B. D.; Albertson, T. M. & Herr, A. J. (2010). DNA replication fidelity and cancer, *Seminars in Cancer Biology*, Vol. 20, No. 5, (October 2010), pp. 281-293, ISSN 1040-579x
- Prewett, M.; Deevi, D. S.; Bassi, R.; Fan, F.; Ellis, L. M.; Hicklin, D. J. & Tonra, J. R. (2007). Tumors established with cell lines selected for oxaliplatin resistance respond to oxaliplatin if combined with cetuximab, *Clinical Cancer Research*, Vol. 13, No. 24 (December 2007), pp. 7432-7440, ISSN 1078-0432
- Psyrris, A.; Yu, Z.; Weinberger, P. M.; Sasaki, C.; Haffty, B.; Camp, R.; Rimm, D. & Burtness, B. A. (2005). Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis, *Clinical Cancer Research*, Vol. 11, No. 16, (August 2005), pp. 5856-5862, ISSN 1078-0432

- Quesada, A. R.; Medina, M. A.; Munoz-Chapuli, R. & Ponce, A. L. (2010). Do not say ever never more: the ins and outs of antiangiogenic therapies, *Current Pharmaceutical Design*, Vol. 16, No. 35, (December 2010), pp. 3932-3957, ISSN 1381-6128
- Rai, K. R.; Moore, J.; Wu, J.; Novick, S. C. & O'Brien, S. M. (2008). Effect of the addition of oblimersen (Bcl-2 antisense) to fludarabine/cyclophosphamide for relapsed/refractory chronic lymphocytic leukemia (CLL) on survival in patients who achieve CR/nPR: Five-year follow-up from a randomized phase III study, *Journal of Clinical Oncology*, Vol. 26, No. 15S, (May 2008), pp. 7008, ISSN 0732-183X
- Rainey, M. D.; Charlton, M. E.; Stanton, R. V. & Kastan, M. B. (2008). Transient inhibition of ATM kinase is sufficient to enhance cellular sensitivity to ionizing radiation, *Cancer Research*, Vol. 68, No. 18, (September 2008), pp. 7466-7474, ISSN 0008-5472
- Raschle, M.; Marra, G.; Nyström-Lahti, M.; Schär, P. & Jiricny, J. (1999). Identification of hMutLbeta, a heterodimer of hMLH1 and hPMS1. *Journal of Biological Chemistry*, Vol. 274, No. 45, (November 1999), pp. 32368-32375, ISSN 0021-9258
- Rotter, V. (1983). p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 80, No. 9, (May 1983), pp. 2613-2617, ISSN 0027-8424
- Rustum, Y. M. (2004). Thymidylate synthase: a critical target in cancer therapy? *Frontiers in Biosciences*, Vol. 9, (September 2004), pp. 2467-2473, ISSN 1093-9946
- Saga, Y.; Suzuki, M.; Mizukami, H.; Urabe, M.; Fukushima, M.; Ozawa, K. & Sato, I. (2002). Enhanced expression of thymidylate synthase mediates resistance of uterine cervical cancer cells to radiation, *Oncology*, Vol. 63, No. 2, (September 2002), pp. 185-191, ISSN 0030-2414
- Sancar, A. (1999). Excision repair invades the territory of mismatch repair. *Nature Genetics*, Vol. 21, No. 3, (March 1999), pp. 247-249, ISSN 1061-4036
- Santarosa, M. & Ashworth, A. (2004). Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way, *Biochimica et Biophysica Acta*, Vol. 1654, No. 2, (June 2004), pp. 105-122, ISSN 0006-3002
- Sarker, A. H.; Tsutakawa, S. E.; Kostek, S.; Ng, C.; Shin, D. S.; Peris, M.; Campeau, E.; Tainer, J. A.; Nogales, E. & Cooper, P. K. (2005). Recognition of RNA Polymerase II and Transcription Bubbles by XPG, CSB, and TFIIH: Insights for Transcription-Coupled Repair and Cockayne Syndrome, *Molecular Cell*, Vol. 20, No. 2, (October 2005), pp. 187-198, ISSN 1097-2765
- Sato, S.; Fujita, N. & Tsuruo, T. (2000). Modulation of Akt kinase activity by binding to Hsp90, *Proceedings of the National Academy of Science of the United States of America*, Vol. 97, No. 20, (September 2000), pp. 10832-10837, ISSN 0027-8424
- Sawai, A.; Chandarlapaty, S.; Greulich, H.; Gonen, M.; Ye, Q.; Arteaga, C. L.; Sellers, W.; Rosen, N. & Solit, D. B. (2008). Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel, *Cancer Research*, Vol. 68, No. 2, (January 2008), pp. 589-596, ISSN 0008-5472
- Schenkein, D. (2002). Proteasome inhibitors in the treatment of B-cell malignancies, *Clinical Lymphoma*, Vol. 3, No. 1, (June 2002), pp. 49-55, ISSN 1526-9655

- Scully, R. & Livingston, D. M. (2000). In search of the tumour-suppressor functions of BRCA1 and BRCA2, *Nature*, Vol. 408, No. 6811, (November 2000), pp. 429-432, ISSN 0028-0836
- Selivanova, G.; Ryabchenko, L.; Jansson, E.; Iotsova, V. & Wiman, K. G. (1999). Reactivation of mutant p53 through interaction of a C-terminal peptide with the core domain, *Molecular and Cellular Biology*, Vol. 19, No. 5, (May 1999), pp. 3395-3402, ISSN 0270-7306
- Seth, P.; Brinkmann, U.; Schwartz, G. N.; Katayosa, D.; Gress, R.; Pastan, I. & Cowan, K. (1996). Adenovirus-mediated gene transfer to human breast tumor cells: an approach for cancer gene therapy and bone marrow purging, *Cancer Research*, Vol. 56, No. 6, (March 1996), pp. 1346-1351, ISSN 0008-5472
- Shah, S. A.; Potter, M. W.; McDade, T. P.; Ricciardi, R.; Perugini, R. A.; Elliott, P. J.; Adams, J. & Callery, M. P. (2001). 26S proteasome inhibition induces apoptosis and limits growth of human pancreatic cancer, *Journal of Cellular Biochemistry*, Vol. 82, No. 1, (April 2001), pp. 110-122, ISSN 0730-2312
- Shao, J.; Zhou, B.; Zhu, L.; Qiu, W.; Yuan, Y. C.; Xi, B. & Yen, Y. (2004). In vitro Characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase, *Cancer Research*, Vol. 64, No. 1, (January 2004), pp. 1-6, ISSN 0008-5472
- Shen, S. X.; Weaver, Z.; Xu, X.; Li, C.; Weinstein, M.; Chen, L.; Guan, X. Y.; Ried, T. & Deng, C. X. (1998). A targeted disruption of the murine Brca1 gene causes gamma-irradiation hypersensitivity and genetic instability, *Oncogene*, Vol. 17, No. 24, (December 1998), pp. 3115-3124, ISSN 0950-9232
- Showalter, S. L.; Showalter, T. N.; Witkiewicz, A.; Havens, R.; Kennedy, E. P.; Hucl, T.; Kern, S. E.; Yeo, C. J. & Brody, J. R. (2008). Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil. Is it time to move forward? *Cancer Biology & Therapy*, Vol. 7, No. 7, (July 2008), pp. 986-994, ISSN 1538-4047
- Shringarpure, R.; Grune, T.; Mehlhase, J. & Davies, K. J. (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome, *Journal of Biological Chemistry*, Vol. 278, No. 1, (January 2003), pp. 311-318, ISSN 0021-9258
- Sorensen, C. S.; Syljuasen, R. G.; Falck, J.; Schroeder, T.; Ronnstrand, L.; Khanna, K. K.; Zhou, B. B.; Bartek, J. & Lukas, J. (2003). Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A, *Cancer Cell*, Vol. 3, No. 3, (March 2003), pp. 247-258, ISSN 1535-6108
- Spears, B. B.; Heath-Chiozzi, M. & Huff, J. (2001). Clinical application of pharmacogenetics, *Trends in Molecular Medicine*, Vol. 7, No. 5, (May 2001), pp. 201-204, ISSN 1471-4914
- Starcevic, D.; Dalal, S. & Sweasy, J. B. (2004). Is there a link between DNA polymerase beta and cancer? *Cell Cycle*, Vol. 3, No. 8, (August 2004), pp. 998-1001, ISSN 1538-4101
- Stebbins, C. E.; Russo, A. A.; Schneider, C.; Rosen, N.; Hartl, F. U. & Pavletich, N. P. (1997). Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent, *Cell*, Vol. 89, No. 2, (April 1997), pp. 239-250, ISSN 0092-8674

- Steelman, L. S.; Stadelman, K. M.; Chappell, W. H.; Horn, S.; Basecke, J.; Cervello, M.; Nicoletti, F.; Libra, M.; Stivala, F.; Martelli, A. M. & McCubrey, J. A. (2008). Akt as a therapeutic target in cancer, *Expert Opinion on Therapeutic Targets*, Vol. 12, No. 9, (September 2008), pp. 1139-1165, ISSN 1472-8222
- Storey, A.; Thomas, M.; Kalita, A.; Harwood, C.; Gardiol, D.; Mantovani, F.; Breuer, J.; Leigh, I. M.; Matlashewski, G. & Banks, L. (1998). Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature*, Vol. 393, No. 6682, (May 1998), pp. 229-234, ISSN 0028-0836
- Strachan, T. & Read, A. P. (1999). Chapter 18: Cancer Genetics, in: *Human molecular genetics 2*, Wiley-Liss, ISBN 0471330612, New York, USA
- Strathdee, G.; MacKean, M. J.; Illand, M. & Brown, R. (1999). A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer, *Oncogene*, Vol. 18, No. 14, (April 1999), pp. 2335-2341, ISSN 0950-9232
- Stubbe, J. & Riggs-Gelasco, P. (1998). Harnessing free radicals: formation and function of the tyrosyl radical in ribonucleotide reductase, *Trends in Biochemical Sciences*, Vol. 23, No. 11, (November 1998), pp. 438-443, ISSN 0968-0004
- Syljuasen, R. G.; Sorensen, C. S.; Nylandsted, J.; Lukas, C.; Lukas, J. & Bartek, J. (2004). Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation, *Cancer Research*, Vol. 64, No. 24, (December 2004), pp. 9035-9040, ISSN 0008-5472
- Tait, D. L.; Obermiller, P. S.; Redlin-Frazier, S.; Jensen, R. A.; Welcsh, P.; Dann, J.; King, M. C.; Johnson, D. H. & Holt, J. T. (1997). A phase I trial of retroviral BRCA1sv gene therapy in ovarian cancer, *Clinical Cancer Research*, Vol. 3, No. 11, (November 1997), pp. 1959-1968, ISSN 1078-0432
- Tait, D. L.; Obermiller, P. S.; Hatmaker, A. R.; Redlin-Frazier, S. & Holt, J. T. (1999). Ovarian cancer BRCA1 gene therapy: phase I and II trial differences in immune response and vector stability, *Clinical Cancer Research*, Vol. 5, No. 7, (July 1999), pp. 1708-1714, ISSN 1078-0432
- Takai, H.; Naka, K.; Okada, Y.; Watanabe, M.; Harada, N.; Saito, S.; Anderson, C. W.; Appella, E.; Nakanishi, M.; Suzuki, H.; Nagashima, K.; Sawa, H.; Ikeda, K. & Motoyama N. (2002). Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription, *The EMBO Journal*, Vol. 21, No. 19, (October 2002), pp. 5195-5205, ISSN 0261-4189
- Tanaka, H.; Arakawa, H.; Yamaguchi, T.; Shiraishi, K.; Fukuda, S.; Matsui, K.; Takei, Y. & Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage, *Nature*, Vol. 404, No. 6773, (March 2000), pp. 42-49, ISSN 0028-0836
- Taniguchi, T.; Tischkowitz, M.; Ameziane, N.; Hodgson, S. V.; Mathew, C. G.; Joenje, H.; Mok, S. C. & D'Andrea A. D. (2003). Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors, *Nature Medicine*, Vol. 9, No. 5, (May 2003), pp. 568-574, ISSN 1078-8956
- Tavtigian, S. V.; Simard, J.; Rommens, J.; Couch, F.; Shattuck-Eidens, D.; Neuhausen, S. & et al. (1996). The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds, *Nature Genetics*, Vol. 12, No. 3, (March 1996), pp. 333-337, ISSN 1061-4036
- Thanigaimani, S.; Kichenadasse, G.; & Mangoni, A. A. (2011). The emerging role of vascular endothelial growth factor (VEGF) in vascular homeostasis: lessons from recent

- trials with anti-VEGF drugs, *Current Vascular Pharmacology*, Vol. 9, No. 3, (May 2011), pp. 358-380, ISSN 1570-1611
- Thompson, D. & Easton, D. F.; The Breast Cancer Linkage Consortium. (2002). Cancer incidence in BRCA1 mutation carriers, *Journal of the National Cancer Institute*, Vol. 94, No. 18, (September 2002), pp. 1358-1365, ISSN 0027-8874
- Thompson, L. H. (2005). Unraveling the Fanconi anemia-DNA repair connection, *Nature Genetics*, Vol. 37, No. 9, (September 2005), pp. 921-922, ISSN 1061-4036
- Thomson, D. M.; Porter, B. B.; Tall, J. H.; Kim, H. J.; Barrow, J. R. & Winder, W. W. (2007). Skeletal muscle and heart LKB1 deficiency causes decreased voluntary running and reduced muscle mitochondrial marker enzyme expression in mice, *American Journal of Physiology. Endocrinology and Metabolism*, Vol. 292, No. 1, (January 2007), pp. E196-202, ISSN 0193-1849
- Tokunaga, E.; Oki, E.; Egashira, A.; Sadanaga, N.; Morita, M.; Kakeji, Y. & Maehara, Y. (2008). Deregulation of the Akt pathway in human cancer, *Current Cancer Drug Targets*, Vol. 8, No. 1, (February 2008), pp. 27-36, ISSN 1568-0096
- Toledo, F. & Wahl, G. M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas, *Nature Reviews. Cancer*, Vol. 6, No. 12, (December 2006), pp. 909-923, ISSN 1474-175X
- Tornaletti, S. (2009). DNA repair in mammalian cells: Transcription-coupled DNA repair: directing your effort where it's most needed, *Cellular and Molecular Life Sciences*, Vol. 66, No. 6, (March 2009), pp. 1010-1020, ISSN 1420-682X
- Torrents, E.; Aloy, P.; Gibert, I. & Rodríguez-Trelles, F. (2002). Ribonucleotide reductases: divergent evolution of an ancient enzyme, *Journal of Molecular Evolution*, Vol. 55, No. 2, (August 2002), pp. 138-152, ISSN 0022-2844
- Turner, N.; Tutt, A. & Ashworth, A. (2005). Targeting the DNA repair defect of BRCA tumours, *Current Opinion in Pharmacology*, Vol. 5, No. 4, (August 2005), pp. 388-393, ISSN 1471-4892
- Tutt, A.; Lord, C. J.; McCabe, N.; Farmer, H.; Turner, N.; Martin, N. M.; Jackson, S. P.; Smith, G. C. & Ashworth, A. (2005). Exploiting the DNA repair defect in BRCA mutant cells in the design of new therapeutic strategies for cancer, *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. 70, pp. 139-148, ISSN 0091-7451
- Tyner, S. D.; Venkatachalam, S.; Choi, J.; Jones, S.; Ghebranious, N.; Igelmann, H.; Lu, X.; Soron, G.; Cooper, B.; Brayton, C.; Hee Park, S.; Thompson, T.; Karsenty, G.; Bradley, A.; & Donehower, L. A. (2002). p53 mutant mice that display early ageing-associated phenotypes, *Nature*, Vol. 415, No. 6867, (January 2002), pp. 45-53, ISSN 0028-0836
- Veis, D. J.; Sorenson, C. M.; Shutter, J. R. & Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair, *Cell*, Vol. 75, No. 2, (October 1993), pp. 229-240, ISSN 0092-8674
- Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2, *Cell*, Vol. 108, No. 2, (January 2002), pp. 171-182, ISSN 0092-8674
- Verdoes, M.; Florea, B. I.; Menendez-Benito, V.; Maynard, C. J.; Witte, M. D.; van der Linden, W. A.; van den Nieuwendijk, A. M.; Hofmann, T.; Berkers, C. R.; van Leeuwen, F. W.; Groothuis, T. A.; Leeuwenburgh, M. A.; Ovaa, H.; Neeffjes, J. J.; Filippov, D. V.; van der Marel, G. A.; Dantuma, N. P. & Overkleeft, H. S. (2006). A fluorescent

- broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo, *Chemistry & Biology*, Vol. 13, No. 11, (November 2006), pp. 1217-1226, ISSN 1074-5521
- Voeller, D.; Rahman, L. & Zajac-Kaye, M. (2004). Elevated levels of thymidylate synthase linked to neoplastic transformation of mammalian cells, *Cell Cycle*, Vol. 3, No. 8, (August 2004), pp. 1005-1107, ISSN 1538-4101
- Wajant, H. (2002). The Fas signaling pathway: more than a paradigm, *Science*, Vol. 296, No. 5573, (May 2002), pp. 1635-1636, ISSN 0036-8075
- Wang, G. T.; Li, G.; Mantei, R. A.; Chen, Z.; Kovar, P.; Gu, W.; Xiao, Z.; Zhang, H.; Sham, H. L.; Sowin, T.; Rosenberg, S. H. & Lin, N. H. (2005). 1-(5-Chloro-2-alkoxyphenyl)-3-(5-cyanopyrazin-2-yl) ureas as potent and selective inhibitors of Chk1 kinase: synthesis, preliminary SAR, biological activities, *Journal of Medicinal Chemistry*, Vol. 48, No. 9, (May 2005), pp. 3118-3121, ISSN 0022-2623
- Wang, S. C.; Nakajima, Y.; Yu, Y. L.; Xia, W.; Chen, C. T.; Yang, C. C.; McIntush, E. W.; Li, L. Y.; Hawke, D. H.; Kobayashi, R. & Hung, M. C. (2006). Tyrosine phosphorylation controls PCNA function through protein stability, *Nature Cell Biology*, Vol. 8, No. 12, (December 2006), pp. 1359-1368, ISSN 1465-7392
- Wang, X.; Zhenchuk, A.; Wiman, K. G. & Albertioni, F. (2009). Regulation of p53R2 and its role as potential target for cancer therapy, *Cancer Letters*, Vol. 276, No. 1, (April 2009), pp. 1-7, ISSN 0304-3835
- White, J. S.; Choi, S. & Bakkenist, C. J. (2008). Irreversible chromosome damage accumulates rapidly in the absence of ATM kinase activity, *Cell Cycle*, Vol. 7, No. 9, (May 2008), pp. 1277-1284, ISSN 1538-4101
- Whitesell, L. & Lindquist, S. L. (2005). Hsp90 and the chaperoning of cancer, *Nature Reviews. Cancer*, Vol. 5, No. 10, (October 2005), pp. 761-772, ISSN 1474-175X
- Whiteside, D.; McLeod, R.; Graham, G.; Steckley, J. L.; Booth, K.; Somerville, M. J. & Andrew, S. E. (2002). A homozygous germ-line mutation in the human MSH2 gene predisposes to hematological malignancy and multiple cafe-au-lait spots, *Cancer Research*, Vol. 62, No. 2, (January 2002), pp. 359-362, ISSN 0008-5472
- Wilson, T. E. & Lieber, M. R. (1999). Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway, *Journal of Biological Chemistry*, Vol. 274, No. 33, (August 1999), pp. 23599-23609, ISSN 0021-9258
- Winder. W. W. (2001). Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle, *Journal of Applied Physiology*, Vol. 91, No. 3, (September 2001), pp. 1017-1028, ISSN 8750-7587
- Wooster, R.; Bignell, G.; Lancaster, J.; Swift, S.; Seal, S.; Mangion, J.; Collins, N.; Gregory, S.; Gumbs, C. & Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2, *Nature*, Vol. 378, No. 6559, (December 1995), pp. 789-792, ISSN 0028-0836
- Wu, J.; Gu, L.; Wang, H.; Geacintov, N. E. & Li, G. M. (1999). Mismatch repair processing of carcinogen-DNA adducts triggers apoptosis, *Molecular and Cellular Biology*, Vol. 19, No. 12, (December 1999), pp. 8292-8301, ISSN 0270-7306
- Xia, B.; Sheng, Q.; Nakanishi, K.; Ohashi, A.; Wu, J.; Christ, N.; Liu, X.; Jasin, M.; Couch, F. J. & Livingston, D.M. (2006). Control of BRCA2 cellular and clinical functions by a

- nuclear partner, PALB2, *Molecular Cell*, Vol. 22, No. 6, (June 2006), pp. 719-729, ISSN 1097-2765
- Xia, W.; Wei, Y.; Du, Y.; Liu, J.; Chang, B.; Yu, Y. L.; Huo, L. F.; Miller, S. & Hung, M. C. (2009). Nuclear expression of epidermal growth factor receptor is a novel prognostic value in patients with ovarian cancer, *Molecular Carcinogenesis*, Vol. 48, No. 7, (July 2009), pp. 610-617, ISSN 0899-1987
- Xu, X.; Weaver, Z.; Linke, S. P.; Li, C.; Gotay, J.; Wang, X. W.; Harris, C. C.; Ried, T. & Deng, C. X. (1999). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon isoform-deficient cells, *Molecular Cell*, Vol. 3, No. 3, (March 1999), pp. 389-395, ISSN 1097-2765
- Yamaguchi, T.; Matsuda, K.; Sagiya, Y.; Iwadate, M.; Fujino, M. A.; Nakamura, Y. & Arakawa, H. (2001). p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint, *Cancer Research*, Vol. 61, No. 22, (November 2001), pp. 8256-8262, ISSN 0008-5472
- Yap, T. A.; Garrett, M. D.; Walton, M. I.; Raynaud, F.; de Bono, J. S. & Workman, P. (August 2008). Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises, *Current Opinion in Pharmacology*, Vol. 8, No. 4, (August 2008), pp. 393-412, ISSN 1471-4892
- Yeaman, T. J.; Mule, J.; Dalton, W. S. & Sullivan, D. (2008). On the eve of personalized medicine in oncology, *Cancer Research*, Vol. 68, No. 18, (September 2008), pp. 7250-7252, ISSN 0008-5472
- Yokomakura, N.; Natsugoe, S.; Okumura, H.; Ikeda, R.; Uchikado, Y.; Mataka, Y.; Takatori, H.; Matsumoto, M.; Owaki, T.; Ishigami, S. & Aikou, T. (2007). Improvement in radiosensitivity using small interfering RNA targeting p53R2 in esophageal squamous cell carcinoma, *Oncology Reports*, Vol. 18, No. 3, (September 2007), pp. 561-567, ISSN 1021-335X
- Yoshino, T.; Shiina, H.; Urakami, S.; Kikuno, N.; Yoneda, T.; Shigeno, K. & Igawa, M. (2006). Bcl-2 expression as a redictive marker of hormone-refractory prostate cancer treated with taxane-based chemotherapy, *Clinical Cancer Research*, Vol. 12, No. 20 (Pt 1), (October 2006), pp. 6116-6124, ISSN 1078-0432
- Zanesi, N.; Pekarsky, Y.; Trapasso, F.; Calin, G. & Croce, C. M. (2010). MicroRNAs in mouse models of lymphoid malignancies, *Journal of Nucleic Acid Investigation*, Vol. 1, No. 1, (January 2010), pp. 36-40, ISSN 2035-6005
- Zhang, Y. W.; Otterness, D. M.; Chiang, G. G.; Xie, W.; Liu, Y. C.; Mercurio, F. & Abraham, R. T. (2005). Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway, *Molecular Cell*, Vol. 19, No. 5, (September 2005), pp. 607-618, ISSN 1097-2765
- Zhang, Y. W.; Brognard, J.; Coughlin, C.; You, Z.; Dolled-Filhart, M.; Aslanian, A.; Manning, G.; Abraham, R. T. & Hunter, T. (2009). The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress, *Molecular Cell*, Vol. 35, No. 4, (August 2009), pp. 442-453, ISSN 1097-2765
- Zhao, H. & Piwnicka-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1, *Molecular and Cellular Biology*, Vol. 21, No. 13, (July 2001), pp. 4129-4139, ISSN 0270-7306

- Zhou, B. B.; Anderson, H. J. & Roberge, M. (2003). Targeting DNA checkpoint kinases in cancer therapy, *Cancer Biology & Therapy*, Vol. 2, No. 4 Supplement 1, (July-August 2003), pp. S16-22, ISSN 1538-4047
- Zollner, T. M.; Podda, M.; Pien, C.; Elliott, P. J.; Kaufmann, R. & Boehncke, W. H. (2002). Proteasome inhibition reduces superantigenmediated T cell activation and the severity of psoriasis in a SCID-hu model, *Journal of Clinical Investigation*, Vol. 109, No. 5, (March 2002), pp. 671-679, ISSN 0021-9738

DNA Repair, Cancer and Cancer Therapy - The Current State of Art

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1. Introduction

Since the demonstration of double stranded DNA structure by Watson and Crick in 1953, knowledge about DNA structure and sequences has accumulated (Alberts et al., 2008). Although DNA contains complex genetic information in a very stable manner, DNA sequence and/or structure are sometimes disrupted. Previous research on the damage and repair mechanisms of DNA complex showed the existence of various types of DNA damage as well as the presence of sophisticated processes utilized by the cells to maintain the integrity of genome. DNA damage can be defined as any changes in the genomic integrity due to the disruptive impact of any exogenous and endogenous factors. Interestingly, it has been shown that damage to DNA is a usual event which is also underlying cause of many disorders including certain mutations and deletions leading to cancer and other inherited or acquired pathologies.

There are many endogenous and exogenous sources which cause DNA damages interfering with genome. Endogenous factors emerge from the DNA replication as well as recombination (Martin, 2008a). Although some of the exogenous factors may directly react with DNA, some of them tend to cause DNA damage by indirect route. Oxidation damage, alkylation of bases, hydrolysis of bases and replication errors are considered to be endogenous factors. Ultraviolet light, ionizing radiation and environmental chemical agents are among the exogenous factors.

1.1 Damage exerted by endogenous factors

1.1.1 Oxidative damage to bases

Normal metabolic events and external factors can lead to the formation of reactive oxygen species (ROS). ROS are molecules that contain an unpaired electron in their utmost outer orbital, which makes them very reactive. Generally, the ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2) and hydroxyl radicals ($\cdot OH$). These agents can oxidize DNA, causing damage such as oxidized bases, single strand and double strand breaks. Oxidatively damaged nucleotides can be found common in cells despite the extensive DNA repair. Not surprisingly, the amount of this damage is higher in cancer cells (Iida et al.,

2001). There are various ways by which the cell can endure the damage or decrease the number of the ROS (Slupphaug et al., 2003). Oxidative stress is observed when the natural antioxidant capacity of the cell cannot tolerate the ROS production. This results in the damage of the macromolecules such as DNA, proteins and lipids. ROS also affects the antioxidant enzymes as well (Tabatabaie et al., 1994). Studies have emphasized that any increase in the endogenous ROS generation or decrease in cellular antioxidants increases the mutation rate and inevitably raise the risk of cancer. Consuming antioxidant- rich diet has been proven to decrease the risk of cancer (Loft & Poulsen, 1996).

1.1.2 Alkylation of bases: Methylation

Oxygen is not the only reason of DNA damage in cells. Alkylating agents such as S-adenosylmethionine (SAM), which is a reactive methyl group donor, play significant roles in DNA damage. SAM methylates DNA, which is important for regulation of gene expression (Holliday & Ho, 1998) (Fig. 1). Endogenous methylation can also be carried out by betaine, choline and simple alkylating agents. Although this may occur endogenously, they might also be obtained from exogenous sources by environmental pollution and/or tobacco.

Most frequently, DNA methylation generates 7-methylguanine and 3-methyladenine. Having no effect on the coding specificity of the base, 7-methylguanine can be considered as less harmful (Zhao et al., 1999). However it can cause the blocking of DNA replication by the destabilization of the glycosyl bond. 3-methyladenine also blocks replication. DNA glycosylase is present in all living cells, removing 3-methyladenine from DNA. However, this action is found to be decreasing by age. 7-methylguanine repair is very insufficient and its accumulation can be observed in mammalian DNA (Atamna et al., 2000).



Fig. 1. Alkylation of bases

1.1.3 Hydrolysis of bases: Deamination, depurination / depyrimidation

Hydrolytic damage causes depurination, depyrimidation, and deamination of bases. The glycosidic bond in DNA structure is prone to breakage under heating, alkylation of bases or N-glycosylase activity (Lindahl et al., 1982). An abasic site is the result of the glycosidic bond cleavage in DNA. Apurinic (AP) sites can be produced spontaneously or by the effect of ROS (Nakamura et al, 2000). Abasic sites are among the most common endogenous lesions estimated to be 10 000 lesions/ cell/day. Hydrolytic deamination takes place frequently in DNA bases; however it is more frequent in single stranded DNA than double stranded. Deamination and methylation processes affect amino containing bases, cytosine, and adenine. In a cell, daily between 100 to 500 cytosines are deaminated to uracil. Deamination and methylation converts cytosine into uracil and/or mutant thymidine leading to wrong base pairs (Lindahl, 1993).

Human cells lose about 5000 adenines or guanines everyday because of spontaneous base-sugar link fissions. Less frequently, adenines spontaneously deaminate to give hypoxanthine (Alberts et al., 2008).

1.1.4 Errors in replication

In humans, 10^7 cells divide every second and it is estimated that one-third of these spontaneous mutations take place. These mutations are caused by mistakes in DNA replication and the copying of damaged templates by DNA polymerases. Scientists think that, errors mostly caused by mispairing of bases with different nature. This means pairing of nontautomeric chemical forms of different bases or pairing of normal bases but mismatch which is caused by a little shift of nucleotide positions. This mispairing is named as wobble and it occurs because of the flexibility of DNA double helix (Crick, 1966).

Any undetected error in replication will lead to mutant cell due to mismatch, (for example, mutant strand containing CG instead of AT). Fortunately, rate of replication error is as low as $1/10^5$ and with proof-reading mechanism this rate reduces up to $1/10^7$ - $1/10^9$ (Johnson et al., 2000).

1.2 Damage exerted by exogenous factors: Exogenous factors are composed of two main sources

1.2.1 Physical factors: UV light and ionizing radiation

Ultraviolet (UV) radiation, as a physical factor, is of solar origin and one of the most powerful exogenous agents disrupting genomic sequence. UV light is composed of three subtypes, UV-A, UV-B, UV-C. All has different wavelengths resulting in various effects on DNA. Compared with UV-A, UV-B has shorter wavelength (280-315 nm) and a more direct effect on DNA. It modifies chemical composition of DNA by forming dimers which disrupts molecular composition (Rastogi, 2010). TT dimerization is the most encountered form of UV-related damage. Thymidine dimers interfere with DNA replication by changing DNA polymerase (Arlett et al., 2006). Fortunately, UV-B radiation occupies a very small part of total solar energy. UV-A has weaker impact on DNA sequence because of its poor absorption by DNA. But, it tends to produce 1O_2 which can disrupt DNA sequence. This way is the indirect damaging of UV-A light on DNA. UV-C does not lead to considerable hazard on cells owing to its high absorption in atmosphere.

Radiation is also known to interfere with genome integrity. The mechanisms of the damage to DNA are as follows ;

- Rupture of the strand: It can divide as single or double strand breaks. Single strand breaks (SSBs) may occur at the phosphodiester bond, or at the bond between the sugar and the base. A large proportion of the SSBs are caused by OH \cdot . Double strand breaks (DSBs) involve breakage of both strands and are directly proportional to the radiation dosage.
- Alteration of bases: Bases can be damaged or destroyed by radiation. Among the bases, pyrimidines (T, C) are more vulnerable to radiation than purines.
- Destruction of sugars.
- Cross-links and formation of dimers (Alberts et al., 2008).

1.2.2 Chemical agents

Over a century, exposure to chemical agents has been known to induce cancer. After many studies on individuals who work with chemicals or exposed to the chemicals, researchers demonstrated the basic principles of chemical carcinogenesis. These chemicals establish

covalent bounds with bases on DNA and create DNA adducts. This structure is accepted as beginning of carcinogenesis (Poirier, 2004). Chemicals can cause other DNA disruption except adducts like strand cross-links, breakages and deletions. Some important chemical agents are; tobacco-specific N-nitrosamine, benzidine, aromatic amines, asbestos, benzene, aflatoxins, polycyclic aromatic hydrocarbons (Poirier, 2004, Loeb & Harris, 2008).

Aflatoxins are toxic metabolites of fungi and are carcinogenic in several animal species though with variable potency (CJ Chen & DS Chen, 2002, Zhang, 2010). Aflatoxin B1 (AFB1) which is a very toxic Aflatoxin type is a secondary metabolite of *Aspergillus flavus* and *Aspergillus parasiticus* and is known to be a human hepatocarcinogen (Zhang, 2010, Wogan & Newberne, 1967, Wogan, 1976, 1987, 1992). It may contaminate various food resources, which include but is not limited to, cereals such as rice, wheat and maize, as well as corn and peanuts which are stored in warm and humid places (Zhang, 2010, Wogan, 1976, Toteja et al., 2006, Matumba et al., 2009). AFB1 forms DNA adducts with guanine in the DNA of human hepatocytes and is thought to cause G: C to T: A transversion mutations, that acts as a cause of hepatocellular carcinogenesis (Hussain et al., 2007)

2. DNA repair mechanisms

In response to genotoxic stress, which can be mainly caused by various chemicals, reactive cellular metabolites and ionizing or UV radiation, DNA repair pathways and cell cycle checkpoints can be activated, allowing the cell to repair or prevent the transmission of damaged or incompletely replicated chromosomes. The balance between cell cycle arrest, DNA repair and the initiation of cell death could determine if DNA damage is compatible with cell survival or requires elimination of the cell by apoptosis. Defects of DNA repair pathways and cell cycle checkpoints may cause susceptibility to DNA damage, genomic defects, hypersensitivity to cellular stress and resistance to apoptosis, which characterize cancer cells (Ishikawa et al., 2006).

Major DNA repair pathways are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombinational repair (HR) and non-homologous end joining repair (NHEJ). These pathways each require a number of proteins and enzymes. By contrast, the direct removal of small alkyl groups (such as methyl groups) specifically from the O⁶ position of guanine and the O⁴ position of thymine in DNA is produced by the action of a single enzyme, O⁶-methylguanine-DNA methyltransferase (MGMT) (Fleck & Nielsen, 2004).

2.1 Base excision repair (BER)

BER is characterized by the excision of nucleic acid base residues in the free form (Friedberg et al., 1995). Conversely, NER removes damaged nucleotides which are approximately 30 nucleotides long. The primary and initiating event of BER is the hydrolysis of the N-glycosyl bond, therefore releasing the free base. This hydrolysis in DNA is catalyzed by a class of enzymes called DNA glycosylases (Fiedberg & Wood, 1996).

BER principally repairs non-bulky lesions produced by oxidation, alkylation or deamination of bases. Cells contain various DNA glycosylases, each of them showing a specific substrate spectrum. After hydrolysis of the N-glycosylic bond by a DNA glycosylase, the damaged base is released and an apurinic or apyrimidinic site (AP site) is produced. An AP site can also form spontaneously and represents damage itself (Fleck & Nielsen, 2004). The repair of base loss in these sites utilizes a specific class of endonucleases designated as AP endonucleases. APE1 is the major human AP endonuclease (Friedberg et al., 1995). Two

pathways can repair the AP site formed by a DNA glycosylase: These are short-patch BER (SP-BER) and long-patch BER (LP-BER) pathways. SP-BER involves a single nucleotide replacement followed by ligation (Hoeijmakers, 2001). DNA ligase III interacts with XRCC1, Pol β and poly(ADP-ribose) polymerase-1 (PARP-1) and is involved only in short-patch BER (Kubota et al., 1996). LP-BER involves DNA synthesis of multiple nucleotides (usually 2–6 nucleotides) (Hoeijmakers, 2001). The LP-BER depends on factors which are normally involved in DNA replication: DNA polymerase δ (POL δ) or ϵ (POL ϵ), proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) (Izumi et al., 2003; Sung & Demple, 2006). In LP-BER, the replaced strand does not exposed to degradation during polymerization but rather is displaced and cut away by DNAase IV or flap endonuclease 1 (FEN1), whereas the ligation step is performed by Ligase I (LIG1). LP-BER is usually used in yeast cells whereas SP-BER is generally used in mammalian cells (Altieri et al., 2008).

2.2 Nucleotide excision repair (NER)

NER mainly repairs bulky DNA adducts, such as UV-light-induced photolesions [(6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs)], intrastrand cross-links, large chemical adducts generated from exposure to aflatoxine, benzo[*a*]pyrene and other genotoxic agents (Christmann et al., 2003). The names of many of the genes found in NER, start with the letters “XP,” because they were first identified in human DNA-repair disease, Xeroderma pigmentosum (XP) (Altieri et al., 2008). NER consists of two repair subpathways: Global genome repair (GGR) and transcription-coupled repair (TCR). GGR removes damage in the whole genome whereas TCR specifically repairs the transcribed strand of active genes (Fleck & Nielsen, 2004).

2.2.1 Global genome repair (GGR)

The XPC/HR23B is the first NER factor to detect a lesion and recruit the repair machinery to the damaged site in GGR (Kusumoto et al., 2001). Another factor which participates the recognition of the damage is UV-DDB (UV-damaged-DNA-binding protein), consisting of two proteins, DDB1 and DDB2 (also known as XPE) (Altieri et al., 2008). The transcription factor transcription factor IIIH (TFIIH) is also recruited to the site of DNA damage (Yokoi et al., 2000). The binding of TFIIH is mediated by its p62 subunit which specifically interacts with XPC/HR23B, thus allowing the recruitment of TFIIH to the damaged site (Altieri et al., 2008). TFIIH harbors DNA helicase activity, which is exerted by its helicase subunits XPB and XPD (Schaeffer et al., 1993; Schaeffer et al., 1994). It is responsible for unwinding of DNA around the lesion (Evans et al., 1997a). Except the unwinding function, it is also responsible for the recruitment of XPA and XPG. The initial unwinding by XPB helicase opens up a small region and permits access of XPA to the damaged region. XPA also interacts with many other NER components like TFIIH, RPA and ERCC. The binding of XPA to TFIIH allows the complete unwinding of DNA helix to generate an open stretch of approximately 20-30 nucleotides, with the action of RPA. RPA is the major single-stranded DNA-binding protein required for eukaryotic metabolism. It is involved in many processes like DNA repair, replication, and recombination. RPA facilitates DNA unwinding by TFIIH through its ssDNA binding activity. After the interaction with XPA, RPA binds to the single, undamaged strand thus protecting it from nuclease attack (Lee et al., 2003). After binding of RPA and XPA, XPC/HR23B is released, allowing its recycling for other damaged sites where the repair mechanism must start (Altieri et al., 2008). After damage recognition and the formation of an open complex, removal of the lesion is performed by bidirectional incisions at determined positions flanking the DNA

damage (Evans et al., 1997b). 3'-incision is carried out by XPG (O'Donovan et al., 1994), and 5'-incision is performed by the XPF-ERCC1 complex (Sijbers et al., 1996). The arising DNA gap is filled in by the action of Pol δ and Pol ϵ and sealed by DNA ligase I and accessory factors (Araujo et al., 2000; Mu et al., 1995).

2.2.2 Transcription-coupled repair (TCR)

Transcription-coupled repair (TCR) is specifically removes DNA lesions in the genomic region where transcription is occurring simultaneously. Nowadays, it is not very clear how repair is coupled with active transcription, but it is generally supposed that a stalled transcript provides a strong signal to attract the repair mechanism. In this situation, the recognition factors intercede the dissociation of RNA polymerase II (RNAPII) from the DNA strand to allow the repair process to continue (Sarasin & Stary, 2007). Slow removal of DNA lesions from transcription templates would prevent efficient transcription. XPB, XPD (as part of TFIIH), XPG, CSA and CSB are essential for TCR (Le Page et al., 2000; Schaeffer et al., 1993). The reduction of transcription in CSB cells upon UV irradiation is caused by blockage of RNAPII at the photoproduct regions (Selby et al., 1997). When RNAPII is inactivated at the site of DNA lesions, CSA and CSB mediate the activation of NER pathway by release of the stalled RNAPII elongation complex from the damaged DNA. GGR and TCR could be related through a direct interaction of CSB and XPG (Iyer et al., 1996). In humans, TCR requires all the proteins needed for GCR except XPC, XPE and HR23B. The following steps of TCR are actually the same as for GCR with the development of the open complex and the lesion demarcation by XPA, RPA, and TFIIH, the excision of damaged strand, the filling by DNA polymerase, and the sealing of DNA fragments by a DNA ligase (Altieri et al., 2008).

2.3 Mismatch repair (MMR)

MMR is a type of DNA repair mechanism that targets base substitution and insertion/deletion mismatches resulting from errors formed during DNA replication and escaped from the proofreading activity of DNA polymerases, an event occurring with a frequency of approximately 1 in 10^9 - 10^{10} base pairs per cell division (Iyer et al., 2006). It is responsible for removal of these base mismatches which are caused by spontaneous and induced base deamination, methylation, oxidation and replication errors (Christmann et al., 2003).

MMR is well understood in *E. coli*, where the core enzymes of the repair system are the products of the mutH, mutL, and mutS genes. MMR can only protect cells from permanent mutations if the parental strand which contains the correct information can be accurately separated from the daughter strand. In *E. coli*, the strand discrimination signal is achieved by adenine methylation in GATC sequences. Newly replicated DNA is not still methylated on the daughter strand (Modrich, 1991; Friedberg et al., 1995). Thus MutH recognizes the temporarily unmethylated newly synthesized DNA strand and cleaves it at hemimethylated GATC sites which are located within 1,000 bp of the mismatch (Altieri et al., 2008). MutL protein mediates communication between the bound MutH and MutS products (Modrich, 1991). MutL also recruits UvrD at the damage site (Altieri et al., 2008). UvrD helicase and RPA generates a ssDNA filament containing the mismatched base, which is then cleaved by nuclease activities. Ultimately, DNA polymerase III refills the gap truly and DNA ligase III seals the last nick (Kunkel & Erie, 2005).

All eukaryotic organisms have MutS and MutL homologues, MSHs and MLHs, respectively. Five MSHs (MSH2-MSH6) are present in both yeast and mammals, whereas MSH1 exists

only in yeast. MSH2 is required for all mismatch correction in nuclear DNA, whereas MSH3 and MSH6 are required for the repair of some distinct types of mismatched DNA during replication. MSH4 and MSH5 are probably involved in meiotic recombination. Mammalian cells have two MutS activities that function as heterodimers and share MSH2 as a common subunit: MutS α (MSH2-MSH6 heterodimer) and MutS β (MSH2-MSH3 heterodimer) (Jascur & Boland, 2006; Modrich, 2006). Eukaryotic cell does not contain MutH protein. In this situation the problem is to find an entry point for the strand excision activities. The solution seems to rely on using nicks or gaps left behind by the progressing replication forks, which may explain the respective role of PCNA in MMR (Kunkel & Erie, 2005). Finally, the excision of the DNA strand which contains the mispaired base is carried out by exonuclease I (Genschel et al., 2002) and the new synthesis by Pol δ (Longley et al., 1997).

2.4 Double strand break repair (DSBR)

DNA DSBs are the most damaging lesions in the genome. They can form as a result of several damaging agents including ionizing radiation (IR) or chemical exposure (Ataian & Krebs, 2006). The major difference between DSBs and many other types of DNA lesions, including SSBs, is that in DSBs both DNA strands are damaged, which impedes the use of the complementary strand as template in the repair process (Genovese et al., 2006).

The two major pathways used by cells to repair DNA DSBs are homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is an error-free pathway which is usually found in simple eukaryotes (Cromie et al., 2001). Conversely, NHEJ is an error-prone pathway which is usually predominates in mammals (Dasika et al., 1999).

2.4.1 Homologous recombinational repair (HR)

HR is initiated by a nucleolytic excision of the DSB in the 5'-3' direction by the MRE11-RAD50-NBS1 (MRN) complex (Christmann et al., 2003). In the development of MRN complex, MRE11 and RAD50 produce the core complex and then they interact with NBS1. MRE11 has got various biochemical properties, such as DNA exonuclease activity, which can be stimulated by RAD50, DNA unwinding activity and single-strand DNA endonuclease activity. Contribution of MRN complex to DSB sites is supported by the binding of NBS1 to phosphorylated histone-H2AX. After DNA strand excision and protein binding, heteroduplex DNA is formed. This process requires BRCA2 and RAD51. BRCA2 is assigned in controlling the recombinase activity of RAD51 and its loading onto single-stranded DNA (Altieri et al., 2008). RAD51 is assisted by a number of protein factors including RAD52, RAD54, BRCA1 and RAD51 prologues (Rad51B, Rad51C, Rad51D, XRCC2, XRCC3) (Fleck & Nielsen, 2004). Afterwards the resulting 3' single-stranded DNA is bound by a heptameric ring complex formed by RAD52 proteins (Stasiak et al., 2000), which protects against exonucleolytic digestion. For binding of DNA ends, RAD52 competes with the Ku complex. This situation may determine whether the DSB is repaired by the HR or the NHEJ pathway (Van Dyck et al., 1999). RPA is another important protein that interacts with RAD51 (Golub et al., 1998). Interaction of RAD51 with RPA stabilizes RAD51-mediated DNA pairing by binding to the displaced DNA strand (Eggler et al., 2002). Thus, RAD51 catalyzes strand-exchange process with the complementary strand in which the damaged DNA invades the undamaged DNA duplex, displacing one strand as D-loop (Baumann & West, 1997; Gupta et al., 1998). After D-loop formation, the annealed 3'-end is then extended by repair synthesis through the original break site to restore the missing sequence

information at the break point. The sister chromatid provides a proper template for such error-free repair synthesis, actually it is the preferred template for homology-directed repair. On the other side of the D-loop, an "X" like structure, known as Holliday junction, is formed at the border between hetero- and homoduplex. If the Holliday junction is transported in the same direction as replication, it will release strand which is newly synthesized. Once repair synthesis is complete, the next step is to release the newly synthesized end, which can be performed by sliding the Holliday junction toward the 3'-end. Then the two broken ends reconnect. This process is facilitated by RAD52 and promoted by the annealing of complementary sequences. This process may generate gaps or flaps, depending on the degree of 3'-end extension during repair synthesis. Flaps can be removed by XPF/ERCC1 complex also remaining gaps are filled and sealed by PCNA-dependent DNA polymerase δ/ϵ and DNA ligase I (Altieri et al., 2008).

2.4.2 Non-homologous end joining repair (NHEJ)

The term "non-homologous" is used to describe this repair pathway. Also in this pathway 1–6 bp region of sequence homology (microhomology) near the DNA end frequently facilitates rejoining (Helleday et al., 2007). In contrast to NHEJ, HR is directed by longer stretches of homology (>100 bp) therefore the major difference between NHEJ and HR is the span of homologous sequences associated with repair process (Altieri et al., 2008).

In the first step heterodimeric complex consisting of the proteins Ku70 (Reeves & Stoeber, 1989) and Ku80 binds to the damaged DNA, thus protecting the DNA from exonuclease digestion (Altieri et al., 2008). Then the DNA–Ku complex attracts and activates the catalytic subunit (DNA-PKcs), a serine/threonine protein kinase. DNA-PKcs is autophosphorylated after juxtaposition of the two DNA ends (Helleday et al., 2007). If further processing of ends is not required, the complex attracts the additional core components, XRCC4, LIG4 and XLF, which together form the ligase complex and seal the DNA ends. In the presence of 3'- and 5'-overhangs, hairpins, gaps and flaps, characterized by single-strand/double-strand transitions DNA end joining requires an additional end processing before sealing (Altieri et al., 2008). Processing of DSBs is mainly performed by the MRN complex (Nelms et al., 1998) which has endonuclease, exonuclease, and helicase activity (Paull and Gellert, 1999) and removes excess DNA at 3' flaps. Flap endonuclease 1 (FEN1) is one of the candidate responsible for removal of 5' flaps (Wu et al., 1999). Other factors which are needed for processing includes structure-specific Artemis nuclease and/or the DNA polymerases POL μ , POL λ , and TdT (Altieri et al., 2008). Artemis acts in a complex with DNA-PK (Moshous et al., 2001). It also displays single-strand-specific exonuclease activity (Ma, 2002). In this process, Ku heterodimer interact with Artemis, and the LIG4/XRCC4 complex, thus organizing the activities and the reversible interaction of the processing factors with the core components (Altieri et al., 2008).

3. Cell cycle checkpoints

Cell cycle checkpoint signaling is activated in response to incomplete DNA replication due to DNA damages induced by both internal and external sources such as UV light, reactive oxygen species, ionizing radiation or DNA damaging chemotherapeutic agents. Active checkpoints prevent further progression during the cell cycle. If the genotoxic stress exceeds repair capacity, additional signaling pathways may cause cell death, probably via apoptosis. (Reinhardt & Yaffe, 2009b). If the damage level is low, the cell can deal with the lesions so it

does not need to activate the checkpoint signaling. If the lesions are not rapidly repairable or if damage level is high, checkpoint signaling mechanisms take place for cell survival and protection of genome integrity (Lazzaro et al., 2009). Cells can arrest at cell cycle checkpoints to allow for: (1) repairing of cellular damage; (2) the dissipation of an exogenous cellular stress signal; or (3) availability of required hormones, growth factors or nutrients (Pientepol & Stewart, 2002). Thus cell cycle checkpoints provide more time for repair of DNA damage before DNA replication and mitosis (Kaufmann & Paules, 1996).

3.1 G₁/S checkpoint

In the presence of DNA damage, the G₁/S checkpoint prevents replication of damaged DNA through two different signal transduction pathways. The first pathway involves the degradation of Cdc25A phosphatase. Chk2 and Chk1 activated by ATM and ATR phosphorylate Cdc25A, which is then degraded by the ubiquitin proteasome pathway (Shimada & Nakanishi, 2006). Cdc25A degradation results from the inactivation of Cdk2 and prevents Cdc45 from loading onto chromatin (Arata et al., 2000). Cdc45 is required for the recruitment of DNA polymerase α , thus lack of Cdc45 incorporation into the chromatin structure inhibits new origin firing. This pathway plays a role in the initial G₁/S checkpoint arrest. In order to maintain this arrest, transcriptional responses are mediated by p53 (Shimada & Nakanishi, 2006). ATM and ATR phosphorylate p53 at Ser15 (Banin et al, 1998; Canman et al, 1998), which inhibits the interaction of p53 with MDM2 (Shieh, 1997), thus p53 is stabilized. ATM also phosphorylates MDM2 on Ser395 and decreases the probability of an interaction between MDM2 and p53, thus p53 is accumulated (Maya et al., 2001) Stabilization and increased transactivation activity of p53 leads to the induction of p21, which inhibits the Cdk2-cyclin E-PCNA complex, thereby inhibiting G₁/S transition (Mailand et al., 2000). p21 also binds to the cyclin D-Cdk4 complex and prevents it from phosphorylating Rb, thus suppressing the Rb/E2F pathway (Nakanishi et al., 2006). Therefore, degradation of cyclin D1 which is a subunit of the cyclin-dependent kinase cdk4, is the critical step in promoting a rapid arrest in G₁. When cyclin D1 disappears, p21 is released from cdk4 complexes and binds rather than cdk2 complexes, preventing progression of cell cycle from G₁ into S phase (Walworth, 2000). In summary, the G₁/S checkpoint response targets two independent and critical tumor suppressor pathways, p53 and pRb, which are most commonly deregulated in cancers (Nakanishi et al., 2006)

3.2 S phase checkpoint

In the S phase, genotoxic stress can arise from DNA-damaging insults or from difficulties with the replication process. S-phase checkpoints are categorized into three types: (1) the replication-dependent intra-S-phase checkpoint (commonly named as replication checkpoint); (2) the replication-independent intra-S-phase checkpoint (generally named as intra-S-phase checkpoint), which can be induced by double strand breaks; and (3) the S-M checkpoint, which is also depends on DNA replication (Bartek et al., 2004). In the initiation of DNA damage checkpoints activity, the first step is the recognition of DNA damage. Studies in yeasts and mammals have demonstrated that RAD9, RAD1, HUS1 and RAD17 are required factors that activate checkpoint signaling (Melo & Toczyski, 2002). RAD9, RAD1 and HUS1 form a heterotrimeric complex which is known as 9-1-1 complex. RAD17 interacts with four small RFC subunits (Rfc2, Rfc3, Rfc4 and Rfc5) to form an RFC-related complex. When DNA is damaged, the 9-1-1 complex is recruited to the damage site under

the regulation of the RAD17 complex (Melo et al., 2001). In this process chromatin-bound 9-1-1 complex facilitates phosphorylation mediated by ATR and ATM (Nakanishi et al., 2006). During the S-phase, damaged DNA inhibits replicative DNA synthesis (intra-S checkpoint). This checkpoint is regulated by two distinct pathways, known as ATM/ATR-Chk1/Chk2-Cdc25A and ATM-NBS1-SMC1 (Falck et al., 2002). Depending on the type of DNA damage, ATM or ATR phosphorylates Chk2 or Chk1, respectively, resulting in the phosphorylation and degradation of Cdc25A (Shimada & Nakanishi, 2006). Downregulation of Cdc25A subsequently causes inactivation of the S-phase-promoting cyclin E/Cdk2 and prevents loading of Cdc45 on replication origins. The phosphorylation of NBS1 on S343 by ATM is required for activation of the MRN complex and the intra-S checkpoint (Lim et al., 2000; Zhao et al., 2000). Depending on the phosphorylation state of NBS1, SMC1 is phosphorylated on Ser-957 and Ser-966 by ATM which is required for the intra-S checkpoint (Kim et al., 2002; Yazdi et al., 2002). Other mediator proteins, such as 53BP1, BRCA1 and MDC1, are also involved in the intra-S checkpoint by regulating the phosphorylation of downstream proteins such as Chk1, Chk2, and NBS1 (Shimada & Nakanishi, 2006).

3.3 G₂/M checkpoint

The G₂/M checkpoint prevents cells from entry into mitosis through the inhibition of cyclinB/Cdc2 kinase by Chk1/Chk2, p38-mediated subcellular sequestration, degradation, and inhibition of the Cdc25 family of phosphatases. The initiation of G₂/M arrest is also carried out with p53 (Shimada & Nakanishi, 2006). After DNA damage, members of the PI-3K family initiate signal transduction pathways that regulate DNA repair and cell cycle progression. Various members of the PI-3K family can directly phosphorylate p53, including DNA-PK, ATM, and ATR. ATM-dependent signaling also results in activation of the Chk1 and Chk2 kinases (Pientepol & Stewart, 2002). Following DNA damage, the ATM-Chk2-Cdc25A and/or the ATR-Chk1-Cdc25A pathways are activated. 53BP1, MDC1, BRCA1 also play roles in the activation of Chk1 and Chk2 (Chan et al., 1999). MDC1 functions as a molecular bridge between histone γ -H2AX and NBS1 in the MRN complex (Nakanishi et al., 2006). Phosphorylated Cdc25A cause the degradation and inactivation of cyclinB/Cdc2. Many studies suggest that Chk1 and Chk2-mediated phosphorylation of p53 may be a crucial role for stabilization of the protein after DNA damage (Hirao et al., 2000; Shieh et al., 2000). The major targets of p53 at G₂/M checkpoint are the Cdk inhibitor p21, GADD45, which causes the dissociation of the Cdc2 and cyclin complex, and 14-3-3 sigma, which sequesters the cyclin B/Cdc2 complex in the cytoplasm (Chan et al., 1999). Two isoforms of MAP kinase, p38 α and γ are also implicated in the G₂/M checkpoint by the regulation of Cdc25B and Chk2, respectively. Cells which require these genes and enzymes exhibit a G₂/M checkpoint defect (Shimada & Nakanishi, 2006).

4. Polymorphisms of DNA repair genes

Many of the hereditary diseases with cancer predisposition are known to be caused by germ-line mutations of DNA repair genes (Paz-Elizur et al, 2008, Au, 2006). DNA repair deficiencies are milder in sporadic cancers than hereditary cases because of absence of germ-line mutations (Paz-Elizur et al, 2008). Generally, the response to DNA damage involves expression of various genes to repair. Susceptibility to the diseases caused by failure of DNA repair can depend on rare mutations in genes involved in DNA repair or on low penetrance single nucleotide polymorphisms (SNPs) (Ripperger et al., 2009). Although no

clinical decisions can be based on their presence or absence, polymorphisms of DNA repair-related genes may modulate cancer predisposition (Mocellin et al., 2009). SNPs occur when a single nucleotide in the genome sequence is altered; they occur every 100–300 bases along the 3 billion base human genome, so they contain about 90% of all human genetic variations that are thought to account for many health-related conditions, such as individualized drug responsiveness and disease predispositions including cancer (Mocellin et al., 2009, Paz-Elizur et al, 2008). Even though risks conferred by individual loci are relatively small, some risky alleles have been thought to act multiplicatively (Ripperger et al., 2009).

There are several different loci studied for various kinds of diseases and cancers. Cell cycle check-point and DNA repair gene polymorphisms are main foci in those studies.

8-oxoguanine formation is one of the major mutagenic oxidative DNA lesions, frequently used as a measure of oxidative stress. To protect DNA from such a damage, prevention, repair or proofreading must be operated; prevention is by avoiding the incorporation by the enzyme MTH1 to hydrolyse 8-oxo-dGTP; repair is by excising 8-oxoguanine from DNA by OGG1-initiated BER; and proofreading is by removing an adenine misincorporated opposite a template 8-oxoG by MUTYH-initiated BER in order to enable conversion of the premutagenic 8-oxoG:A mispair into a 8-oxoG:C base pair. BER is applied on oxoguanine primarily by 8-oxoguanine DNA glycosylase 1 (OGG1). The most common SNP studied in this gene is *OGG1*Ser326Cys thought to change the phosphorylation status of the enzyme. Studies on polymorphism in this gene region and others involved in BER of oxidative DNA damage, such as *APE1* or *XRCC1*, concluded no associations with cancer risk (Paz-Elizur et al, 2008). *XRCC* does not related to their biochemical functions; these genes only represent components of different damage recovery pathways (Basso et al., 2007). SSBs can be repaired by PARPs and *XRCC1* (Basso et al., 2007, Ladiges, 2006). *XRCC1* is known to have a large number of SNPs with its relative high frequency in the population (Basso et al., 2007, Au , 2006, Ladiges, 2006). *XRCC1* has no enzymatic activity, but has three interactive domains: the N-terminal domain (NTD) is the site for POL b binding and also the site for direct binding to gapped or nicked DNA. Previously, 27 gene variations of *XRCC1* were detected, the most frequent ones are R399Q and R194W (Ladiges, 2006). The *XRCC1* 194W allele was recently found to have a protective role against tobacco-related cancers. And the *XRCC1* 399Q allele was shown to behave as a risk factor for tobacco-related cancers in light smokers, but as a protective factor in heavy smokers (Basso et al., 2007).

The Xeroderma pigmentosum complementation group C (XPC) is one of the eight genes in the NER pathway; the others are *ERCC1*, *XPA*, *XPB*, *XPB*, *XPD*, *XPE*, *XPF* and *XPG*. XPC is involved in the DNA damage recognition and DNA repair initiation in the NER pathway, this is important because the binding of XPC to damaged DNA is the rate-limiting step of NER. Normal XPC gene is found to be critical for the cells to complete excision repair of bulky DNA lesions including smoking-induced DNA adducts. The results of studies about the association of XPC polymorphisms with cancer risk are contradicting (Qui et al., 2008)

XPD gene product is the adenosine triphosphate-dependent DNA helicase component of the transcription factor, TFIIH. The defects in *XPD/ERCC2* (the xeroderma pigmentosum group D (*XPD*) gene, also called the excision repair cross-complementing rodent repair deficiency group 2 (*ERCC2*) gene) are the cause of an autosomal recessive skin disorder characterized by solar hypersensitivity of the skin exposed to direct sunlight and so it has high risk for developing epithelial cancers and melanoma (Mocellin et al., 2009, Manuguerra et al., 2006). *XPD/ERCC2* is thought to be associated with the likelihood of harboring melanoma. The *XRCC3* protein is important in DNA DSBs/recombinational repair as a member of Rad51-

related protein family that is participating in HR to maintain chromosome stability and repair DNA damage (Manuguerra et al., 2006).

Breast cancer is the most common malignancy in women with an average lifetime risk of 8–10%. Breast cancer risk doubles in the women with a first degree relative with the same problem (Ripperger et al., 2009). Germline BRCA1 or BRCA2 mutations account for 20–40% of breast cancer in families, and they are associated with a high lifetime risk of 60–85% for breast cancer as well as an increased risk for ovarian cancer (Ripperger et al., 2009, Basso et al., 2007). In high risk families, some other genes are investigated to find their risk usually concentrating on genes involved in DNA repair as CHEK2, RAD50, BRIP1 and PALB2. Cell cycle checkpoint kinase 2 (CHEK2) is a signalling component of DNA repair phosphorylating BRCA1. Currently, there are several different SNPs in genes or chromosomal loci that have been identified in genome-wide association studies and a common SNP in CASP8 was found to reduce breast cancer risk (Ripperger et al., 2009).

Breast cancer cells are thought to be deficient in DSB repair (Ralhan et al., 2007). The products of key breast cancer susceptibility genes, BRCA1, BRCA2 (XRCC11), ATM and TP53, play important roles in DSB repair and chromosome stability (Ralhan et al., 2007, Basso et al., 2007). ATM mutation (7271T→G) has been suspected to be associated with an increased risk of breast cancer in relatives. The active ATM monomers phosphorylate various DSB repair and genome surveillance factors such as Artemis, NBS1, BRCA1, Fanconi anemia complementation group D2 protein (FANCD2), p53, p53-binding protein 1 (53BP1). Recently RAD50 germline mutations have been related to breast cancer susceptibility. P53 has several responses simultaneously, modulating DNA repair, blocking the cell cycle or inducing apoptosis in irreparably damaged cells. It inhibits strand exchange mediated by RAD51, binds Holliday junctions and detects mismatches in heteroduplex junction DNA (Ralhan et al., 2007).

The polymorphism studies are usually focused on cancer cases. Since the development of cancer involves the induction of multiple mutations, recent investigations are usually about the interactions of multiple susceptibility genes. But cancer is polygenic and single genetic variants are usually insufficient to predict risk of cancer. Consequently, the functional significance of these SNPs is still largely unknown. Using established common cancer susceptibility SNPs, there are hundreds of possible combinations of genotypes for each kinds of cancer in different populations. The studies for SNPs must be enlarged for various populations to be reliable.

5. DNA repair disorders

DNA repair disorders are usually characterized by X-ray sensitivity, cancer susceptibility, immunodeficiency, neurological abnormalities. DNA repair disorders can be in either of the repair types. Defects in the NER mechanism are responsible for several genetic disorders, including XP (hypersensitivity to sunlight/UV, resulting in increased skin cancer incidence and premature aging), Cockayne syndrome (hypersensitivity to UV and chemical agents), Trichothiodystrophy (sensitive skin, brittle hair and nails). The latter two usually accompanies with mental retardation. XP is an autosomal recessive hereditary disease with a prevalence of approximately 1–4 in 10⁶ live births characterized by severe predisposition to skin cancer, mainly squamous cell and basal cell carcinoma (Basso et al., 2007, Au, 2006). XP cells are defective in NER and known responsible genes are XP-A to XP-G (Basso et al., 2007).

Other DNA repair disorders include Werner's syndrome with growth retardation and premature aging; Bloom's syndrome with skin hypersensitivity and high incidence of malignancies and Ataxia telangiectasia (Louis-Bar syndrome) (ATM) with sensitivity to some chemicals and ionizing radiation. ATM mutations are found to be responsible for the autosomal recessive disease, ataxia telangiectasia, characterized by cerebellar ataxia, telangiectases, immune defects and predisposition to various malignancies (Table 1) (Ripperger et al., 2009). The most common DSB repair defects result from deficiencies in the ATM and NBS genes. The defects in NBS and DNA Ligase IV genes are chromosomal instability syndromes associated with various chromosomal aberrations and translocations (Nahas & Gatti, 2009). All those diseases are "progeria syndromes" means "accelerated aging diseases" because these patients suffer from aging-related diseases at an abnormally young age, while not manifesting all the symptoms of old age.

Hereditary breast cancer, hereditary colon cancer and Fanconi's anemia are also DNA repair diseases. Hereditary breast and ovarian cancer is the most frequent autosomal dominant disorder associated with mutations in BRCA1 or BRCA2. Additionally, there are several diseases increasing breast cancer risk. For instance, Peutz-Jeghers syndrome, caused by heterozygous germline mutations in STK11, is increasing the risk of breast cancer. It is mainly a polyposis syndrome characterized by melanocytic macules of the lips, digits as well as multiple hamartomatous polyps of gastrointestinal tract. Cowden syndrome is another one characterized by multiple hamartomas in skin, gastrointestinal tract, endometrium, breast and brain and it is associated with an increased breast cancer risk of up to 30–50% by the age of 70 years (Ripperger et al., 2009). Hereditary colon cancer is occurring by another defective DNA repair pathway, MMR, causing the predisposition to cancer. This condition leads to microsatellite instability (MSI) and frameshift mutations (Basso et al., 2007). MSI is also a common finding in colorectal tumors of Lynch syndrome patients (Basso et al., 2007, Ripperger et al., 2009). *MUTYH*-associated polyposis (MAP) is another disease with strong predisposition to a hereditary form of colorectal cancer, germ-line biallelic mutations in the *MUTYH* gene has been found to be responsible (Paz-Elizur et al, 2008). Another interesting disease is Fanconi Anemia, characterized by progressive bone marrow failure and multiple congenital abnormalities, which has been suggested to be caused by defects in coordination of NER, HR and translesional DNA synthesis (TLS). Fanconi anemia can be either autosomal recessive or X-linked recessive cancer susceptibility syndrome (Basso et al., 2007, Nahas & Gatti, 2009). Cells taken from Fanconi anemia patients exhibit hypersensitivity to mitomycin C, the DNA crosslinking agent. This hypersensitivity to cross-linking agents increases the risk to create chromosomal abnormalities. The characteristic feature for cell lines of patients deficient in DNA repair and chromatin maintenance proteins is the increased chromosomal aberration frequency (Nahas & Gatti, 2009). Another syndrome, Li-Fraumeni, is caused by germline TP53 mutations, has a high prevalence in breast cancer, soft tissue sarcomas, leukaemia and brain tumors in young population (Ripperger et al., 2009). There are several different genetic syndromes related to DNA repair, and because of the complexity of the repair pathways, various genes are found to be responsible from each. There are still some DNA repair syndromes without any known defective gene region. The XCIND syndrome comprises the chromosomal instability syndromes, the cancer susceptibility syndromes, the DNA DSB repair disorders, and the some primary immunodeficiencies (Nahas & Gatti, 2009).

SYNDROME	GENE	PRIMARY PATHOGENESIS	PATHWAY	PREDISPOSITION
Ataxia Telangiectasia	ATM	ATM protein kinase	HR, NHEJ	Immunodeficiency, cancer
Fanconi Anemia	Fanconi anemia genes (A, B, C, D1, D2, E, F, G, I, J, L, M), Rad50	Replication Fork/ Cell Cycle Checkpoint	Multiple pathways, crosslinking repair	myelofibrosis, leukemia, other cancers
X-linked Agammaglobulinemia (Bruton)	BTK	BTK gene function	NER	Immunodeficiency
Lynch Syndrome (Hereditary non-polyposis colorectal cancer : HNPCC)	MLH1, MSH2, MSH6, PMS1, PMS2	DNA repair/cell cycle checkpoint	MMR	Colon cancer (70-85%) Endometrial carcinoma (50%) Other cancers (15%)
Peutz-Jeghers	STK11 (LKB1)	Cell cycle checkpoint	Multiple pathways	Gastrointestinal hamartomatous polyps, breast cancer, other cancers
SCID -ADA	ADA	Toxicity of deoxyadenosine	NHEJ	Immunodeficiency
SCID-Artemis	artemis	DNA end-joining repair	NHEJ	Immunodeficiency
Xeroderma Pigmentosum	XP-A, XP-B		NER	UV-induced skin cancers
Nijmegen breakage Syndrome	NBS1	Double strand DNA repair	Multiple pathways, DSB cell signalling	Immunodeficiency, microcephaly, lymphoid malignancy
Multiple colorectal adenomas and carcinomas with no germline APC defect	MUTYH	Base excision repair	BER	Indicating mutations in BER genes are involved in cancer.
Werner's Syndrome	WRN RecQ helicase	Cell cycle checkpoint	HR, TLS	Premature aging, cancer
Bloom's Syndrome	BLM RecQ helicase	Cell cycle checkpoint	HR, TLS	Premature aging, cancer

Table 1. Genetic disorders involved in DNA repair pathways (Pollard & Gatti, 2009, Howlett et al., 2006, Donahue & Campbell, 2004, Mastrocola & Heinen, 2010, Pichierri et al., 2011, Masai, 2011)

The cause of the most of those XCIND syndromes is not determined, suggesting many new DNA repair proteins have yet to be identified. Advanced researches will determine those and perhaps even new paths of DNA repair. Those diseases are important to be resolved both for their susceptibility for various cancers and their illustrating capacity to understand cancer mechanisms and also aging.

6. DNA repair and cancer therapy

The genome is continually exposed to mutagenic stress from endogenous and exogenous insults that damage DNA (Martin et al., 2008b, Moeller et al., 2009, Liang et al., 2009). DNA repair mechanisms play a central role to overcome these damaging effects and maintain DNA integrity. Deregulation of the DNA repair mechanisms is associated with the development of cancer as well as other diseases (Amir et al., 2010, Megnin-Chanet et al., 2010). DNA damage repair mechanisms are required to prevent cancer. However, incomplete efficiency of these repair mechanisms is also required for genotoxic treatments (i.e. chemotherapy and/or radiotherapy) to achieve cure since DNA repair mechanisms greatly affect the response to cytotoxic treatments (Moeller et al., 2009, Rowe & Glazer, 2010). Most of the anticancer therapies lead to DNA damage to trigger death signals in cancer cells. The efficacy of cancer therapy is extensively influenced by DNA repair capacity. Based on this rationale, inhibitors of DNA repair proteins have been developed in cancer therapy, mostly to potentiate the effects of cytotoxic agents (Martin et al., 2008b).

6.1 DNA repair inhibitors as monotherapy (*Synthetic Lethality*)

When mutation of two genes in isolation is compatible with viability, but simultaneous mutation is lethal, these two genes are synthetically lethal (Martin et al., 2008b, Moeller et al., 2009, Mangerich & Burkle, 2011, Reinhardt et al., 2009a, Rowe & Glazer PM, 2010, Helleday et al., 2008). Accordingly, targeting a gene that is synthetic lethal to a cancer-relevant mutation should kill only malignant cells and preserve normal cells (Mangerich & Burkle, 2011). DNA repair is an ideal target for inhibition in cancer cells as the inhibitors should be exclusively toxic to cancer cells and be associated with minimal adverse effects for patients. Therefore, DNA repair inhibitors have been shown to work as single agents in patients with DNA repair defective tumors. The most remarkable example is the use of PARP inhibitors to treat patients with inherited breast and ovarian cancers that lack wild-type copies of BRCA1 and BRCA2 genes. PARP was discovered in 1963 by Chambon and his group. It is a multifunctional nuclear protein implicated in detection and signaling of DNA strand breaks introduced by oxidative stress, ionizing radiations and cytotoxic agents. PARP is involved in multiple cellular processes, such as DNA repair and maintenance of genomic integrity, regulation of transcription, epigenetic regulation, chromatin remodeling, death via necrosis and apoptosis, regulation of cellular replication and differentiation, inflammation, regulation of telomerase activity and protein degradation via ubiquitination. (Martin et al., 2008b, Sodhi et al., 2010, Rassool & Tomkinson, 2010, Moeller et al., 2009).

PARP-1 is the most studied and the founding member of the PARP family. It is a 116 kDa protein having substantially conserved structural and functional organization including an N-terminal double zinc finger DNA-binding domain (DBD), a nuclear localization signal, a central auto modification domain and a C-terminal catalytic domain (Sodhi et al., 2010, Megnin-Chanet et al., 2010, Mangerich & Burkle, 2011). Zinc-finger DBD detects and binds to sites of single-stranded DNA damage. PARP1 utilizes NAD⁺ as a substrate and catalyzes

the addition of ADP-ribose polymer side chains to itself, DNA ligase III, DNA polymerase- β , XRCC1, and other repair components, by that means recruiting and regulating the effectors of BER. The presence of PARP1 has been demonstrated to be required for efficient functioning of BER (Kupper JH et al., 1997, Sodhi et al., 2010, Mangerich & Burkle, 2011, Amir et al., 2010, Rowe & Glazer, 2010). Cells with defective BRCA-1 and BRCA-2 become highly dependent on other alternative repair pathways. One of those alternative routes is BER. This repair mechanism help prevent the development of DSBs in order to compensate for the inability of BRCA-mutant cells to repair DSB in an error-free manner. Inhibition of this pathway via PARP1 inhibitors increases the number of unrepaired SSBs, which eventually cause the collapse of the replication fork and produces DSBs. As a result, BRCA-defective cells are hypersensitive to the blockade of BER by the inhibition of PARP1 due to dysfunction of DSB repair. The non-tumor cells are better able to tolerate the PARP inhibition because their HR pathway is intact (Farmer et al., 2005, McCabe et al., 2005, Martin et al., 2008b, Amir et al., 2010, Rowe & Glazer, 2010).

Cells that are defective in recombination-related proteins other than BRCA1 or BRCA2, such as RAD51, RAD54, XRCC2, XRCC3, DSS1, replication protein A1, ATM, ATR, CHK1, CHK2, NBS1 and components of the Fanconi anaemia repair pathway, also show increased sensitivity to PARP inhibition. This suggests that PARP inhibitors might also be used in treating several types of tumors with defects in HR (Bryant et al., 2005, McCabe et al., 2006, Bryant & Helleday, 2006, Helleday et al., 2008).

Several phase I and II trials using PARP inhibitors for patients with breast, ovarian, and a variety of other malignancies are currently under way. Olaparib (AZD2281, KU-0059436, KuDOS Pharmaceuticals/AstraZeneca, Cambridge, UK) shows low toxicity, and there are suggestions of significant antitumor activity, as assessed by radiography and by measurement of tumor biomarkers (Yap et al., 2007, Martin et al., 2008b, Rassool & Tomkinson, 2010, Mangerich & Burkle, 2011, Amir et al., 2010, Rowe & Glazer, 2010, Megnin-Chanet et al., 2010, Helleday et al., 2008). Also, BSI-201 (BiPar Sciences/Sanofi-aventis, San Francisco, California), ABT-888 (Abbott Labs, Chicago, IL), AG-014699 (Agouron Pharmaceuticals/Pfizer Inc., La Jolla, CA), MK-4827 (Merck & Co Inc, Whitehouse Station, NJ) and Ccp-9722 (Abbott Labs, Chicago, IL) are strong inhibitors of PARP-1 and they are currently undergoing phase I or II testing both as monotherapy as well as in combination with a variety of different chemotherapy regimens.

Another synthetic lethal interaction has been determined between ATM and p53. Loss of ATM or Chk2 strongly increased the sensitivity of p53-deficient cells to doxorubicin-induced cell death. Inhibition of ATM/Chk2 in p53-deficient tumors provides an elegant synthetic lethality-based strategy to sensitize these tumors for DNA-damaging chemotherapy (Reinhardt et al., 2007, 2009a).

The major challenge in the area of synthetic lethal approaches to cancer treatment is the identification of new synthetic lethal pairs. Genome-wide RNAi screening and next generation sequencing of cell lines and primary tumors should allow the systematic search for new synthetic lethal relationships (Rowe & Glazer, 2010). With the exploitation of new synthetic lethal approaches it is possible that novel therapeutics can be identified that show strong selectivity for tumor cells, yield better response rates and lower toxicity.

6.2 DNA repair inhibitors in combination therapy

Several clinical and preclinical studies using PARP inhibitors in combination with cytotoxic agents including alkylating agents, topoisomerase inhibitors, DNA-crosslinking agents and

ionizing radiation (IR) have been conducted. The data showed that PARP inhibitors sensitize malignant cells to all of these agents and to IR. (Martin et al., 2008b, Sodhi et al., 2010, Mangerich & Burkle A, 2011, Rowe & Glazer PM, 2010, Megnin-Chanet et al., 2010, Helleday et al., 2008).

6.2.1 Alkylating agents

Temozolomide (TMZ) is an alkylating agent which is used as a single agent or in combination with IR in the therapy of glioblastoma multiforme and melanomas (Stupp et al., 2005, Mangerich & Burkle, 2011). TMZ can cross the blood brain barrier effectively and display limited bone marrow toxicity (Plummer et al., 2005, Mangerich & Burkle, 2011, Helleday et al., 2008). The therapeutic benefit of TMZ depends on its ability to methylate DNA which occurs mostly at N-7 and O-6 position of guanine residues. TMZ also methylates N-3 position of adenine. This methylation damages DNA and triggers the death of tumor cells. However, some tumor cells show resistance to TMZ by repairing this type of DNA damage and therefore diminish therapeutic efficacy of the drug. Tumor cells express O-6 methylguanine-DNA methyltransferase (MGMT) in response to drug and subsequently repair O6-methylguanine. (Hegi et al., 2005, Martin et al., 2008b, Mangerich & Burkle, 2011, Rowe & Glazer, 2010, Megnin-Chanet et al., 2010). A potent oral inhibitor of MGMT O-6(4bromothienyl) guanine has been used in combination with TMZ with Phase II trials ongoing in metastatic melanoma and colorectal cancer (Hegi et al., 2005). TMZ has been also used in combination with PARP inhibitors due to its mechanism of action. Methylation products of TMZ are repaired efficiently by BER. PARP activity increases after TMZ administration because of DNA damage induction. As PARP inhibition blocks BER, increased cytotoxic lesions become lethal via induction of apoptosis. However, TMZ resistance develops if there is a deficiency in the MMR, which contributes to TMZ cell killing when functional. MMR is required for the induction of DNA strand breaks after the formation of methyl products. PARP inhibitor AG14361 has been indicated to restore sensitivity to TMZ in MMR-deficient human colon and ovarian cancer cells (Curtin N et al., 2004).

6.2.2 Platinum drugs

Cisplatin, carboplatin and oxaliplatin are the most commonly used chemotherapeutic compounds in cancer patients (Helleday et al., 2008). These drugs cause inter- and intrastrand crosslinks that are repaired by NER. It has been suggested that upregulation of ERCC1 expression, is a key enzyme in NER, is associated with the resistance to platinum-based therapy. ERCC1 inhibitors have therefore been developed to deal with the resistance to platinum therapies (Altaha et al., 2004).

Another resistance to platinum drugs develops due to the silencing of MMR genes by hypermethylation. The toxicity of agents such as cisplatin depends on functional MMR. For this reason, DNA demethylating agents such as 2'-deoxy-5-azacytidine (decitabine; MGI Pharma, Bloomington, Minnesota, USA) have been combined with platinum compounds to reverse drug resistance. Preclinical data from xenograft models and translational studies from drug-resistant cells and tissues that are MMR-deficient owing to MLH1 hypermethylation have demonstrated increased chemotherapeutic efficacy when a demethylating agent is combined with platinum chemotherapy (Gifford et al., 2004, Plumb et al., 2000). Decitabine is currently being tested in combination with carboplatin in a phase II clinical trial in patients with ovarian cancer.

PARP1 has a direct role in the repair of damages triggered by platinum compounds. Thus, PARP1 inhibitors potentiate the effect of platinum compounds (Bartsch et al., 2010). PARP-1 inhibitors, in conjunction with platinum derivatives, were found to exhibit significant survival benefit over monotherapy in a relatively small phase II study. In this study, data were reported from a randomized phase II study of combination chemotherapy with carboplatin and gemcitabine with or without PARP1 inhibitor (BSI-201) in patients with triple negative breast cancer (O'Shaughnessy et al., 2009). In combined chemotherapy, PARP-inhibition is highly attractive, as carboplatin will cause DNA strand-breaks while BSI-201 will block PARP1-dependent repair (Bartsch et al., 2010). BSI-201 is currently in phase III clinical trials for breast cancer and squamous cell lung cancer therapy in combination with gemcitabine/carboplatin.

6.2.3 Topoisomerase inhibitors

Topoisomerases are a group of enzymes that resolve torsional strains enforced on the double helix during DNA replication. Topoisomerase 1 (Topo 1) induces transient SSBs by forming a covalent DNA-Topo 1 complex (Wang, 2002). Resealing of these breaks restores DNA integrity. (Koster et al., 2007, Pommier, 2006). Camptothecins are the inhibitors of Topo 1 which target Topo 1-DNA intermediate (Pommier, 2006, Waardenburg et al., 2004). Topotecan and irinotecan are the analogs of Camptothecins and they are used as anticancer agents in patients with ovarian, cervical and small cell lung cancer (Pommier, 2006). These inhibitors reversibly stabilize the covalent Topo 1- DNA intermediate by inhibiting DNA relegation. Topo 1-DNA-drug intermediates are converted to lethal lesions due to DSBs during replication (Waardenburg et al., 2004).

PARP1 induces Topo 1 activity in response to DNA damage. Thus, combined therapy of Topo1 inhibitors with PARP1 inhibitors may potentiate cytotoxic effects of Topo 1 inhibitors (Mangerich & Burkle, 2011, Bowman et al., 2001, Delaney et al., 2000).

In vitro combination experiments using platinum compounds with Topo 1 inhibitors showed a synergic effect in various cell lines (Waardenburg et al., 2004).

6.2.4 Ionizing radiation

DNA-dependent protein kinase (DNA-PK) is an essential enzyme in repairing DSBs by NHEJ following IR. DNA-PK is a member of phosphatidylinositol-3-kinase (PI3K) superfamily. After cellular exposures, DNA-PK is autophosphorylated, which is crucial for efficient NHEJ. Studies show that cells defective in DNA-PK are highly sensitive to IR which makes it an attractive molecular target for cancer therapies (Collis et al., 2005). Currently, a number of potent and selective DNA-PK inhibitors are available including Vanillin, Su11752, IC87102, IC87361, NU7441, NU7026 and Salvicine (Salles et al., 2006, Hollick et al., 2003, Leahy et al., 2004, Ismail et al., 2004).

PARP1 inhibition might have radiosensitizing effect following IR therapy which creates SSBs and DSBs since its inhibition introduces additional cytotoxicity to tumor cells (Dungey et al., 2008, Noel et al., 2006).

6.2.5 ATM inhibition

Two kinases from (PI3K)-related protein kinase family, ATM and ATR are central to cellular response to DSBs. Once the kinases are activated, many proteins are phosphorylated by ATM and ATR which initiates a cascade inducing cell-cycle arrest and facilitates DNA

repair. ATM inhibition makes tumor cells more sensitive to agents that cause DSBs (Helleday et al., 2008). KU55933, an inhibitor of ATM kinase activity, is currently in preclinical development.

PARP-deficient cells have been shown to be sensitive to KU55933 and NU7026, which is a DNA-PK inhibitor. Based on this data, a relationship between PARP1, ATM and DNA-PK may have value in terms of combination therapy in cancer patients (Bryant & Helleday, 2006).

It now seems likely that an understanding of how DNA damage contributes to tumorigenesis and how this damage is repaired can be used to design novel therapeutic approaches to cancer. In BRCA-associated cancers, the inhibition of BER with agents such as the PARP inhibitors may provide an effective synthetic lethality approach resulting in tumor cell death with minimal toxicity to normal tissues.

Even though the use of PARPi in cancer therapy has received much attention in recent years, some issues remain to be addressed carefully in the near future:

An important question is the issue of long-term safety. A major drawback in the systemic long-term treatment with PARP inhibitors is the damage to DNA repair and genomic stability in normal cells, which may lead to secondary tumors at later age. Basic research into obtaining a more complete picture of all DNA repair pathways and their interplay is crucial for solving the existing problems as well as for the future of DNA repair inhibitors in cancer therapy.

7. References

- Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K. & Walter P. (2002). *Molecular Biology of The Cell* Fourth ed. New York: Garland Science. pp. 235-298.
- Altaha, R.; Liang, X.; Yu, JJ. & Reed E. (2004). Excision repair cross complementing-group 1: gene expression and platinum resistance. *Int J Mol Med*, 14, pp. 959-970.
- Altieri, F.; Grillo, C.; Maceroni, M. & Chichiarelli, S. (2008). DNA Damage and Repair: From Molecular Mechanisms to Health Implications. *Antioxid Redox Signal*, 10(5), pp. 891-937.
- Amir, E.; Seruga, B.; Serrano, R. & Ocano, A. (2010). Targeting DNA repair in breast cancer: A clinical and translational update. *Cancer Treat Rev*, 36, pp. 557-565.
- Arata, Y.; Fujita, M.; Ohtani, K.; Kijima, S. & Kato J.Y. (2000) Cdk2-dependent and-independent Pathways in E2F-Mediated S Phase Induction. *J Biol Chem*, 275(9), pp. 6337-6345.
- Araujo, S.J.; Tirode, F.; Coin, F.; Pospiech, H.; Syvaaja, J.E.; Stucki, M.; Hubscher, U.; Egly, J.M. & Wood, R.D. (2000). Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes Dev*, 14, pp. 349-359.
- Arlett, CF.; Plowman, PN.; Rogers, PB.; Parris, CN.; Abbaszadeh, F.; Green, MH.; McMillan, TJ.; Bush, C.; Foray, N. & Lehmann, AR. (2006). Clinical and cellular ionizing radiation sensitivity in a patient with xeroderma pigmentosum. *Br J Radiol*, 79(942), pp. 510-7.
- Ataian, Y. & Krebs, J. (2006). Five Repair Pathways in One Context: Chromatin Modification during DNA Repair. *Biochem Cell Biol*, 84, pp. 490-504.

- Atamna, H.; Cheung, I. & Ames, BN. (2000). A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. *Proc Natl Acad Sci USA*, Jan 18; 97(2), pp. 686-91.
- Au, WW. (2006). Heritable susceptibility factors for the development of cancer. *J Radiat Res* (Tokyo), 47 Suppl B: B13-7.
- Banin, S.; Moyal, L.; Shieh, S; Taya, Y; Anderson, C.W.; Chessa, L.; Smorodinsky, N.I.; Prives, C.; Reiss, Y.; Shiloh, Y. & Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, 281, pp. 1674-1677.
- Bartek, J.; Lukas, C. & Lukas, J. (2004). Checking on DNA damage in S phase. *Nat Rev Mol Cell Biol*, 5, pp.792-804.
- Bartsch, R.; Ziebermayr, R.; Zielinski, CC. & Steger, GG. (2010). Triple-negative breast cancer. *Wien Med Wochenschr*. 160(7-8), pp. 174-181.
- Basso, D.; Navaglia, F.; Fogar, P.; Zambon, CF.; Greco, E.; Schiavon, S.; Fasolo, M.; Stranges, A.; Falda, A.; Padoan, A.; Fadi, E.; Pedrazzoli, S. & Plebani, M. (2007). DNA repair pathways and mitochondrial DNA mutations in gastrointestinal carcinogenesis. *Clin Chim Acta*, 381, pp. 50-55.
- Baumann, P. & West, S.C. (1997). The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A. *EMBO J*, 16, pp. 5198-5206.
- Bowman, K.J.; Newell, D.R.; Calvert, AH. & Curtin, N.J. (2001). Differential effects of the poly (ADP-ribose) polymerase (PARP) inhibitor NU1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells *in vitro*. *Br J Cancer*, 84, pp. 106-112.
- Bryant, H.E.; Schultz, N.; Thomas, H.D.; Parker, K.M.; Flower, D.; Lopez, E.; Kyle, S.; Meuth, M.; Curtin, N.J. & Helleday, T.(2005). *Nature*, 434, pp. 913-917.
- Bryant, H.; & Helleday, T. (2006). Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. *Nucleic Acids Res*, 34(6), pp. 1685-1691.
- Canman, C.E.; Lim, D.S.; Cimprich, K.A.; Taya, Y.; Tamai, K.; Sakaguchi, K.; Appella, E.; Kastan, M.B. & Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, 281, pp. 1677-1679.
- Chan, T.A.; Hermeking, H.; Lengauer, C.; Kinzler, K.W., & Vogelstein, B. (1999). 14-3-3 Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature*, 401, pp. 616-620.
- Chen, CJ & Chen, DS. (2002). Interaction of hepatitis B virus, chemical carcinogen, and genetic susceptibility: multistage hepatocarcinogenesis with multifactorial etiology. *Hepatology*, 36,pp. 1046-1049.
- Christmann, M.; Tomicic, M. T.; Roos, W. P. & Kaina, B. (2003). Mechanisms of Human DNA Repair: An Update. *Toxicology*, 193, pp. 3-34.
- Collis, SJ.; Deweese, TL.; Jeggo, PA. & Parker, AR. (2005). The life and death of DNA-PK. *Oncogene*, 24, pp. 949-961.
- Crick, FH. (1966). Codon-anticodon pairing: The wobble hypothesis. *J Mol Biol*, 19, pp. 548-555.
- Cromie, G.A.; Connelly, J.C. & Leach, D.R. (2001). Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. *Mol. Cell*, 8, pp. 1163-1174.

- Curtin, N.J.; Wang, L.Z.; Yiakouvaki, A.; Kyle, S.; Arris, C.A.; Canan-Koch, S.; Webber, S.E.; Durkacz, B.W.; Calvert, H.A.; Hostomsky, Z. & Newell, D.R. (2004). *Clin Cancer Res*, 10(3), pp. 881-9.
- Dasika, G. K.; Lin, S. J.; Zhao, S.; Sung, P.; Tomkinson, A. & Lee, E. Y. P. (1999). DNA Damage-induced Cell Cycle Checkpoints and DNA Strand Break Repair in Development and Tumorigenesis. *Oncogene*, 18, pp. 7883-7899.
- Delaney, C.A.; Wang, L.Z.; Kyle, S.; White, A.W.; Calvert, A.H.; Curtin, N.J.; Durkacz, B.W.; Hostomsky, Z. & Newell, D.R. (2000). Potentiation of temozolomide and topotecan growth inhibition and cytotoxicity by novel poly(adenosine diphosphoribose) polymerase inhibitors in a panel of human tumor cell lines. *Clin Cancer Res*, 6, pp. 2860-2867.
- Donahue, S.L. & Campbell, C. (2004). A Rad50-dependent pathway of DNA repair is deficient in Fanconi anemia fibroblasts. *Nucl. Acids Res.* 32, (10), pp. 3248-3257.
- Dungey, F.A.; Loser, D.A. & Chalmers, A.J. (2008). Replication-dependent radiosensitization of human glioma cells by inhibition of poly(ADP-Ribose) polymerase: mechanisms and therapeutic potential. *Int J Radiat Oncol Biol Phys* 2008, 72, pp. 1188-97.
- Eggler, A. L.; Inman, R. B. & Cox, M. M. (2002). The Rad51-dependent Pairing of Long DNA Substrates is Stabilized by Replication Protein A. *J Biol Chem*, 277, pp. 39280-39288.
- Evans, E.; Fellows, J.; Coffey, A. & Wood, R.D. (1997a). Open Complex Formation Around a Lesion During Nucleotide Excision Repair Provides a Structure for Cleavage by Human XPG Protein. *EMBO J*, 16, pp. 625-638.
- Evans, E.; Moggs, J. G.; Hwang, J. R.; Egly, J. M. & Wood, R. D. (1997b). Mechanism of Open Complex and Dual Incision Formation by Human Nucleotide Excision Repair Factors. *EMBO J*, 16, pp. 6559-6573.
- Falck, J, Petrini, J.H.; Williams, B.R.; Lukas, J. & Bartek J. (2002). The DNA Damage-dependent Intra-S Phase Checkpoint is Regulated by Parallel Pathways. *Nat Genet*, 30, pp. 290-294.
- Farmer, H.; McCabe, N.; Lord, C.J.; Tutt, A.N.; Johnson, D.A.; Richardson, T.B.; Santarosa, M.; Dillon, K.J.; Hickson, I.; Nightingale, C.; Martin, N.M.; Jackson, S.P.; Smith, G.C. & Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, 434(7035), pp.917-21.
- Fleck, O. & Nielsen, O. (2004). DNA Repair. *J Cell Sci*, 117, pp. 515-517.
- Friedberg, E.C.; Walker, G.C. & Siede, W. (1995). DNA repair and mutagenesis. *ASM Press*, Washington, D.C.
- Friedberg, E. C. & Wood R. (1996). DNA Excision Repair Pathways. *DNA Replication in Eukaryotic Cells*, Melvin L. DePamphilis, pp. 249-269, Cold Spring Harbor Laboratory Press, USA.
- Genschel, J.; Bazemore, L.R. & Modrich, P. (2002). Human exonuclease I is required for 5' and 3' mismatch repair. *J Biol Chem*, 277, pp. 13302-13311.
- Genovese, C.; Trani, D.; Caputi, M. & Claudio, P. P. (2006). Cell Cycle Control and Beyond: Emerging Roles for the Retinoblastoma Gene Family. *Oncogene*, 25, pp. 5201-5209.
- Gifford, G.; Paul, J.; Vasey, P. A.; Kaye, S. B. & Brown, R. (2004). The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. *Clin. Cancer Res*, 10, pp. 4420-4426.

- Golub, E.I.; Gupta, R.C.; Haaf, T.; Wold, M.S. & Radding, C.M. (1998). Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. *Nucleic Acids Res*, 26, pp. 5388–5393.
- Gupta, R.C.; Golub, E.I.; Wold, M.S. & Radding, C.M. (1998). Polarity of DNA strand exchange promoted by recombination proteins of the RecA family. *Proc Natl Acad Sci USA*, 95, pp. 9843–9848.
- Hegi, M.E.; Diserens, A.C.; Gorlia, T.; Hamou, M.F.; de Tribolet, N.; Weller, M.; Kros, J.M.; Hainfellner, J.A.; Mason, W.; Mariani, L.; Bromberg, J.E.; Hau, P.; Mirimanoff, R.O.; Cairncross, J.G.; Janzer, R.C. & Stupp, R. (2005). *N Eng J Med*, 352, pp.997-1003.
- Helleday, T.; Lo, J.; Van Gent, D. C. & Engelward, B. P. (2007). cDNA Double-strand Break Repair: From Mechanistic Understanding to Cancer Treatment. *DNA Repair*, 6, pp. 923–935.
- Helleday, T.; Peterman, E.; Lundin, C.; Hodgson, B. & Sharma, RA. (2008). DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer*, 8, pp. 193-204.
- Hirao, A.; Kong, Y.Y.; Matsuoka, S.; Wakeham, A.; Ruland, J.; Yoshida, H.; Liu, D.; Elledge, S.J. & Mak, T.W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science*, 287, pp. 1824-1827.
- Hoeijmakers, J.H. 2001. Genome maintenance mechanisms for preventing cancer. *Nature*, 411, pp. 366–374.
- Hollick, J.J.; Golding, B.T.; Hardcastle, I.R.; Martin, N.; Richardson, C.; Rigoreau, L.J. et al. (2003). 2,6-disubstituted pyran-4-one and thiopyran-4-one inhibitors of DNA-Dependent protein kinase (DNA-PK). *Bioorg Med Chem Lett*, 13, pp. 3083–6.
- Holliday, R.; & Ho, T. (1998) Gene silencing and endogenous DNA methylation in mammalian cells. *Mutat Res*. May 25; 400(1-2), pp. 361-8.
- Howlett, N.G.; Scuric, Z.; D'Andrea, A.D. & Schiestl, R.H. (2006). Impaired DNA double strand break repair in cells from Nijmegen breakage syndrome patients. *DNA Repair*, 5, pp. 251-257.
- Hussain, SP.; Schwank, J.; Staib, F.; Wang, XW. & Harris, CC. (2007). TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*, 26, pp. 2166-2176.
- Iida, T.; Kijima, H.; Urata, Y.; Goto, S.; Ihara, Y.; Oka, M.; Kohno, S.; Scanlon, KJ. & Kondo, T.; (2001). Hammerhead ribozyme against gamma-glutamylcysteine synthetase sensitizes human colonic cancer cells to cisplatin by down-regulating both the glutathione synthesis and the expression of multidrug resistance proteins. *Cancer Gene Ther*, Oct; 8(10), pp. 803-14.
- Ishikawa, K.; Ishii, H. & Saito, T. (2006). DNA Damage-Dependent Cell Cycle Checkpoints and Genomic Stability. *DNA and Cell Biology*, 25(7), pp. 406-411.
- Ismail, I.H.; Martensson, S.; Moshinsky, D.; Rice, A.; Tang, C.; Howlett, A.; et al. (2004). SU11752 inhibits the DNA-dependent protein kinase and DNA double-strand break repair resulting in ionizing radiation sensitization. *Oncogene*, 23, pp. 873–82.
- Iyer, N.; Reagan, M. S.; Wu, K. J.; Canagarajah, B. & Friedberg, E.C. (1996). Interactions Involving the Human RNA Polymerase II Transcription/nucleotide Excision Repair Complex TFIIH, the Nucleotide Excision Repair Protein XPG, and Cockayne Syndrome Group B (CSB) Protein. *Biochemistry*, 35, pp. 2157–2167.
- Iyer, R.R.; Pluciennik, A.; Burdett, V. & Modrich PL. (2006). DNA mismatch repair: functions and mechanisms, *Chem Rev*, 106, pp. 302–323.

- Izumi, T.; Wiederhold, L.R.; Roy, G.; Roy, R.; Jaiswal, A.; Bhakat, K.K.; Mitra, S. & Hazra, T.K. (2003). Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage. *Toxicology*, 193, pp. 43-65.
- Jascur, T. & Boland, C.L. (2006). Structure and function of the components of the human DNA mismatch repair system. *Int J Cancer*, 119, pp. 2030-2035.
- Johnson, R.E.; Washington, M.T.; Prakash, S. & Prakash, L. (2000). Fidelity of human DNA polymerase. *J Biol Chem*, 275, pp.7447-7450.
- Kaufmann, W. K. & Paules R. S. (1996). DNA Damage and Cell Cycle Checkpoints. *FASEB J*, 10, pp. 238-247.
- Kim, S.T.; Xu, B. & Kastan, M.B. (2002). Involvement of the cohesion protein, Smc1, in Atm dependent and independent responses to DNA damage. *Genes Dev*, 15, pp. 560-570.
- Koster, D. A.; Palle, K.; Bot, E. S. M.; Bjornsti, M.A. & Dekker, N. H. (2007). Antitumour drugs impede DNA uncoiling by topoisomerase I. *Nature*, 448, pp. 213-217.
- Kubota, Y.; Nash, R.A.; Klungland, A.; Schar, P.; Barnes, D.E. & Lindahl, T. (1996). Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. *EMBO J*, 15, pp. 6662-6670.
- Kunkel, T.A. & Erie, D.A. (2005). DNA mismatch repair. *Annu Rev Biochem*, 74, pp. 681-710.
- Kupper, J.H.; Wolf, I. & Burkle, A. (1997). NAD⁺ loading of mammalian cells by electrotransfection leads to increased poly (ADP-ribosyl)ation capacity. *Biochimie*, 79(4), pp.175-8.
- Kusumoto, R.; Masutani, C.; Sugasawa, K.; Iwai, S.; Araki, M.; Uchida, A.; Mizukoshi, T. & Hanaoka, F. (2001). Diversity of the Damage Recognition Step in the Global Genomic Nucleotide Excision Repair *in vitro*. *Mutat Res*, 485, pp. 219-227.
- Ladiges, W.C. (2006). Mouse models of XRCC1 DNA repair polymorphisms and cancer. *Oncogene*, Mar 13;25(11), pp. 1612-9.
- Lazzaro, F.; Giannattasio, M.; Puddu, F.; Granata, M.; Pellicoli, A.; Plevani, P. & Muzi-Falconi, M. (2009). Checkpoint Mechanisms at the Intersection between DNA Damage and Repair. *DNA Repair*, 8, pp. 1055-1067.
- Leahy, J.J.; Golding, B.T.; Griffin, R.J.; Hardcastle, I.R.; Richardson, C.; Rigoreau, L. et al. (2004). Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. *Bioorg Med Chem Lett*, 14, pp. 6083-7.
- Lee, J. H.; Park, C. J.; Arunkumar, A. I.; Chazin, W.J. & Choi, B. S. (2003). NMR Study on the Interaction Between RPA and DNA Decamer Containing cis-syn Cyclobutane Pyrimidine Dimer in the Presence of XPA: Implication for Damage Verification and Strand-Specific Dual Incision in Nucleotide Excision Repair. *Nucleic Acids Res*, 31, pp. 4747-4754.
- Le Page, F.; Kwoh, E.E.; Avrutskaya, A.; Gentil, A.; Leadon, S.A.; Sarasin, A. & Cooper, P.K.. (2000). Transcription-coupled repair of 8-oxoguanine: requirement for XPG, TFIIH, CSB and implications for Cockayne syndrome. *Cell*, 101, pp. 159-171.
- Liang, Y.; Lin, S.Y.; Brunicardi, F.C.; Goss, J. & Li, K. (2009). DNA damage response pathways in tumor suppression and cancer treatment. *World J Surg*, 33, pp. 661-666.
- Lim, D.S.; Kim, S.T.; Xu, B.; Maser, R.S.; Lin, J.; Petrini, J.H. & Kastan, M.B. (2000). ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature*, 404, pp. 613-17.

- Lindahl, T.; Rydberg, B.; Hjelmgren, T.; Olsson, M. & Jacobsson, A. (1982). Cellular defense mechanisms against alkylation of DNA. *Basic Life Sci*, 20, pp. 89-102.
- Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature*, Apr 22; 362(6422), pp. 709-15.
- Loeb, LA & Harris, CC. (2008). Advances in chemical carcinogenesis: A historical review and prospective. *Cancer Res*, 68, pp. 6863-72.
- Loft, S.; & Poulsen, HE. (1996). Cancer risk and oxidative DNA damage in man. *J Mol Med*, Jun; 74(6), pp. 297-312.
- Longley, M. J.; Pierce, A. J. & Modrich, P. (1997). DNA Polymerase Delta is Required for Human Mismatch Repair *in vitro*. *J Biol Chem*, 272, pp. 10917-10921
- Ma, Q.; Baldwin, K.T.; Renzelli, A.J.; McDaniel, A. & Dong, L. (2001). TCDD-inducible poly (ADP-ribose) polymerase: a novel response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem Biophys Res Commun* 289, pp. 499-506.
- Mailand, N.; Falck, J.; Lukas, C.; Syljuasen, R.G.; Welcker, M.; Bartek, J. & Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. *Science*, 288, pp. 1425-1429.
- Mangerich, A.; & Burkle, A. (2011). How to kill tumor cells with inhibitors of poly (ADP-ribose)ylation. *Int J Cancer*, 128, pp. 251-265.
- Manuguerra, M.; Saletta, F.; Karagas, MR.; Berwick, M.; Veglia, F.; Vineis, P. & Matullo, G. (2006). XRCC3 and XPD/ERCC2 single nucleotide polymorphisms and the risk of cancer: a HuGE review. *Am J Epidemiol*. Aug 15, 164(4), pp. 297-302.
- Martin, LJ. (2008a) DNA damage and Repair: Relevance to mechanism of Neurodegeneration. *J Neuropathol Exp Neurol*, 67(5), pp. 377-387.
- Martin, SA.; Lord, CJ. & Ashworth, A. (2008b). DNA repair deficiency as a therapeutic target in cancer. *Curr Opin Genet Dev*, 18, pp. 80-86.
- Masai, H. (2011). RecQL4: a helicase linking formation and maintenance of a replication fork. *J Biochem*, [Epub ahead of print].
- Mastrocola, A.S. & Heinen, C.D.(2010). Lynch syndrome-associated mutations in MSH2 alter DNA repair and checkpoint response functions in vivo. *Hum Mutat*. 10, pp. 1699-1708.
- Matumba, L.; Monjerezi, M.; Chirwa, E.; Lakudzala, D.; Mumba, P.(2009) Natural occurrence of AFB1 in maize and effect of traditional maize flour production on AFB1 reduction in Malawi. *African Journal of Food Science* Vol 3.(12) pp. 413-425.
- Maya, R.; Balass, M.; Kim, S.T.; Shkedy, D.; Leal, J.F.; Shifman, O.; Moas, M.; Buschmann, T.; Ronai, Z.; Shiloh, Y.; Kastan, M.B.; Katzir, E. & Oren, M. (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev*, 15, pp. 1067-1077.
- McCabe, N.; Lord, C.J.; Tutt, A.N.; Martin, N.M.; Smith, G.C. & Ashworth, A. (2005). BRCA2 deficient CAPAN-1 cells are extremely sensitive to the inhibition of Poly(ADP-ribose)polymerase: an issue of potency. *Cancer Biol Ther*, 9, 934-6.
- McCabe, N.; Turner, N.C.; Lord, C.J.; Kluzek, K.; Bialkowska, A.; Swift, S.; Giavara, S.; O'Connor, M.J.; Tutt, A.N.; Zdzienicka, M.Z.; Smith, G.C. & Ashworth, A.(2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose)polymerase inhibition. *Cancer Res*, 66, pp. 8109-8115.
- Megnin-Chanet, F.; Bollet, MA. & Hall, J. (2010). Targeting poly (ADP-ribose) polymerase activity for cancer therapy. *Cell Mol Life Sci*, 67, pp. 3649-3662.

- Melo, J.; Cohen, J. & Toczyski, D. (2001). Two Checkpoint Complexes are Independently Recruited to Sites of DNA Damage *in vivo*. *Gene Dev*, 15, pp. 2809–2821.
- Melo, J. & Toczyski, D. A unified view of the DNA-damage checkpoint. (2002). *Curr Opin Cell Biol*, 14, pp. 237–45.
- Mocellin, S.; Verdi, D. & Nitti, D. (2009). DNA repair gene polymorphisms and risk of cutaneous melanoma: a systematic review and meta-analysis. *Carcinogenesis*, Oct; 30(10), pp. 1735–43.
- Modrich, P. Mechanisms in eukaryotic mismatch repair. (2006). *J Biol Chem*, 281, pp. 30305–30309.
- Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. *Annu Rev Genet*, 25, pp. 229–253.
- Moeller, B.J.; Pasqualini, R. & Arap, W. (2009). Targeting cancer-specific lethality in double-strand DNA break repair. *Cell Cycle*, 8(12), pp. 1872–1876.
- Moshous, D.; Callebaut, I.; de Chasseval, R.; Corneo, B.; Cavazzana-Calvo, M.; Le Deist, F.; Tezcan, I.; Sanal, O.; Bertrand, Y.; Philippe, N.; Fischer A. & Villartay J.P. (2001). Artemis, a Novel DNA Double-strand Break Repair/V(D)J Recombination Protein, is Mutated in Human Severe Combined Immune Deficiency. *Cell*, 105, pp. 177–186.
- Mu, D.; Park, C.H.; Matsunaga, T.; Hsu, D.S.; Reardon, J.T. & Sancar, A. (1995). Reconstitution of human DNA repair excision nuclease in a highly defined system. *J Biol Chem*, 270, pp. 2415–2418.
- Nahas, S.A.; & Gatti, R.A. (2009). DNA double strand break repair defects, primary immunodeficiency disorders, and 'radiosensitivity'. *Curr Opin Allergy Clin Immunol*. Dec;9(6), pp. 510–6.
- Nakamura, J.; La, D.K. & Swenberg J.A. (2000). 5'-nicked apurinic/apyrimidinic sites are resistant to beta-elimination by beta-polymerase and are persistent in human cultured cells after oxidative stress. *J Biol Chem*, Feb 25, 275(8), pp. 5323–8.
- Nakanishi, M.; Shimada, M. & Niida, H. (2006). Genetic Instability in Cancer Cells by Impaired Cell Cycle Checkpoints. *Cancer Sci*, 97(10), pp. 984–989.
- Nelms, B.E.; Maser, R.S.; MacKay, J.F.; Lagally, M.G. & Petrini, J.H. (1998). In situ visualization of DNA double-strand break repair in human fibroblasts. *Science*, 280, pp. 590–592.
- Noel, G.; Godon, C.; Fernet, M.; Giocanti, N.; Megnin-Chanet, F. & Favaudon, V. (2006). Radiosensitization by the poly(ADP-ribose) polymerase inhibitor 4-amino-1,8-naphthalimide is specific of the S phase of the cell cycle and involves arrest of DNA synthesis. *Mol Cancer Ther*, 5, pp. 564–74.
- O'Donovan, A.; Davies, A.A.; Moggs, J.G.; West, S.C. & Wood, R.D. (1994). XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature*, 371, pp. 432–435.
- O'Shaughnessy, J.; Osborne, C.; Pippen, J.; et al. (2009). Efficacy of BSI-201, a poly (ADP-ribose) polymerase-1 (PARP1) inhibitor, in combination with gemcitabine/carboplatin (G/C) in patients with metastatic triple-negative breast cancer (TNBC): Results of a randomized phase II trial. *J Clin Oncol*, 27(Suppl 15) pp. 793S.
- Paull, T.T. & Gellert, M. (1999). Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev*, 13, pp. 1276–1288.

- Paz-Elizur, T.; Sevilya, Z.; Leitner-Dagan, Y.; Elinger, D.; Roisman, L. & Livneh Z. (2008). DNA repair of oxidative DNA damage in human carcinogenesis Potential application for cancer risk assessment and prevention. *Cancer Lett* July 18; 266(1): 60-72.
- Pichierri, P.; Ammazalorso, F.; Bignami, M. & Franchitto, A. (2011). The Werner syndrome protein: linking the replication checkpoint response to genome stability. *Aging (Albany NY)*, 3, pp. 311-8.
- Pietenpol, J. A. & Stewart, Z. A. (2002). Cell Cycle Checkpoint Signaling: Cell Cycle Arrest versus Apoptosis. *Toxicology*, Vol. 181-182, pp. 475-481.
- Plumb, J. A.; Strathdee, G.; Sludden, J.; Kaye, S. B. & Brown, R. (2000). Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res*, 60, pp. 6039-6044.
- Plummer, E.R.; Middleton, M.R.; Jones, C.; Olsen, A.; Hickson, I.; McHugh, P.; Margison, G.P.; McGown, G.; Thorncroft, M.; Watson, A.J.; Boddy, A.V.; Calvert, A.H.; Harris, A.L.; Newell, D.R. & Curtin, N.J. (2005), *Clin Cancer Res*, 11, pp.3402-9.
- Poirier, MC. (2004). Chemical-induced DNA damage and human cancer risk. *Nat Rev Cancer* 4, 630-637.
- Pollard, J.M.; & Gatti, R.A. (2009). Clinical Radiation Sensitivity with DNA Repair Disorders An Overview. *Int J Radiat Oncol Biol Phys*, 74(5), pp.1323-1331.
- Pommier, Y. (2006). Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer*, 6:789-802.
- Qiu, L.; Wang, Z.; Shi, X. & Wang, Z. (2008). Associations between XPC polymorphisms and risk of cancers: A meta-analysis. *Eur J Cancer*. Oct;44(15):2241-53.
- Ralhan, R.; Kaur, J.; Kreienberg, R.; Wiesmuller, L. (2007). Links between DNA double strand break repair and breast cancer: accumulating evidence from both familial and nonfamilial cases. *Cancer Lett*, Apr 8;248(1):1-17.
- Rassool, FV. & Tomkinson, AE. (2010). Targeting abnormal DNA double strand break repair in cancer. *Cell Mol Life Sci*, 67:3699-3710.
- Rastogi, RP.; Kumar, A.; Tyagi, MB. & Sinha, RP. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *J Nucleic Acids*. ,pp. 592980.
- Reeves, W.H. & Sthoeger, Z.M. (1989). Molecular cloning of cDNA encoding the p70 (Ku) lupus autoantigen. *J Biol Chem*, 264, pp. 5047-5052.
- Reinhardt, H.C.; Aslanian, A.S.; Lees, J.A. & Yaffe, M.B. (2007). P53-deficient cells rely on ATM-and ATR-mediated checkpoint signaling through the p38 MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell*, 11(2), pp. 175-89.
- Reinhardt, HC.; Jiang, H.; Hemann, MT. & Yaffe, MB. (2009a). Exploiting synthetic lethal interactions for targeted cancer therapy. *Cell Cycle*, 8(19), pp. 3112-3119.
- Reinhardt, H C. & Yaffe, M. B. (2009b). Kinases that Control the Cell Cycle in Response to DNA Damage: Chk1, Chk2, and MK2. *Current Opin Cell Biol*, 21, pp.245-255.
- Ripperger, T.; Gadzicki, D.; Meindl, A. & Schlegelberger, B. (2009). Breast cancer susceptibility: current knowledge and implications for genetic counselling. *Eur J Hum Genet*, 17(6), pp. 722-31.
- Rowe, BP. & Glazer, PM. (2010) Emergence of rationally designed therapeutic strategies for breast cancer targeting DNA repair mechanisms. *Breast Cancer Res*, 12, pp. 203
- Sarasin, A. & Stary, A. (2007). New Insights for Understanding the Transcription-coupled Repair Pathway. *DNA Repair*, 6, pp. 265-269.

- Schaeffer, L.; Roy, R.; Humbert, S.; Moncollin, V.; Vermeulen, W.; Hoeijmakers, J. H.; Chambon, P. & Egly, J.M. (1993). DNA Repair Helicase: a Component of BTF2 (TFIIH) Basic Transcription Factor. *Science*, 260, pp. 58–63.
- Schaeffer, L.; Moncollin, V.; Roy, R.; Staub, A.; Mezzina, M.; Sarasin, A.; Weeda, G.; Hoeijmakers, J.H. & Egly, J.M. (1994). The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *EMBO J*, 13, pp. 2388–2392.
- Selby, C.P.; Drapkin, R.; Reinberg, D. & Sancar, A. (1997). RNA Polymerase II Stalled at a Thymine Dimer: Footprint and Effect on Excision Repair. *Nucleic Acids Res*, 25, pp. 787–793
- Shieh, S.Y.; Ikeda, M.; Taya, Y. & Prives, C. (1997). DNA damage induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, 91, pp. 325–334.
- Shieh, S.Y.; Ahn, J.; Tamai, K.; Taya, Y. & Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage inducible sites. *Genes Dev*, 14, pp. 289–300.
- Shimada, M. & Nakanishi, M. (2006). DNA Damage Checkpoints and Cancer. *J Mol Histol*, 37, pp. 253–260.
- Sijbers, A.M.; de Laat, W. L.; Ariza, R. R.; Biggerstaff, M.; Wei, Y. F.; Moggs, J. G.; Carter, K. C.; Shell, B. K.; Evans, E.; de Jong, M. C.; Rademakers, S.; De Rooij, J.; Jaspers, N. G.; Hoeijmakers, J. H. & Wood, R. D. (1996). Xeroderma Pigmentosum Group F Caused by a Defect in a Structure-specific DNA Repair Endonuclease. *Cell*, 86, pp. 811–822.
- Slupphaug, G.; Kavli, B. & Krokan, H.E. (2003). The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res*, Oct 29; 531(1-2), pp. 231–51.
- Sodhi, R.K.; Singh, N. & Jaggi, A.S. (2010). Poly (ADP-ribose) polymerase-1 (PARP-1) and its therapeutic implications. *Vascular Pharm*, 53, pp. 77–87.
- Stasiak, A.Z.; Larquet, E.; Stasiak, A.; Muller, S.; Engel, A.; Van Dyck, E.; West, S.C. & Egelman, E.H. (2000). The Human Rad52 Protein Exists as a Heptameric Ring. *Curr Biol*, 10(6), pp. 337–340.
- Stupp, R.; Mason, W.P.; et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Eng J Med*, 352, 987–996.
- Sung, J.S. & Dimple, B. (2006). Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. *FEBS J*, 273, pp. 1620–1629.
- Tabatabaie, T. & Floyd, R.A. (1994). Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and the protective effect of spin trapping agents. *Arch Biochem Biophys*, Oct; 314(1), pp. 112–9.
- Toteja, G.S.; Mukherjee, A.; Diwakar, S.; Singh, P.; Saxena, B.N.; Sinha, K.K.; Sinha, A.K.; Kumar, N.; Nagaraja, K.V.; Bai, G.; Prasad, C.A.; Vanchinathan, S.; Roy, R.; Parkar, S. (2006). Aflatoxin B1 contamination in wheat grain samples collected from different geographical regions of India: A multicenter study. *J Food Prot*. Jun; 69(6):1463–7.
- Waardenburg, R.C.; Jong, L.A.; Eijndhoven, M.A.; Verseyden, C.; Pluim, D.; Jansen, L.E.; Bjornsti, M. & Schellens, J.H. (2004). Platinated DNA adducts enhance poisoning of DNA Topoisomerase I by Camptothecin. *J Biol Chem*, 279(52), pp. 54502–54509.
- Van Dyck, E.; Stasiak, A.Z.; Stasiak, A. & West, S.C. (1999). Binding of Double-strand Breaks in DNA by Human Rad52 Protein. *Nature*, 398, pp. 728–731.
- Walworth, N.C. (2000). Cell-cycle Checkpoint Kinases: Checking in on the Cell Cycle. *Curr Opin Cell Biol*, 12, pp. 97–704.

- Wang, J. C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Rev Mol Cell Biol*, 3, pp. 430-440.
- Wogan, G.N & Newberne P.M. (1967). Dose-response characteristics of aflatoxin B1 carcinogenesis in the rat. *Cancer Res*, 27, pp. 2370-2376.
- Wogan, G.N. (1976). Aflatoxins and their relationship to hepatocellular carcinoma. In: Okuda K and Peters RL., editor. *Hepatocellular Carcinoma*. New York: John Wiley and Sons; pp. 25-42.
- Wogan, G.N. (1987) IARC. Monographs on the evaluation of carcinogenic risk to humans. Lyon, France: IARC Publications.
- Wogan, G.N. (1992). Aflatoxins as risk factors for hepatocellular carcinoma in humans. *Cancer Res*, 52, pp. 2114-2118.
- Wu, X., Wilson, T.E. & Lieber, M.R. (1999). A role for FEN-1 in nonhomologous DNA end joining: the order of strand annealing and nucleolytic processing events. *Proc Natl Acad Sci USA*, 96, pp. 1303-1308.
- Yap, T.A.; Boss, D.S.; Fong, P.C.; Roelvink, M.; Tutt, A.; Carmicheal, J.; O'Connor, M.J.; Kaye, S.B.; Schellens, J.H. & Bono, J. First in human phase I pharmacokinetic (PK) and pharmacodynamic (PD) study of KU-0059436 (Ku), a small molecule inhibitor of poly ADP-ribose polymerase (PARP) in cancer patients, including BRCA1/2 mutation carriers. (2007), *J Clin Oncol*, 25, pp.3529.
- Yazdi, P.T.; Wang, Y.; Zhao, S.; Patel, N.; Lee, E.Y. & Qin, J. (2002). SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev*, 16, pp. 571-582.
- Yokoi, M.; Masutani, C.; Maekawa, T.; Sugawara, K.; Ohkuma, Y. & Hanaoka, F. (2000). The Xeroderma Pigmentosum Group C Protein Complex XPC-HR23B Plays an Important Role in the Recruitment of Transcription Factor IIIH to Damaged DNA. *J Biol Chem*, 75, pp. 9870-9875.
- Zhang YJ. (2010). Interactions of chemical carcinogens and genetic variation in hepatocellular carcinoma. *World J Hepatol*, 2(3), pp. 94-102.
- Zhao, C.; Tyndyk, M.; Eide, I. & Hemminki, K. (1999). Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents. *Mutat Res Mar* 8; 424(1-2), pp. 117-25.
- Zhao, S.; Weng, Y.C.; Yuan, S.S.; Lin, Y.T.; Hsu, H.C; Lin, S.C.; Gerbino, E.; Song, M.H.; Zdzienicka, M.Z.; Gatti, R.A.; Shay, J.W.; Ziv, Y.; Shiloh, Y. & Lee, E.Y. (2000). Functional link between ataxiatelangiectasia and Nijmegen breakage syndrome gene products. *Nature*, 405, pp. 473-7.

Application of UV-Induced Unscheduled DNA-Synthesis Measurements in Human Genotoxicological Risk Assessment

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1. Introduction

1.1 Environmental endogenous DNA damages

Cancer development is a long-term, multistep process with a complex interplay between genes and environment. The magnitude of environmental effects depends on the presence or absence of genetic susceptibility of the subjects to certain cancer types. Molecular epidemiological studies in cancer have proved that besides target cell genetic instability, the presence of triggering environmental exposure is critical in cancer development [Albertini & Hayes 1997, Newby & Howard 2005]. The biomarker responses, exposure character and the route of exposure of different environmental factors (chemicals, physical agents and biological agents) are also important in causing tumors especially in the cases of occupational cancer [Ward 1995]. The EPA Guidelines for carcinogen Risk Assessment [EPA 2005] is based on the mode of action of chemicals, such as interaction with DNA, cytotoxicity, or binding to the receptors modifying signal pathways. There are several natural compounds - so called chemopreventive agents- which are able to modify the genotoxic or mutagenic response (Ames 1983) in different organisms. These vitamins, antioxidants, phytochemicals, micro nutrients are available on the market without knowing their mode of action. Mutagenesis caused by environmental chemicals or physical agents can be prevented by protection of the cell's DNA replication, increasing the repair capacity or delaying cell replication to allow enough time to make a complete repair of damaged cells. Antioxidants are able to protect the cells from oxidative stress, and stimulate the phase I reactions including oxidation, reduction, and hydrolysis of xenobiotics by the monooxygenase detoxicating key enzymes, such as CYP450 [Xu et al. 1996, Poulsen & Loft 1998]. These changes increase the polarity of these molecules and help to conjugate them in phase II to glucuronic acid, acetic acid and sulfuric acid which are the physiological ways to eliminate active metabolites that are genotoxic to the target cells. The best studied crucial early event in carcinogenesis is chromosomal aberration, including microsatellite instability, abnormal number of chromosomes (aneuploidy), gene amplification or the loss of heterozygosity of tumor suppressor genes. By reducing chromosomal mutation via chemoprevention, the cell may be able to survive the genotoxic effects without any permanent damage, or it is able to go through the physiological pathway of apoptosis, without mutation occurring in the P53 gene [Lowe & Lin 2000].

1.2 The role of DNA repair in gene-environmental interactions

The measurement of UV-induced DNA repair is recommended in the risk assessment of environmental exposure to harmful chemicals (Reg. 440/2008/EC). Data obtained on prokaryota organisms suggest that exposure to chemicals as e.g. free oxygen radicals can interact with UV-induced DNA repair mechanisms (Chandor-Proust et al, 2008). Among the repair mechanisms existing in higher eukaryota, base excision repair (BER) seems to be the main mechanism involved in the removal of lesions produced by alkylation, deamination or oxidation (Rastogi et al, 2010). Orelli et al. (2009) demonstrated recently that nucleotide excision repair (NER) also plays an important role in the development of cisplatin resistance. UV-induced DNA damages can induce the so called three prime exonuclease1 (trex1), as a response to genotoxic stress. Beside thymine dimer production, UV irradiation can also produce reactive oxygen species. Benzo(a)pyrene (BaP) and hydrogen peroxide may, similarly to UV, induce the so-called three prime exonuclease1 (trex1) involved in the repair pathways of UV-induced DNA lesions, and cells deficient in trex1 show reduced recovery from UV and BaP replication inhibition, and increased sensitivity to towards genotoxins compared to the isogenic control (Christmann et al, 2010). These data suggest that both main mechanisms can be involved in the total repair of environmental chemical-induced genotoxic stress. Such mechanisms can probably explain the observed UDS reduction in some of our groups exposed to various chemicals but not UV.

A second question is whether decreased UDS can be related to an increase in apoptotic capacity? Cells deficient in the repair of UV-induced DNA damage can be more susceptible to a G1 arrest after UV treatment than cells with normal repair capacity or those cells which have completed their DNA repair prior to movement from G1 to S phase (Geyer et al, 2000). Zampetti-Bosseler and Scott (1981) demonstrated a prolonged mitotic delay in repair deficient ataxia teleangiectasia and retinoblastoma fibroblasts after X-ray irradiation compared to normal human fibroblasts, also suggesting a general key role of cell cycle check points beside DNA repair in preservation of genome stability (Kaufman, 1995). Skin fibroblasts from derived ataxia teleangiectasia patients are also more sensitive to UV-induced mutagenesis than those taken from healthy subjects (Hannan et al, 2002), and their results suggested a relationship between cell cycle control and DNA repair pathways in human cells. Genotoxic chemicals can also delay cellular proliferation in DNA repair-deficient cell clones more significantly than in wild type cells, by interfering with DNA replication, thereby inducing DNA damage (Kyunghee et al, 2009). The recently discovered cell cycle checkpoint activation mechanisms are discussed in detail by Rastogi et al (2010). In the present study the so-called premature centromere division (PCD) was used as a cytogenetic indicator of abnormalities in cell cycle regulation (Méhés 1978, Vig, 1981, Major et al, 1999). PCD yields were increased among cytostatic drug producers, anesthesiologists using halothane, and in exposures to formaldehyde, benzene and PAHs. PCD can be involved in the pathomechanism of aneuploidy, it seems to be a possible manifestation of chromosome instability also in human chromosome breakage syndromes and it can be connected with carcinogenesis (for review, c.f. Major et al, 1999).

2. Cancer development and DNA repair

We don't know exactly what the cause of cancer is; therefore we have several mechanisms and theories to explain it. One of them is shown in Fig.1.

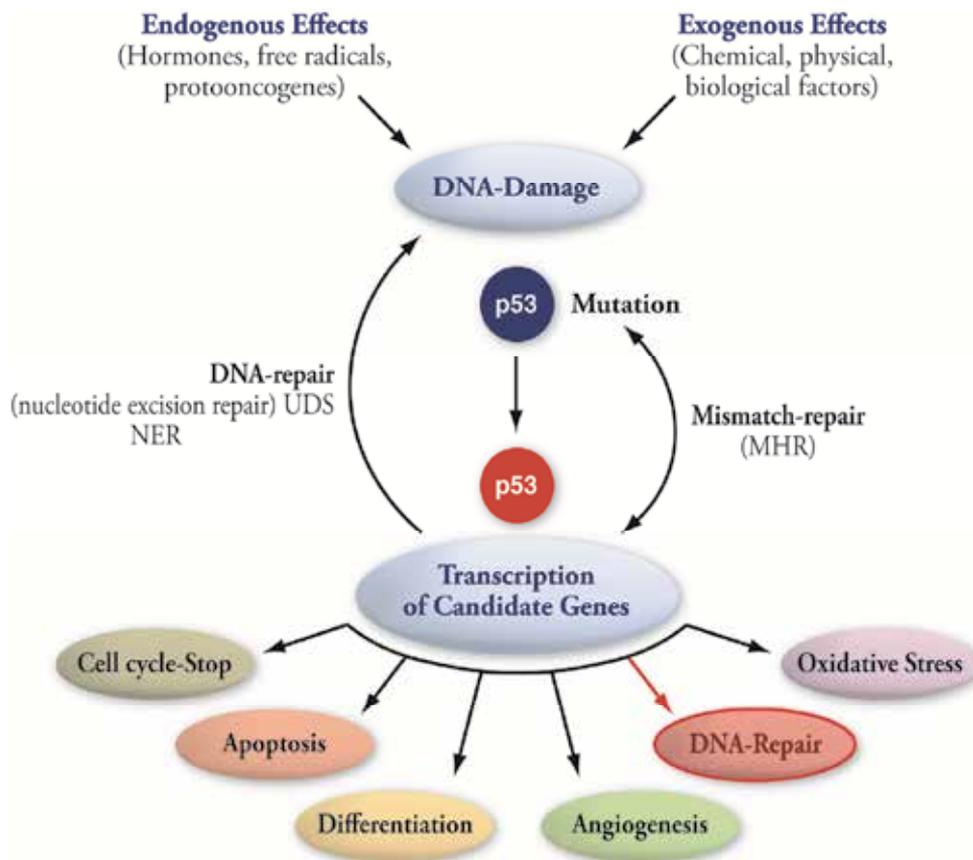


Fig. 1. Basic mechanism of cancer development

Figure 1. explains the genotoxic mechanism of cancer development, in which the P53 gene mutation is caused by DNA damage, and the consequence of this mutation leads to an inhibition of cell cycle arrest, or in differentiating cells, induces angiogenesis and inhibits the apoptotic activity of mutated cells. These changes are randomly mixed in target cells influencing clonal proliferation. The development of cancer is known to be a multistep process that is theoretically divided into initiation, promotion and progression (Fig. 2). Accumulation of mutational events necessarily leads to immortalizing the target cell. During this process the cells express several changes in phenotype. Most attractive changes are chromosomal aberrations (numerical and structural), easily detectable in cells, such as peripheral blood lymphocytes (PBL). Several epigenetic mechanisms are involved in cell initiation and promotion, eg. inhibition of DNA methyl-transferases, or DNA-repair enzymes (Ames 1989). Genotoxicity occurs when xenobiotics modify the DNA structure causing DNA damages which can lead to cytotoxicity or mutagenesis. DNA repair mechanisms are responsible for keeping the DNA in normal conformation and removing the lesions by enzymatic reactions. The damaging agents are divided into two main categories: endogenous and environmental agents. The endogenous factors are generated during normal metabolism; therefore these DNA damages are unavoidable and are related to sporadic and hereditary cancer (Valko et al. 2004, Bartkova et al. 2005). Usually the

physiological activities of DNA-repair and antioxidant systems are sufficient to keep these damages in balance, except when this machinery is already genetically altered. Although these damages are crucial in cancer development (Bardelli et al.2001), several other epigenetic events may lead to genomic instability, which initiate spontaneous chromosome breakage. Many other methods are used as biomarkers for DNA damage such as DNA strand breaks, chromosome aberrations (CA), micronucleus assay (MN), DNA-adduct, point mutation (HPRT) and epigenetic markers like DNA-methylation status, or the examination of the slow acetylation status among dye workers. These biomarkers are used in risk assessment of occupational and environmental cancer (Sorsa 1984, Forni 1987, Norppa 1997, Tompa et al. 2007) and they are important tools in analytical epidemiological studies, when intervention is necessary to avoid cancer development in the future (Hayes 1992).

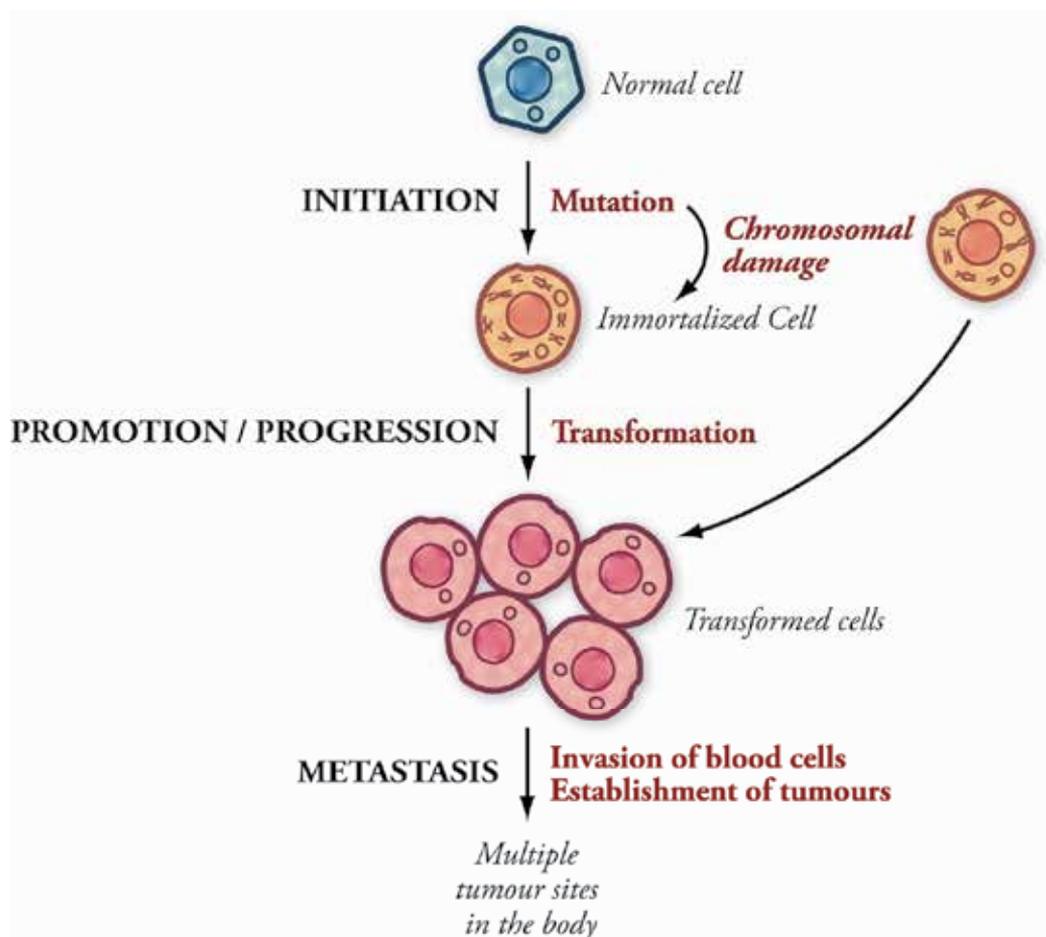


Fig. 2. Main steps of the multistep theory of cancer development: initiation, promotion and progression

In the case of high cancer risk, chemoprevention can be indicated with the help of these biomarkers. Several cohort follow up studies have shown a 2-3 fold increase in cancer risk among those individuals who have previously had a permanent high level of

chromosomal aberrations, (Bonassi 2000) compared to controls, who have low level of CAs [Nordic Study Group 1990]. Diet is able to influence the base-line mutation in DNA since folic acid and other antioxidants and selenium supplementation can prevent DNA amplification and double stranded DNA breaks (Fenech 2001, Crott et al. 2001) caused by different alkylating agents e.g. methotrexate. Several micronutrients such as zinc, magnesium, folic acid and vitamin B12 are required as a co-factor in normal DNA metabolism. Not only direct carcinogen exposure can be genotoxic, but the deficiency of these protective factors can also cause chromosomal aberrations, genetic instability and gene mutations in somatic cells which can lead to cancer. The supplementation of these chemopreventive agents (given with indication, based on the evidence of low serum levels), may give us a future perspective in anti-cancer treatment in the early stage of cancer development (Klein, Thompson, 2004). The balance of micronutrients, antioxidants and any other chemopreventive agents are regulated and kept very precisely at individually specific levels, and adopted by the optimal balance according to the functional requirements. Reactive oxygen species (ROS) do not necessarily play a negative role in cell metabolism. The white blood cells, such as neutrophils and macrophages produce a great amount of ROS during phagocytosis (Meydani et al. 1995). Unnecessary antioxidant treatment may inhibit immune surveillance and can cause immune suppression during chemoprevention. Without the measurement of antioxidant status this treatment is probably more hazardous than helpful. The presence of individual susceptibility markers of cancer development, like chromosomal mutation, DNA-repair capacity, or HPRT-point mutation must be tested parallel to the detection of antioxidant status. Chemopreventive action may be indicated on the basis of the positivity of the investigated biomarkers. The basic concept, first introduced by Brewer (1971) and Motulsky (1991), ie. genetic variations affect the adaptation to any kind of environmental agent, created the new expression "ecogenetics", explaining the reasons for individual susceptibility. Genetic polymorphism is the variation of normal phenotypes in the population, which usually does not alter the basic function of genes, but may modify the inducibility of the synthesis of the coded protein.

When gene expression changes without DNA sequence change, it is considered as "epigenetic" carcinogenesis. These heritable changes may include the methylation of cytosine bases in the DNA, or the modifications of histone proteins (acetylation, methylation, and phosphorylation). Hypermethylation of CpG-rich promoter regions are one of the most common epigenetic changes during carcinogenesis [Ames 1985, 1993, Baylin& Ohm 2006].

In physiological conditions the mammalian genome is often methylated at the C5 position of the cytosine by DNA methyltransferases. This mechanism plays a critical role in epigenetic gene silencing. When the methylation occurs in a different position on the DNA, this process may lead to serious DNA damage without mutation. S-adenosylmethionine (SAM) is a major methyl donor in various biosynthesis processes in normal cells. It is able to donate methyl groups to the DNA without an enzymatic reaction. Methionine deficiency may cause hypomethylation of DNA, which causes higher vulnerability of DNA replication during the cell cycle. In the absence of DNA methylation, there is increased nuclear clustering of pericentric heterochromatin and extensive changes in primary chromatin structure and global levels of histone H3 methylation and acetylation also become altered. This is one of the reasons why altered methylation of DNA can decrease the mobility of chromatin structure and nuclear

organization. In general DNA methylation is important in the control of gene transcription and chromatin structure. The complexities of this process are just beginning to be elucidated in relationship to other epigenetic mechanism of cancers [Feinberg et al. 2002]. Other histone modifications, such as acetylation and phosphorylation, affecting histone methylation also appear to be highly reliant on chromatin remodeling enzymes. The chemopreventive effects of sodium selenite and benzyl thiocyanate and their inhibitory effect on methyltransferase activity was demonstrated on human cultured colon carcinoma cells (Fiala et al. 1998).

3. Reactive oxygen species (ROS) and cancer chemoprevention

All cells of every organism are continuously exposed to free radicals, or reactive oxygen species (ROS) produced by oxidation that is an integral part of physiological metabolism, and controlled by physiological antioxidant mechanisms like phase II enzymes (superoxide dismutase, catalase, glutathione peroxidase). Oxidative stress arises, when the level of ROS exceeds the cell antioxidant capacity. Generation of ROS in different individuals is roughly correlated with life span, and defines the rate of aging and age related diseases like cancer (Klaunig et al. 1998). Several cellular defense mechanisms are available to protect the cellular compartments from oxidative damages, like superoxide dismutase and catalase and vitamins E and C which function to terminate lipid chain reactions involving free radicals. Many environmental xenobiotics induce free radicals reacting with DNA, RNA, proteins and lipids, forming adducts with nucleic acids. Chemoprevention of free radical formation is one of the best scientifically established ways of cell protection against mutagenic agents. Vegetarian food and different food supplements have enough antioxidants to prevent oxidative damage of macromolecules. Consumption of mediterranean food, olive oil, fish, vegetables, citrus fruits, green tea etc. caused differences in statistical appearance of cancer types and incidences, as well as in other chronic diseases (Trichopoulou et al. 2000). All of these beneficial effects are related to the antioxidant contents of diet and the relaxed life style.

Several *in vivo* and *in vitro* studies described the beneficial effects of antioxidants like polyphenols, terpenoids or vitamins in preventing cancer development or cell transformation. Although some human studies have described failure to prevent lung cancer among smokers and miners in long-term chemoprevention trials (Omenn et al. 1996). In a smoker group the supplementation with synthetic beta-carotene even increased the incidence of lung cancer, because the high dose caused a prooxidant effect during liver metabolism (Hennekens et al. 1996). In some other human trials, selenium, vitamin E and D, cyclooxygenase-2 inhibitors, lycopene and green tea were useful in reducing prostatic cancer development among PSA positive patients, except in those individuals, who had already *in situ* carcinoma (Mayer et al. 2005, Klein & Thompson, 2004).

Approximately every fifth cancer case is related to chronic inflammation; therefore anti-inflammatory agents are also used in chemoprevention, especially in the case of gastrointestinal cancer. Aspirin, piroxicam, ibuprofen or the naturally occurring sulindac has been shown to lead to a total regression of colorectal adenomatous polyps in patients with familiar adenomatosis (FAP). Vitamin D is also used as a chemopreventive agent, because it increases the apoptotic pathway through the inhibition of proliferation signals at the *bcl2* gene expression level, as it is shown on Fig. 3. (Weitsman et al. 2003).

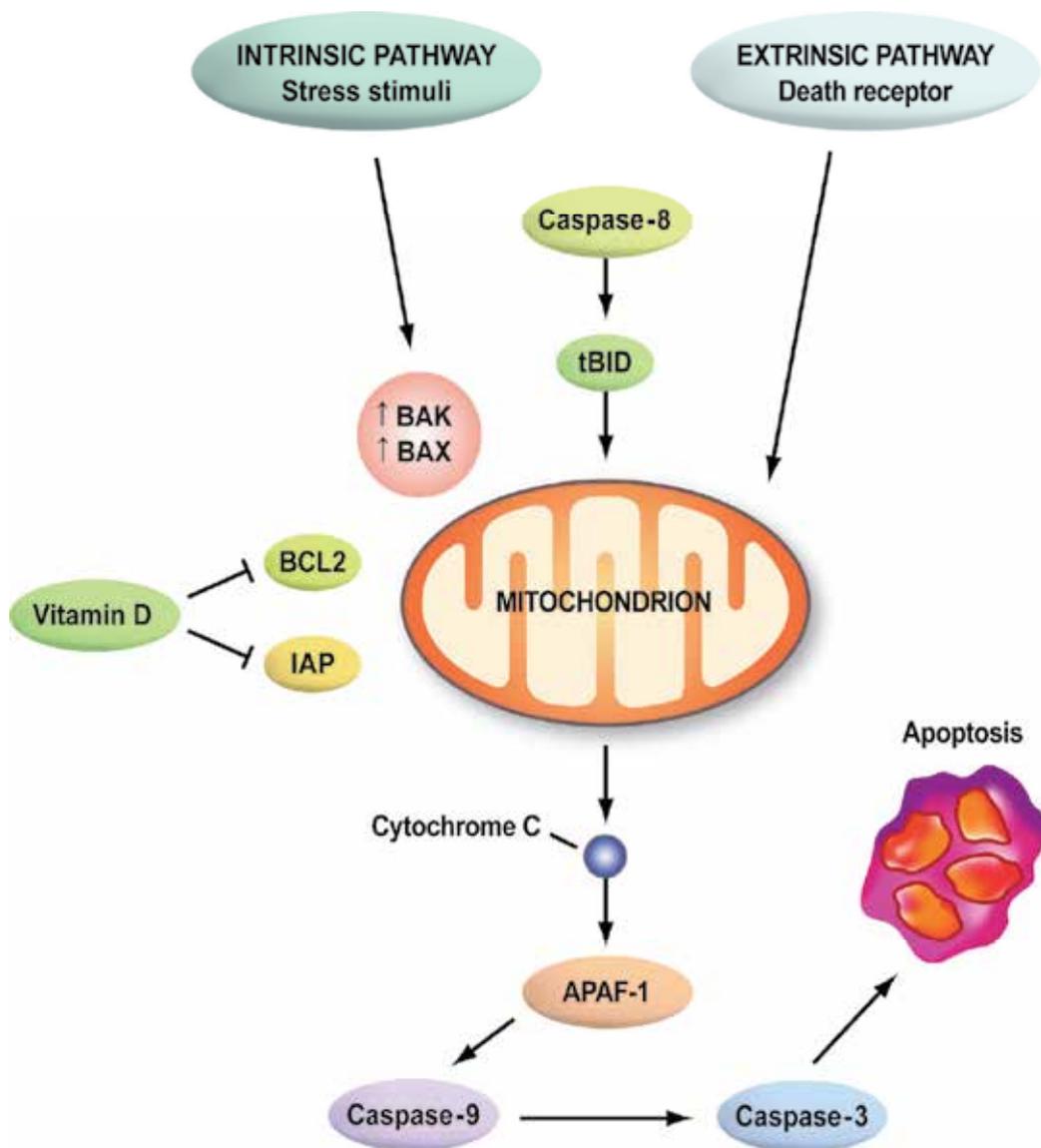


Fig. 3. Protective effect of Vitamin D through inhibition of unlimited cell growth and prevention of pro-caspase activity

4. The role of DNA repair in cancer chemoprevention

The principle of chemoprevention is based on the fact, that the treatment is able to interrupt the biological mechanisms that are involved in early carcinogenesis. It is important to know the mechanism of carcinogenesis, not only to understand the mode of action, but this knowledge gives potential for the development of novel chemopreventive agents, for future

perspectives. Chemoprevention may modify the progression of early molecular and morphological changes in the target tissues, like oncogene activation, chromosomal aberrations, mismatch-repair, and dysplasia, down regulation of DNA-repair enzymes, hyperplasia, angiogenesis, telomerase activity or anti-apoptotic effect of carcinogens. Cancer development is a long-term, multi-step process which consists of several genetic and epigenetic changes before the development of invasive cancer. The above mentioned intermediate biomarkers may serve as good tools in the indication of chemopreventive intervention.

5. Basic mechanisms of UV-induced DNA repair

The role of UV-induced DNA repair in the etiology of various malignancies has been demonstrated. Some inherited syndromes with altered DNA repair capacity increased sensitivity to mutagens manifested e.g. in abnormal sister chromatid exchange (SCE; Major et al, 1985) and have increased cancer risk (Au et al, 1996) as e.g. Down's syndrome or xeroderma pigmentosum. Wei et al (2003) studying the role of UV-induced DNA repair in the etiology of cutaneous malignant melanoma of patients with xeroderma pigmentosum (XP) suggested that reduced DNA repair capacity may contribute to susceptibility to sunlight-induced malignant melanomas among the general population as well.

UV exposure can also induce skin cancer partly by inducing immune suppression e.g. via the isomerization of *trans*-urocanic acid occurring naturally in the outermost layer of the skin, to the *cis*-isomer which can convert UV radiation into a biologically recognizable signal that activates immune suppression (Harriott-Smith & Halliday, 1988). The receptor of the *cis*-isomer is most probably the serotonin receptor itself (Walterscheid et al, 2006). Sreevidya et al (2010) demonstrated recently that platelet activating factor and serotonin receptor antagonists, known to reverse photocarcinogenesis and photoimmune suppression, can regulate DNA repair. The authors conclude that repairing DNA damage, neutralizing the activity of *cis*-urocanic acid, and reversing oxidative stress abrogates UV-induced immune suppression and cancer induction, suggesting that DNA, urocanic acid and lipid photo-oxidation serve as UV photoreceptors

UV irradiation induced DNA damage can be repaired by two major pathways: nucleotide excision repair (NER) is the pathway for removal lesions that distort DNA such as UV-induced thymine dimers, while base excision repair (BER) removes lesions resulted from exposure to exogenous or endogenous reactive oxygen species (for review, c.f. Legrand et al, 2008 and Asagoshi et al, 2010). For a detailed review of the molecular mechanisms of UV-induced DNA damage and repair c.f. Rastogi et al. (2010). NER is initiated by two distinct DNA damage sensing mechanisms: transcription coupled repair which removes damage from the active strand of transcribed genes, and global genome repair which removes damage present elsewhere in the genome (for review, c.f. Lans et al, 2010). For an efficient NER, modification of histones by acetylation and remodeling of nucleosomes is necessary (Guo et al, 2011). Genetic polymorphism may also affect the NER or BER repair capacity as it was demonstrated in case of repair enzymes XRCC1, XPA and XPD (Chang et al, 2010). The earlier dogma strictly separating the repair mechanisms of double and single strand DNA breaks seems to be outdated, since recent studies have presented increased evidence that various DNA repair mechanisms are well interlinked, as e.g. NER and mismatch repair can be involved in double strand DNA repair (for review, c.f. Ye Zhang et al, 2009).

6. Analysis of biomarkers in blood samples

Biomarkers for DNA damages and risk assessment

Considering the basic mechanism of cancer development, the most acceptable predictors of cancer risks are the DNA-damage biomarkers (see Table 3.). These damages can be provoked by exogenous or endogenous agents when DNA repair or mis-repair is in dysfunction. The unrepaired DNA damage can reduce the basic cell functions eg. maintenance of genetic integrity, triggering of cell cycle arrest, apoptosis, uncontrolled growth and other functionalities. Ultimately, damaged repair capacity leads to an increase in somatic mutations and cancer.

Methods	Target cells
Unscheduled DNA synthesis (UDS)	Lymphocytes, hepatocytes
DNA strand breaks (SGE or Comet assay)	Any living cells, germ cells
Chromosomal aberrations	Tumor cells, lymphocytes, germ cells
Micronucleus assay	Lymphocytes, bone marrow cells
Aneuploidy	Tumor cells, lymphocytes, germ cells
Telomere shortening	Any living cells
DNA-adducts and oxidation, methylation	Lymphocytes, germ cells
Nuclear p53	Any living cells
Point mutation (HPRT)	Any living cells
Mitochondrial DNA mutation	Any living cells

Table 1. Biomarkers of DNA damage

7. The use of UV-induced DNA repair for risk assessment

For assessing DNA repair capacity in human subjects exposed to various genotoxic agents in order to assess the health risk, probably the most frequently used method is the determination of UV-induced DNA repair which measures the unscheduled DNA synthesis (UDS) in cells with inhibited total repair (Bianchi et al, 1982). DNA repair measurement in liver cell lines, is also recommended by the European Union for the risk assessment of harmful chemicals, as it appears in the Regulation 440/2008/EC Part B (B.39.) and its amendments. The Regulation also allows the use of cells other than hepatocytes. The detection of a UDS response depends on the number of DNA bases excised and replaced at the site of the damage. The Regulation recommends the UDS test for the detection of substance-induced "longpatch repair" (20-30 bases), while, in contrast, the test can also detect "shortpatch repair" (1-3 bases) although with much lower sensitivity. The Regulation warns the users, that mutagenic events may be a result of non-repair, misrepair or misreplication of DNA lesions. The extent of the UDS response gives no indication about the fidelity of the repair process. In addition, it is possible that a mutagen reacts with DNA but the DNA damage is not repaired via an excision repair process. The lack of specific information on mutagenic activity provided by the UDS test is compensated for by the potential sensitivity of this endpoint because it is measured in the whole genome (Reg. 440/2008/EC).

Eldridge et al (1992) suggested a role of unscheduled DNA synthesis (UDS) in the development of human breast cancer. Kopanja et al (2009) demonstrated that cells with a deletion of the Cul4A gene which encodes a core component of cullin-based E3 ubiquitin ligase complex being over-expressed in breast cancers and correlating with poor prognosis, exhibit aberrant cell cycle regulation and reduced levels of UDS. On the other hand, nucleotide excision repair, a major mechanism involved in UV-induced DNA repair pathways can contribute to the development of resistance against drugs like cisplatin in cancer cells (Orelli et al, 2009). UV-induced UDS can reflect only a part of the total repair capacity of human cells. An easy method for the measurement of the total repair capacity can be the single cell gel electrophoresis (Comet assay) (Collins et al. 1997). A cytogenetic phenomenon, the sister chromatid exchange (SCE) can also be considered as a representative of post-replication repair (Okada et al, 2005). In the multiple end-point genotoxicology monitoring system using peripheral blood lymphocytes for the assessment of genotoxic (and leukocytes for the immune toxic) effects of environmental exposure to harmful chemicals, the repair capacity of the cells is measured by UDS, SCE and recently the Comet assay. Studies of cigarette smokers, groups of workers exposed to various chemicals e.g. uranium, butadiene (Au et al, 1996), benzene, and cytostatic drugs (Tompa et al, 1994, 2005, 2006), suggest that exposed populations can have a mutagen-induced abnormal DNA repair response. Repair mechanisms involved in the development of malignancies suggest an important role of DNA repair studies in cancer risk assessment. In an early study, Eldridge et al. (1992) demonstrated by an assay using UDS induced by chemicals and UV irradiation in early passage cultures of normal mammary epithelial cells derived from 5 different women, that UDS may be used in addressing the role of environmental agents in the development of human breast cancer.

Studies of DNA repair in populations exposed to mutagenic chemicals need to integrate chromosome aberration and other relevant assays for a more precise prediction of health risk (Au et al, 1996). When applying the so called multiple end-point genotoxicological monitoring system in Hungary, beside the use of UV-induced unscheduled DNA synthesis (UDS), we also included other biomarkers such as structural and numeric chromosome aberrations (CA), sister-chromatid exchange (SCE), mutations in the HPRT loci, early centromere separation (CS), and apoptotic capacity (AC) (Tompa, Sapi, 1989, Jakab et al, 2010, Major et al, 1999). In the present multiple end-point genotoxicology monitoring system run in Hungary (Tompa et al, 2006, c.f., Fig. 4.). DNA repair is investigated at three levels: Comet assay, UV-induced unscheduled DNA synthesis (UDS), and sister-chromatid exchange (SCE), representing the total repair capacity, the nucleotide excision repair, and the post-replication repair, respectively. Here we present data of UDS (and SCE) obtained in groups of subjects exposed to cytostatic drugs, anesthetic gases, formaldehyde, heavy and precious metals, benzene and polycyclic aromatic hydrocarbons compared to industrial controls.

8. Methods

8.1 The measurement of UV induced unscheduled DNA synthesis (UDS) in PBLs

The measurement of UDS was done according to Bianchi et al.(1982), as previously described (Tompa et al., 2005). Briefly, the separation of peripheral blood lymphocytes (PBLs) of citrated blood samples was performed by Ficoll-Hypaque density centrifugation. PBLs were irradiated in open petri dishes by UV light (24 J/m²) and then incubated for 3 h

- Methods for the multiple endpoint geno- and immune toxicology monitoring using peripheral blood lymphocytes and leukocytes in Hungary**
- Information, investigation only with written consent,
 - Anamnesis,
 - Physical and clinical laboratory check-up,
 - Blood sampling (by venipuncture), Lymphocyte separation
 - Processing (standard methods)
 - Genetic toxicology (genotype):
 - Primary DNA lesions and total DNA repair (Comet assay)
 - Gene mutations in HPRT loci
 - Structural and numeric chromosome aberrations (CA)
 - Sister-chromatid exchanges (SCE)
 - UV-induced DNA repair capacity (UDS),
 - Apoptotic capacity
 - Immune toxicology (phenotype):
 - Response to lectine stimulation,
 - Phenotypic characterisation of lymphocyte subpopulations
 - Leukocyte functional tests
 - Risk assessment and risk communication

Fig. 4. Biomarkers used for the multiple geno- and immuno toxicological monitoring in Hungary.

with 10 $\mu\text{Ci/ml}$ $^3\text{H-TdR}$ (activity: 37 MBq/ml, Amersham) in the absence or presence of 2.5 mM hydroxyurea. The degree of 'de novo' UDS was measured by scintillometry based on $^3\text{H-TdR}$ incorporation in separated lymphocytes. UDS was calculated as the difference between radioactivities of the incorporated $^3\text{H-TdR}$ in UV irradiated and control cultures (relative units).

8.2 Determination of CA and SCE frequencies

Whole blood samples were processed for studies of CA and SCE. The cell culture methods were identical in both protocols: samples of 0.8 ml heparinized blood were cultured in duplicate at 37°C, in 5% CO₂ atmosphere, in 10 ml RPMI-1640 (Sigma-Aldrich) supplemented with 20% fetal calf serum serum (Gibco Invitrogen Corporation) and 0.5 % Phytohemagglutinin-P (PHA, Gibco Invitrogen Corporation), without antibiotics. For CA and SCE analyses, the cultures were incubated for 50 hr and 72 hr, respectively. 5-Bromo-2-deoxyuridine (BrdU, Sigma-Aldrich) used in SCE analysis to identify the first and subsequent metaphases, was added at a concentration of 5 $\mu\text{g/ml}$ at 22 hr of culture. Culture harvest, slide preparation and staining were made following standard methods using 5% Giemsa stain (Fluka) for CA (Moorhead et al., 1960), and according to the Fluorescent-Plus-Giemsa method of Perry and Wolff (1974) for SCE. All microscopic analyses were blindly performed by permanent staff. CA characterization was carried out in 100 metaphases with 46 \pm 1 chromosomes per subject according to Carrano and Natarajan (1988). Mitoses containing only achromatic lesions (gaps) and/or aneuploidy (mitoses with 45 or 47 chromosomes) were not considered aberrant. The frequencies of total premature

(early) centromere divisions (PCD i.e. the separation of centromeres during prophase/metaphase of the mitotic cycle) were scored according to Méhes & Bajnóczky (1981). Mitoses with more than three chromosomes with PCD were considered as PCD/CSG (centromere separation general).

9. Flow cytometric analysis of apoptosis and cell proliferation in PBLs

For the measurement of the percentage of apoptosis and S-phase, PBLs were separated from the blood samples on Histopaque 1077 gradients (Sigma-Aldrich) and cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 20% fetal calf serum (Gibco Invitrogen Corporation) and 0.5 % PHA (Gibco Invitrogen Corporation) for 50 hours without antibiotics in a standard thermostat at 37°C in humidified atmosphere containing 5% CO₂. One hour prior to the termination of the cultures, 5 µg/ml BrdU (Sigma-Aldrich) was added to the cultures. Cells were washed twice with PBS, and fixed in 1 ml of ice-cold 70% ethanol and stored at -20 °C until further processing.

DNA denaturation prior to propidium iodide (PI, Sigma-Aldrich) and fluorescein isothiocyanate (FITC)-labeled monoclonal anti-BrdU (Becton-Dickinson) staining was performed at room temperature with 2M HCl containing 0.2 mg/ml pepsin (Sigma-Aldrich), according to the method of Piet van Erp et al. (1988). DNA was stained with PI and the incorporated BrdU was detected by flow cytometry with FITC-labeled monoclonal antibody.

Flow cytometric analysis was performed on a FACS Calibur (Beckton-Dickinson) flow cytometer. Data for at least 10000 lymphocytes per sample were acquired; CellQuestPro Software was used for analysis. Statistical analysis was made using the GraphPad Prism 3.02 software (GraphPad Software, Inc.), differences between the studied groups and the control were tested using the Student's t-test, $p < 0.05$ was considered as statistically significant.

10. Sample selection

Here we present the mean values (\pm SE) of the results of the genotoxicological investigations in 55 donors from 3 production units in the oil industry. Together with the results of the genotoxicological investigations completed with measurements of apoptosis and cell proliferation in altogether 275 subjects from oncology health care units, workers from the pharmaceutical industry, pathology and anesthesiology units, goldsmiths and galvanizers from the metal industry producing coins and mints. All subjects took part in the study voluntarily with prior informed consent, and were interviewed by a physician to collect data on age, medication, life-style (smoking and drinking habits), as well as medical and work histories in relation to known, or suspected chemical mutagens and/or to exposure to ionizing radiation.

Blood was collected by venipuncture from each of the investigated subjects: 18 ml blood in 2 VACUETTE® Coagulation tubes filled with 1 ml of 0.109 mol/l (3.2%) buffered tri-sodium citrate (Ref. No. 455322, Greiner Bio-One) for the measurements of UV induced unscheduled DNA synthesis (UDS), and 9 ml blood in 1 VACUETTE® Heparin tube coated with the anticoagulant sodium-heparin (Ref. 455051, Greiner Bio-One) for the determination of CA and SCE frequencies, and for the flow cytometric analysis of apoptosis and cell proliferation. The samples were processed immediately after blood collection. Only active smokers were considered to be "smokers". None of the individuals were addicted to alcohol, subjects

considered as "drinkers" consumed less than the equivalent of 80 g pure alcohol daily. All subjects took part in a routine clinical checkup, including hematology, liver and kidney function tests. The results were compared with control subjects without any known occupational exposure to genotoxic agents.

11. Demographic and exposure data

11.1 Exposure to aromatic hydrocarbons

Among oil industry workers, in the first group we have investigated 27 workers exposed mainly to benzene from a plant producing aromatic compounds, such as benzene (26 men and 1 woman, 186 investigations). The second and third groups comprised of 14 bitumen producers (13 men and 1 woman, 107 investigations) and 14 coke producing workers mainly exposed to PAH's (only men, 87 investigations), respectively. Mean ages were 34.7 ± 1.6 years (range 24-55) for the benzene producers, 40.4 ± 2.4 years (range 26-55) for the bitumen exposed workers, and 32.1 ± 1.4 years (range 25-42) for the coke producers, respectively. Mean percentages of current smokers were 22.2 % among benzene producers, 50.0 % among bitumen exposed workers, and 28.6% among the coke producers, respectively. Mean frequencies of "drinkers" were 81.5% in the benzene exposed group, 78.6% among bitumen producers and 71.4 among coke producers, respectively.

11.2 Exposure to cytostatic drugs

Altogether 138 subjects of hospital staff from health care units exposed to various cytostatics during the treatment of cancer patients, were divided into two groups. The first group of health care workers working without adequate protection consisted of 23 subjects (1 man and 22 women, 45 investigations), while the other group of the health care workers using protective devices during work consisted of 115 subjects (8 men, 107 women, 131 investigations), respectively. In the group of the pharmaceutical industry workers producing cytostatics there were 36 subjects (4 men, 32 women, 97 investigations). Mean ages were 38.9 ± 2.1 years (range 24-57) for the health care personnel without and 33.7 ± 0.93 years (range 20-62) with protection, respectively. Mean age among pharmaceutical industry workers exposed to cytostatics was in the range of 20-55 years (mean 36.0 ± 1.6 years). Mean percentages of active smokers were 47.8 % among health care personnel without protection, 54.8 % among health care personnel with protection, and 44.4% among pharmaceutical industry workers, respectively. Mean frequencies of "drinkers" in the above listed groups were 13.0 % among health care personnel without protection, 52.2 % among health care personnel with protection, 44.4% among pharmaceutical industry workers, respectively.

11.3 Exposure to anesthetic gases and formaldehyde

Hospital staff from anesthesiology units were also divided into two groups: the first consisted of 30 subjects exposed to the anesthetic gas halothane (4 men and 26 women, 34 investigations), while in the other 28 workers were exposed to anesthetic gases isoflurane and sevoflurane (2 men and 26 women, 28 investigations). Pathology staff consisted of 21 subjects (only women, 21 investigations) exposed to formaldehyde. Mean ages of anesthesiology unit workers exposed to halothane and anesthetic gases other than halothane were 39.0 ± 1.8 and 40.4 ± 1.36 years (ranges 23-57 and 29-55), respectively. Mean age in the groups of pathology staff was 43.3 ± 2.0 years (range 26-60). The frequencies of active smokers were 16.7% among anesthesiologists exposed to halothane, 35.7% among

anesthesiologists exposed to anesthetic gases other than halothane and 23.8 % in pathology workers, respectively. The frequencies of 'drinkers' were 46.7% among anesthesiologists exposed to halothane, 64.3% among anesthesiologists exposed to anesthetic gases other than halothane and 57.1 % in pathology workers, respectively.

11.4 Exposure to heavy metals

In the group of the 22 goldsmiths' and galvanizers there were 14 men and 8 women (22 investigations). The mean age in the group of goldsmiths and galvanizers was 51.5 ± 1.6 years (range 34-60). There were 31.8% smokers and 45.5% 'drinkers' in this group.

12. Control subjects

The results of the investigated subjects in oil industry groups were compared with 87 industrial controls (53 men and 34 women), selected from the administrative staff in the oil industry, without known previous occupational exposure to genotoxic agents. Mean age was 38.6 ± 1.1 years (range 20-67) for this group of the industrial controls. There were 42.5% current smokers and 50.6% 'drinkers' among industrial controls.

In case of the investigated health care, pharmaceutical and metall industry subjects the results were compared with 57 industrial controls without known previous occupational exposure to genotoxic agents (11 men and 46 women). The controls were selected from health care personnel and from the administrative staff in the metal industry producing coins and mints, without known previous occupational exposure to cytostatics and other genotoxic agents. The mean age was 44.1 ± 1.7 years (range 22-69). The percentages of active smokers and 'drinkers' were 24.6% and 45.6% among industrial controls, respectively.

13. Results

The results of the UDS measurements and the mean frequencies of SCE and CA in the workers in the oil industry are summarized in Table 2A. UDS was significantly decreased among benzene ($p=0.00067$) and bitumen ($p=0.00788$) exposed donors. Similarly, a significantly decreased UDS ($p=7.04E-8$) was also observed among coke producers. CA was significantly increased in each group of the exposed donors. Similarly to CA, an increase in the mean values of SCE could be observed in each group of the exposed, although the increases were only significant among the benzene and bitumen exposed ($p=0.000602$ and $p=0.001204$, respectively).

Table 2B summarizes the cytogenetic parameters in cultured PBLs among the oil industry workers. Mean frequencies of cells with aberrations (aberrant cells, AB.C) were increased in all groups of the oil industry workers. The aberrations in all groups were mainly of the chromatid type breaks. Similarly to the CA and AB.C values, mean PCD(CSG) values were also significantly increased in all groups of oil industry workers ($p=1.238E-21$, $p=9.7E-15$ and $p=1.61E-13$, respectively).

The results of flow cytometric and UDS measurements, and the mean frequencies of SCE and CA are summarized in Table 3A. Mean apoptosis values were significantly increased in two groups of the cytostatics exposed subjects (health care personnel without protection, $p=0.0047$ and pharmaceutical industry workers, $p=0.0056$), in anesthesiologists exposed to halothane ($p=0.02451$) and in formaldehyde exposed subjects ($p=0.00066$). Apoptosis was also increased among anesthesiologists exposed to anesthetic gases other than halothane,

but this increase was only at the 10% level ($p=0.09427$). In contrast, apoptosis was significantly reduced among goldsmiths and galvanizers ($p=0.02203$). Cell proliferation (the percentage of S-phase) was significantly decreased in both groups of health care personnel exposed to cytostatics ($p=0.00079$ and $p=3.65E-8$, respectively) and in both groups of anesthesiologists ($p=7.42E-8$ and $p=0.003324$, respectively). In the group of the pharmaceutical industry workers, S-phase showed a significant increase ($p=3.21E-10$). A statistically significant decrease in UDS was observed in the groups of health care personnel exposed to cytostatics without protection ($p=0.057927$) and the workers from the pharmaceutical industry ($p=0,04959$). SCE was only significantly increased in the group of the health care personnel without adequate protection ($p=0.000416$). CA was significantly increased in the groups of the pharmaceutical industry workers ($p=0.01515$) and the pathologists exposed to formaldehyde ($p=0,053$). Among anesthesiologist exposed to halothane, CA was also increased, but the significance was only at the 10% level ($p=0.08429$).

Groups	Exposure	No of investigations	UDS rel.unit		SCE 1/mitoses		CA %	
			mean	±SE	mean	±SE	mean	±SE
Industrial controls	-	87	7.11	0.37	5.71	0.12	1.60	0.24
Benzene producers	Benzene	186	5.63*	0.21	6.20*	0.08	2.47*	0.17
Bitumen producers	Bitumen	107	6.00*	0.33	6.43*	0.10	2.98*	0.26
Coke producers	PAHs	87	4.42*	0.30	5.90	0.11	2.49*	0.22

*Significant to the industrial controls (Student's t-test. $p<0.05$)

Table 2. a. Mean values (\pm SE) of UV induced unscheduled DNA synthesis (UDS, relative units), the frequencies of sister chromatid exchanges (SCE, 1/mitoses) and chromosome aberrations (CA, %) in cultured peripheral lymphocytes among oil industry workers

Groups	Exposure	Number of investigations	AB.C %		CHT %		CHS %		PCD(CSG) %	
			mean	±SE	mean	±SE	mean	±SE	mean	±SE
Industrial controls	-	87	1.58	0.25	1.14	0.20	0.42	0.11	0.95	0.25
Benzene producers	Benzene	186	2.41*	0.16	1.63*	0.14	0.84*	0.09	6.18*	0.40
Bitumen producers	Bitumen	107	2.75*	0.24	1.64*	0.16	1.34*	0.18	5.33*	0.43
Coke producers	PAHs	87	2.40*	0.22	1.87*	0.20	0.67	0.11	6.05*	0.56

*Significant to the controls (Student's t-test. $p<0.05$)

Table 2. b. Mean values (\pm SE) of the frequencies of cells with chromosomal aberrations (AB.C, %), chromosomal aberrations of the chromatide (CHT, %) and chromosome type (CHS, %) and the frequencies of premature centromere divisions with centromere separation general (PCD/CSG, %) in cultured peripheral lymphocytes among oil industry workers

Groups	Exposure	Number of investigations	Apoptosis %		S-phase %		UDS rel.unit		SCE 1/mitoses		CA %	
			mean	±SE	mean	±SE	mean	±SE	mean	±SE	mean	±SE
Controls	-	57	5.85	0.27	20.93	1.20	5.99	0.35	6.09	0.11	1.81	0.24
Health care personnel without protection	Cytostatics	45	8.39*	0.83	15.07*	1.29	4.92*	0.44	6.94*	0.20	2.47	0.43
Health care personnel with protection	Cytostatics	131	5.90	0.33	12.79*	0.57	5.83	0.25	6.36	0.07	1.61	0.22
Pharmaceutical industry	Cytostatics	97	8.78*	0.94	41.81*	2.19	4.72*	0.30	6.16	0.14	2.62*	0.22
Anesthesiologists	Halothane	34	7.87*	0.81	11.16*	1.11	5.92	0.41	6.25	0.13	2.62**	0.39
Anesthesiologists	Other than halothane	28	8.31	1.42	15.41*	1.35	5.57	0.49	6.36	0.15	1.30	0.27
Pathology staff	Formaldehyde	21	10.46*	1.17	25.24	2.38	4.63	0.86	6.36	0.26	3.05*	0.62
Goldsmiths and galvanizers	Heavy and precious metals	22	4.84*	0.36	22.84	2.14	5.21	0.55	6.14	0.13	1.77	0.38

* Significant to the controls (Student's t-test. $p < 0.05$)

**Significant to the controls (Student's t-test. $p < 0.1$)

Table 3. a. Mean values (\pm SE) of apoptosis induction (%), cell proliferation (S-phase), UV induced unscheduled DNA synthesis (UDS, relative units), the frequencies of sister chromatid exchanges (SCE, 1/mitoses) and chromosome aberrations (CA, %) in cultured peripheral lymphocytes. The investigated groups were: health care personnel and workers in the pharmaceutical industry exposed to cytostatics, anesthesiologists, pathology unit personnel exposed to formaldehyde and goldsmiths and galvanizers in the metal industry exposed to heavy and precious metals

Table 3B represents the cytogenetic data of donors exposed to cytostatics, anesthetic gases, formaldehyde and metals. Aberrations were mainly of the chromatid type, with the exception of health care personnel without protection, where a nearly equal frequency of chromatid and chromosome type aberrations were scored. PCD/CSG was significantly increased in parallel to the increases of CAs and AB.C., among workers from the pharmaceutical industry ($p = 0.00356$) and pathologists exposed to formaldehyde ($p = 0.004608$). However, mean percentages of PCD/CSG were (not significantly) increased among cytostatics exposed health care personnel with protection and anesthesiologists exposed to anesthetic gases other than halothane, although the mean values of CAs and AB.C were not increased in these groups. On the contrary, in case of the anesthesiologists exposed to halothane, PCD/CSG was not increased, but CAs and AB.C were.

14. Discussion

14.1 Gene and environmental interactions

In the present study, the level of environmental genotoxic stress was characterized by the frequencies of chromosomal aberrations (CA). Au et al. (1996) suggested that the

Groups	Exposure	Number of investigations	AB.C %		CHT %		CHS %		PCD(CSG) %	
			mean	±SE	mean	±SE	mean	±SE	mean	±SE
Controls	-	57	1.63	0.22	1.25	0.22	0.56	0.14	4.71	0.55
Health care personnel without protection	Cytostatics	45	2.20	0.33	1.20	0.30	1.27*	0.23	3.67	0.50
Health care personnel with protection	Cytostatics	131	1.47	0.19	0.83	0.14	0.78	0.14	5.71	0.48
Pharmaceutical industry	Cytostatics	20	2.55*	0.21	1.90*	0.18	0.71	0.12	7.07*	0.39
Anesthesiologists	Halothane	34	2.38**	0.33	1.53	0.29	1.09*	0.26	3.41	0.75
Anesthesiologists	Other than halothane	30	1.19	0.27	0.74	0.21	0.44	0.15	6.43	1.61
Pathology staff	Formaldehyde	21	2.80*	0.61	2.35*	0.46	0.70	0.26	8.80*	1.07
Goldsmiths and galvanizers	Heavy and precious metals	22	1.50	0.33	1.18	0.35	0.59	0.23	3.95	0.75

* Significant to the controls (Student's t-test. $p < 0.05$)

** Significant to the controls (Student's t-test. $p < 0.1$)

Table 3. b. Mean values (\pm SE) of the frequencies of cells with chromosomal aberrations (AB.C, %), chromosomal aberrations of the chromatide (CHT, %) and chromosome type (CHS, %) and the frequencies of premature centromere divisions with centromere separation general (PCD/CSG, %) in cultured peripheral lymphocytes. The investigated groups were: health care personnel and workers in the pharmaceutical industry exposed to cytostatics, anesthesiologists, pathology unit personnel exposed to formaldehyde and goldsmiths and galvanizers in the metal industry exposed to heavy and precious metals

measurement of CA yields should be integrated in the assessment of health risk when DNA repair responses are studied. In the Hungarian multiple end-point genotoxicology monitoring system introduced in the late 1980s, the two key biomarkers have been the frequencies of gene mutations in the hprt loci (Tompa, A., Sápi, E., 1989) and CAs (Tompa, A., et al, 1994). In the present study, CA yields were increased in exposures to cytostatic drugs, halothane, formaldehyde, benzene and PAHs, as compared to controls, indicating a genotoxic stress in these populations (see *Tables 2A and 3A*). Chromatid type aberrations (CHT) representing rather the damages of DNA bases and single strand breaks, and chromosome type aberrations (CHS) representing double stranded DNA breaks that formed in cells mostly prior to entering the cell cycle, were increased in groups exposed to cytostatic drugs, formaldehyde, heavy and precious metals, benzene, and PAHs (see *Tables 2B and 3B*). An Italian team led by Bonassi in 2000 and the Nordic Study Group correlated the occurrence of chromosomal aberrations in human PBL cells with cancer risk in human populations. These prospective cohort studies have shown a significant (2.3-2.6 fold) increase in cancer in those individuals, who had permanent high level of chromosomal

aberrations. This seems to verify the hypothesis; that an increase of chromosomal aberration in itself may increase cancer risk. Therefore the intervention should take place in advance, when these alterations have just appeared in the peripheral blood lymphocytes (PBL). Genetic polymorphisms, eg. mutations of detoxification enzymes glutathione S-transferase (GST, GSTP1, and GSTM1) seem to be a risk factor for lung, head and neck cancer. Sequence variation in a DNA-repair gene, i.e. XPD have been associated with high lung cancer incidence. Chromosomal aberrations and loss of heterozygosity (LOH), especially 3p and 9p losses are important in all types of lung cancer too. Nuclear p53 mutation is a predictor of cancer, because the mutant cells are not able to respond properly to apoptotic signals and daughter cells inherit the mutation and genetic instability with the message of cancer development (Gretarsdottir 1998).

14.2 DNA repair capacity in the exposure groups

UV-induced DNA repair capacities (UDS) in PBLs were decreased, while CA yields (including the chromosome type aberrations) were increased in each groups exposed to aromatic hydrocarbons (benzene and PAHs) when compared to the controls in accordance with our earlier observations (Tompa et al 2005). Exposure to benzene, which was present in the ambient air could produce DNA strand breaks and oxidation of the bases in correlation with the level of its metabolite phenylmercapturic acid in urine (Sorensen et al 2003). It means that the higher is benzene in the ambient air, the higher can be the level of benzene-induced DNA damage. It can trigger the repair mechanisms such as BER, NER and double strand break DNA repairs involved in the removal of benzene-induced DNA lesions (Hartwig, 2010). Ambient air PAH levels and their diol epoxide metabolites also correlate with DNA base oxidation and strand breaks in the exposed human populations (Ruchirawat et al 2010; Zhong et al 2010). However, several studies demonstrated reduction in DNA repair capacity (measured by different methods) among benzene or PAHs exposed workers (Tompa et al 2005; Keretsete et al 2008; Chanvaivit et al 2007; Ruchirawat et al 2010). Using the Comet assay for the characterization both of the level of primary DNA lesions and repair in PBLs of petrol attendants with occupational exposures longer than 1 year, Keretsete et al (2008) demonstrated that the genotoxic volatile organic compounds such as benzene and certain PAHs can inhibit the repair of single-strand breaks. The reduction of the excision repair capacity is influenced by the polymorphism of the involved repair genes (Shi et al 2004; Pavanello et al 2005).

DNA repair capacities of PBLs were also reduced while CA yields were increased among the hospital nurses and pharmaceutical industry workers occupationally exposed to various anticancer drugs producing DNA strand breaks or base alterations, compared to the controls and to the subjects with proper protection. The same was observed in the case of pathology unit staff members exposed to formaldehyde. Exposure to halothane, although increased the CA frequencies among the exposed anesthesiologists as compared to the controls, did not affect the UDS levels. Among workers exposed to heavy or precious metals with potency to induce oxidative damages of DNA, no alterations were observed in the investigated biomarkers when compared to the controls. In an early study, Celotti et al (1990) demonstrated increased UV-induced UDS in the PBLs of patients treated with antineoplastic drugs, while no alteration compared to the controls were observed among the nurses handling and administering the drugs. Studies demonstrated that careless handling of antineoplastic drugs can lead to exposure of personnel (Sorsa & Anderson 1996; Tompa et

al 2006). Again reduced UDS capacity was observed among hospital nurses occupationally exposed to ethylene oxide and ^{222}Rn from the local tap-water (Tompa et al 1999; Major et al 1996). In contrary, UDS was increased compared to the control among nurses using horizontal air flow cabinets for the preparation of cytostatic infusions (Jakab et al 1999). Polymorphism of the repair genes XRCC1 and XRCC3 can contribute to increase the genetic damage in susceptible subjects with chronic exposure to cytostatic drugs (Laffon et al 2005; Cornetta et al 2008). Formaldehyde, an endogenous cellular aldehyde often used in pathology and anatomy laboratories is a well-known human mutagenic carcinogen although evidence of genotoxic effects in human PBLs is insufficient (Costa et al 2008; Jakab et al 2010). In our study a significant increase of CA yields and apoptotic capacity was observed among the exposed donors (Jakab et al 2010). Formaldehyde is a capacious inducer of DNA-protein crosslinks beside base modifications. Speit et al (2000) investigating the base excision and crosslink repairs suggested that a disturbed excision repair can have more severe consequences with regards of CA formation after formaldehyde exposure than a disturbed crosslink repair. Costa et al (2008) found no significant effect of genetic polymorphism of DNA repair enzymes on the investigated genotoxic end points (micronuclei, SCE, and primary DNA lesions determined by the comet assay).

15. DNA-repair and apoptosis

UDS was reduced; however apoptotic capacity was increased in some groups exposed to genotoxic chemicals such as anticancer drugs, benzene and polycyclic aromatic hydrocarbons (PAHs) but not UV (see *Tables 2A and 3A*) during the monitoring indicating an exposure-related decrease in UV-induced excision DNA repair capacity among these donors, and suggesting a relationship between UV-induced repair and apoptotic capacities of peripheral blood lymphocytes. However, sister-chromatid exchanges (SCE), probably reflecting post-replication repair events mediated by homologous recombination (Okada et al, 2005), therefore considered as biomarkers of total DNA repair, were increased among hospital nurses exposed to cytostatics, and workers exposed to benzene and PAHs, compared to the controls.

Apoptosis is energy dependent and programmed cell death is regulated by several biochemical mechanisms [Evan & Vousden 2001]. Three main pathways are known of apoptotic events. One is stimulated by the death receptors and caspase 8 activation, the second is an intrinsic pathway with activation of mitochondrial changes through caspase 9 and 3 activation. The third mechanism occurs through cytotoxic T cells with the help of perforin production, and granzyme A and B stimulation with caspase 10 activation. Each pathway activates its initiator caspase. Only granzyme A works independently of caspases. According to our present knowledge, approximately 14 caspases have been identified as initiators of apoptosis and proteolytic enzymes. Inhibition of apoptotic processes can be a significant cause of cancer development or autoimmune diseases. Excessive apoptosis is present in neurodegenerative diseases or in HIV infection. In contrast, tumor cells can resist apoptotic signals leading to an unlimited growth of malignant cells, production of anti-apoptotic proteins like Bcl-2, mutated P53, or down regulation of pro apoptotic Bax protein. P53 mutation is very common in human cancers; more than 50% of malignant tumors express mutant P53 cells. During cell replication the DNA repair is able to recognize DNA damages and keep the cell in G1/S phase. If the damage is irreparable, the apoptotic signal

is activated, although damaged or mutated P53 does not respond properly to this physiological signal (see Fig. 5.)

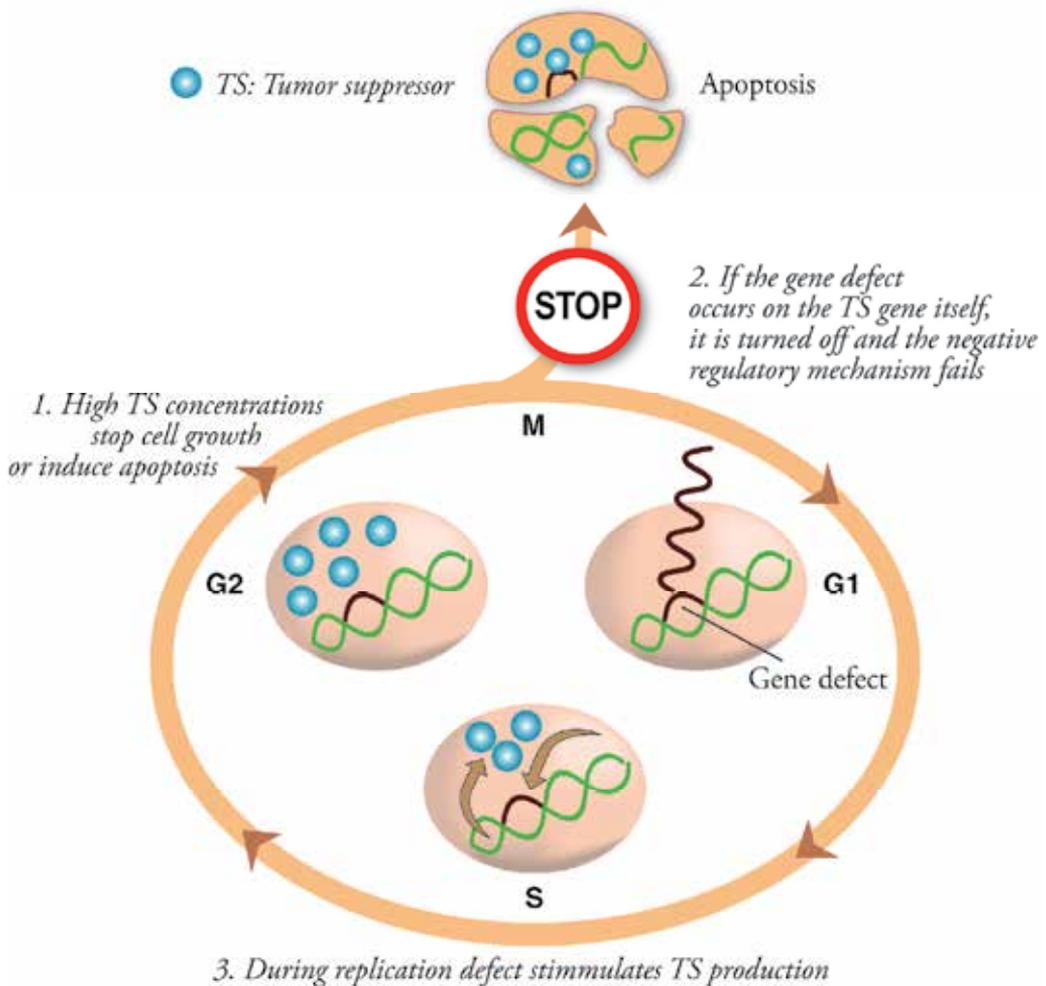


Fig. 5. The cell cycle and apoptosis

The gene p53 can play a key role in response to DNA damage by activating a G1 cell cycle arrest (Geyer et al, 2000). Squires et al. (2004) studying the DNA structure of replication forks in normal human and NER-deficient XP cells observed that replication associated DNA double strand breaks do not accumulate in p53 proficient human cells and proposed the prevention of DSB accumulation at long lived single stranded DNA regions in stalled-replication forks as a major mechanism of maintenance of genome stability by p53. Geyer et al (2000) reported a G1 to S phase delay of the mitotic cell cycle after UV treatment in GM6419 cells expressing dominant negative p53 mutations and suggested that unrepaired DNA damage was the signal for the stabilization of p53 and a subsequent G1 phase cell cycle arrest in UV-irradiated cells. A homeostatic regulator, the wild-type p53-induced phosphatase (Wip1) which is induced by p53 in response to e.g. UV-induced DNA damage is also involved in DNA repair and cell cycle

checkpoint pathways. Wip1 can be activated via both the JNK c-Jun and p38 MAPK-p53 signaling pathways, and a temporal induction of Wip1 depends largely on the balance between c-Jun and p53, which compete for JNK binding (Song et al, 2010). In wild-type but not in c-Jun (and c-Fos) null human cells a clear up-regulation of trex1 was observed after UV irradiation, and upon genotoxic stress a translocation of trex1 into the nucleus was suggested (Christmann et al, 2010) also indicating a strong relationship between UV-induced DNA damage and apoptotic capacity of human cells. Protein p21 is also a key component in p53 regulated cell cycle control and apoptosis, directing an anti apoptotic response following DNA damage as a major transcriptional target of p53 (Hill et al, 2008). Moreover, UV irradiation can also trigger p21 proteolysis, which seems to be in correlation with increased apoptosis (Soria et al, 2008). Data obtained on p53 binding on the p21 promoter suggest that the nature of DNA damage is itself the key factor for p53-regulated expression of target genes such as p21 and the subsequent cellular outcome (Hill et al, 2008).

16. Conclusions

During the multistep process of carcinogenesis several genetic and epigenetic changes accumulate in the target tissue through mutations, alkylation and formation of DNA and protein adducts. The modifications in cell cycle, proto-oncogenes, oncogenes and induction of chromosomal aberrations represent the arsenal of biomarkers showing early signs of cell transformation. Chemoprevention of carcinogenesis is based upon knowledge of the mechanisms of carcinogenesis, eg. inhibition of cell proliferation, signal transduction, increases in tumor suppression, activation of antipromotion, changes in metabolic activation and enhancement of apoptotic activity. Chemopreventive agents are usually selected according to cancer type (lung, colon, breast, oral cavity, bladder and prostate) or on the known mechanism of cancer development. The other, most effective approach to prevent cancer is to avoid carcinogenic agents (primary prevention). Biomarkers can be utilized as indicators of exposures, effects and individual susceptibility to cancer. Proper selection of biomarkers in relation to exposure may have a great impact on the reliability of mechanism of action. Recent developments in genomics provide an opportunity to investigate several oncogenes, tumor-suppressor genes, phenotypic changes in proteins simultaneously. Biomarkers such as the occurrence of high level of chromosomal aberrations can also indicate the need of intervention in high risk groups. An introduction of chemoprevention in order to avoid or delay cancer development can be advised in those cases, where removal of environmental hazards have not been efficient and the subjects have already suffered irreversible genetic damages.

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18. References

Albertini, R.J. & Hayes, R.B. (1997). Somatic Cell Mutations in Cancer Epidemiology. IARC Sci. Publ. Vol.142, pp. 159-184, ISBN 9283221427

- Albertini, R.J. (1999). Biomarker Responses in Human Populations: Towards a Worldwide Map. *Mutation Research*. Vol. 428, pp. 217–226, ISSN 0027-5107
- Albertini, R.J.; Anderson, D.; Douglas, G.R.; Hagmar, L.; Hemminki, K.; Merlo, F.; Natarajan, A.T.; Norppa, H.; Shuker, D.E.; Tice, R.; Waters, M.D. & Aitio, A. (2000). IPCS Guidelines for the Monitoring of Genotoxic Effects of Carcinogens in Humans. International Programme on Chemical Safety. *Mutation Research*, Vol. 463, pp.111–172, ISSN 0027-5107
- Ames, B.N. (1983). Dietary Carcinogens and Anticarcinogens. *Science*, Vol. 221, pp.1256–1264, ISSN 0036-8075
- Ames, B.N. (1989). Endogenous DNA Damage as Related to Cancer and Aging. *Mutation Research*, Vol. 214, pp. 41–46, ISSN 0027-5107
- Asagoshi, K.; Liu, Y.; Masaoka, A.; Lan, L.; Prasad, R.; Horton, J.K.; Brown, A.R.; Wang, X.; Bdour, H.M.; Sobol, R.W.; Taylor, J.S.; Yasui, A. & Wilson, S.H. (2010). DNA Polymerase β -dependent Long Patch Base Excision Repair in Living Cells. *DNA repair*, Vol. 9, No. 2, pp. 109-119, ISSN 1568-7864
- Au, W.W.; Wilkinson, G.S.; Tyring, S.K.; Legator, M.S.; Zein, E.R.; Hallberg, L. & Heo, M.Y. (1996). Monitoring Populations for DNA Repair Deficiency and for Cancer Susceptibility. *Environmental Health Perspectives*, Vol. 104, supplement 3, pp. 579-584, ISSN 0091-6765
- Bardelli, A.; Cahill, C.P.; Lederel, G.; Speicher, M.R.; Kinzler, K.W.; Vogelstein, B. & Lengauer, C. (2001). Carcinogen-specific Induction of Genetic Instability. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 98, No. 10, pp. 5770–5775, ISSN 0027-8424
- Bartkova, J.; Horejsi, Z.; Koed, K.; Kramer, A.; Tort, F.; Zieger, K.; Guldborg, P.; Sehested, M.; Nesland, J.M.; Lukas, C.; Ontoft, T.; Lukas, J. & Bartek, J. (2005). DNA Damage Response as a Candidate Anti-cancer Barrier in Early Human Tumorigenesis. *Nature*, Vol.434, No. 7035, pp. 864-870, ISSN 0028-0836
- Bartsch, H.; Kadlubar, F. & O'Neill, I. (1992). Biomarkers in Human Cancer: Part I. Predisposition and Use in Risk Assessment. *Environmental Health Perspectives*, Vol. 98, pp. 1–286, ISSN 0091-6765
- Bartsch, H.; Kadlubar, F. & O'Neill, I. (1993). Biomarkers in Human Cancer: Part II. Exposure Monitoring and Molecular Dosimetry. *Environmental Health Perspectives*. Vol. 99, pp. 1–390, ISSN 0091-6765
- Baylin, S.B. & Ohm, J.E. (2006). Epigenetic Gene Silencing in Cancer- a Mechanism for Early Oncogenic Pathway Addiction? *Nature Reviews/Cancer*, Vol.62, No. 2, pp.107-116, ISSN 1474-175X
- Bianchi, V.; Nuzzo, F.; Abbondandolo, A.; Bonatti, S.; Capelli, E.; Fiorio, R.; Guilotto, E.; Mazzacarro, A.; Stefanini, M.; Zaccaro, L.; Zantedeschi, & Levis, A.G. (1982). Scintillometric Determination of DNA Repair in Human Cell Lines: A Critical Appraisal. *Mutation Research*, Vol. 93, No.2, pp. 447-463, ISSN 0921-8262
- Biomarkers Definitions Working Group: (2001). Biomarkers and Surrogate Endpoints: Preferred Definitions and Conceptual Framework. *Clinical Pharmacology & Therapeutics*, Vol. 69, No.3, pp. 89-95, ISSN 0009-9236
- Bonassi, S.; Abbondandolo, A.; Camurri, L.; Dal Fra, A.; De Ferrari, M.; Degraffi, F.; Cole, B.F.; Baron, J.A.; Sandler, R.S.; Haile, R.V.; Ahnen, D.J.; Bresalier, R.S.; McKeown-Eyssen, G.; Summers, R.W. (2007). Folic Acid for the Prevention of Colorectal

- Adenomas, a Randomized Clinical Trials. *Journal of the American Medical Association*, Vol. 297, pp. 2351-2359, ISSN 0002-9955
- Bonassi, S.; Hagmar, L.; Stromberg, U.; Montagud, A.H.; Tinneberg, H.; Formi, A.; Heikkila, P.; Wanders, S.; Wilhardt, P.; Haasten, I.L.; Knudsen, L.E. & Norppa, H. (2000). Chromosomal Aberrations in Lymphocytes Predict Human Cancer Independently of Exposure to Carcinogens. *Cancer Research*, Vol. 60, pp.1619-1625, ISSN 0008-5472
- Brogger, A.; Hagmar, L.; Hansteen, I.L.; Heim, S.; Hogstedt, B.; Knudsen, L.; Lambert, B.; Linnainmaa, K.; Mitelman, F.; Nordenson, I.; Reutelwall C.; Salomaa, S.; Skerfving, S. & Sorsa, M (1990). A Nordic Data Base on Somatic Chromosome Damage in Humans. *Mutation Research*, Vol. 241, pp. 325-337, ISSN 0027-5107
- Brogger, A.; Hagmar, L.; Hansteen, I.L.; Heim, S.; Hogstedt, B.; Knudsen, L.; Lambert, B.; Linnainmaa, K.; Mitelman, F.; Nordenson, I.; Reutelwall, C.; Salomaa, S.; Skerfving, S. & Sorsa, M. (1990). An Inter-Nordic Prospective Study on Cytogenetic Endpoints and Cancer Risk. *Cancer Genetics and Cytogenetics*, Vol. 45, pp. 85-92, ISSN 0165-4608
- Carrano, A.V. & Natarajan. A.T. (1988). Considerations on Population Monitoring Using Cytogenetic Techniques. ICPENC Publ., No.14. *Mutation Research*, Vol. 204, No. 3, pp. 379-406, ISSN 0921-8262
- Celotti, L; Biasin, R.; Ferraro, P. & Fiorentino, M. (1990). Effects of In Vivo Exposure to Antineoplastic Drugs on DNA Repair and Replication in Human Lymphocytes. *Mutation Research*, Vol. 245, No. 3, pp. 217-222. ISSN 0027-5107
- Chaing, C.; Tsai, Y.; Bau, D.; Cheng, Y.; Tseng, S.; Wang, R. & Tsai, F. (2010). Pterygium and Genetic Polymorphisms of the DNA Repair Enzymes XRCC1, XPA, and XPD. *Molecular Vision*, Vol. 16, No. 79, pp. 698-704, ISSN: 1090-0535
- Chandor-Proust, A.; Berteau, O.; Douki, T.; Gasparutto, D.; Ollagnier-de-Choudens, S.; Fontecave, M. & Atta, M. (2008). DNA Repair and Free Radicals, New Insights into the Mechanism of Spore Photoproduct Lyase Revealed by Single Amino Acid Substitution. *Journal of Biological Chemistry*, Vol. 283. No. 52, pp. 36361-36368. ISSN 0021-9258
- Chanvaivit, S.; Navasurmit, P.; Hunsonti, P., Autrup, H. & Ruchirawat, M. (2007). Exposure Assessment of Benzene in Thai Workers, DNA-Repair Capacity and Influence of Genetic Polymorphism, *Mutation Research*, Vol. 626, No. 1-2. pp. 79-87, ISSN 0921-8262
- Chrismann, M.; Tomicic, M.T.; Aasland, D.; Berdelle, N. & Kaina, B. (2010). Three Prime Exonuclease I (TREX1) is Fos/AP-1 Regulated by Genotoxic Stress and Protects against Ultraviolet Light and Benzo(a)pyrene-Induced DNA Damage. *Nucleic Acids Research*, Vol.38, No. 19, pp. 6418-6432, ISSN 0305-1048
- Collins, A.; Dusinska, M. & Franklin, M. (1997). Comet Assay in Human Biomonitoring Studies: Reliability, Validation, and Application. *Environmental and Molecular Mutagenesis*, Vol. 30, pp. 149-156, ISSN 0893-6692
- Cornetta, T.; Padua, L.; Testa, A., Ievoli, E.; Festa, F.; Tranfo, G.; Bacceliere, L. & Cozzi, R. (2008). Molecular Biomonitoring of a Population of Nurses Handling Antineoplastic Drugs. *Mutation Research*, Vol. 638. No. 1-2. pp. 75-82. ISSN 0027-5107

- Costa, S.; Coelho, P.; Costa, C.; Silva, S.; Maxan, O.; Santos, L.S.; Gaspar, L & Teixeira, J.P. (2008). Genotoxic Damage in Pathology Anatomy Laboratory Workers Exposed to Formaldehyde. *Toxicology*, Vol. 252. No. 1-3. pp. 40-48. ISSN 0300-483X
- Crott, J.W.; Mashiyama, S.T.; Ames, B.M.; Fenech, M. (2001). The Effect of Folic Acid Deficiency and MTHFR C677T Polymorphism on Chromosome Damage in Human Lymphocytes in Vitro. *Cancer Epidemiology Biomarkers & Prevention*, Vol. 10, pp. 1089-1096, ISSN 1055-9965
- Cunningham, M.J.; Liang, S.; Fuhrman, S.; Seilhamer, J.J. & Somogyi, R. (2000). Gene Expression Microarray Data Analysis for Toxicology Profiling. *Annals of the New York Academy of Sciences*, Vol. 919, pp. 52-67, ISSN 0077-8923
- Davis, K.J.A. (2000). Oxidative Stress, Antioxidant Defenses, and Damage Removal, Repair and Replacement Systems. *IUBMB Life*, Vol. 50, No. 4-5, pp. 279-289, ISSN 1521-6551
- Eldridge, S.R.; Gould, M.N. & Butterworth, B.E. (1992). Genotoxicity of Environmental Agents in Human Mammary Epithelial Cells. *Cancer Research*, Vol. 52, No. 20, pp 5617-5621, ISSN 0008-5472
- EPA:U.S Environmental Protection Agency (2005). *Guidelines for Carcinogen Risk Assessment*, U.S. Environmental Protection Agency, Washington D.C.
- Evan, G.I. & Vousden, K.H. (2001). Proliferation, Cell Cycle and Apoptosis in Cancer. *Nature*, Vol. 411, No. 6835, pp. 342-348, ISSN 0028-0836
- Feinberg, A.P.; Oshimura, M. & Barrett, J.C. (2002) Epigenetic Mechanisms in Human Diseases. *Cancer Research*, Vol. 62, pp. 6784-6787, ISSN 0008-5472
- Fenech, M. & Rinaldi, J. (1994). The Relationship between Micronuclei in Human Lymphocytes and Plasma Levels of Vitamin-C, Vitamin-E, Vitamin B-12 and Folic Acid. *Carcinogenesis*, Vol.15, No. 7, pp.1405-1411, ISSN 0143-3334
- Fenech, M. (2001). Recommended Dietary Allowances (RDAs) for Genomic Stability. *Mutation Research*, Vol. 480-481, pp.51-54, ISSN 0027-5107
- Fenech, M. (2001). The Role of Folic Acid and Vitamin B12 in Genomic Stability of Human Cells. *Mutation Research*, Vol. 475, pp.57-68, ISSN 0027-5107
- Fiala, E.S.; Staretz, M.S.; Pandya, G.A.; El-Bayoumy, K. & Hamilton, S.R. (1998). Inhibition of DNA Cytosine Methyltransferase by Hemopreventive Selenium Compounds, Determined by an Improved Assay for DNA Cytosine Methyltransferase and DNA Cytosine Methylation. *Carcinogenesis*, Vol. 19, No. 4, pp. 597-604, ISSN 0143-3334
- Forni, A. (1987). Cytogenetic Methods for Assessing Human Exposure to Genotoxic Chemicals, In: *Occupational and Environmental Chemical Hazards-Chemical and Biochemical Indices for Monitoring Toxicity*, V. Foa, E. A. Emmet, M. Maroni, & A. Colombi, (eds.), 403-410, Ellis Horwood. Ltd, ISBN 0136298907 Chichester, United Kingdom
- Forni, A.; Lamberti, L.; Lando, C.; Padovani, P.; Sbrana, I; Vecchio, D & Puntoni, R. (1995). Are Chromosome Aberrations in Circulating Lymphocytes Predictive of a Future Cancer Onset in Humans? Preliminary Results of an Italian Cohort Study. *Cancer Genetics Cytogenetics*, Vol.79, pp. 133-135, ISSN 0165-4608
- Friedberg, E.C. (2003). DNA Damage and Repair. *Nature*, Vol. 421, No. 6921, pp. 436- 440, ISSN 0028-0836

- Geyer, R.K.; Nagasawa, H; Little, J.B. & Maki, C.G. (2000). Role and Regulation of p53 During an Ultraviolet Radiation-Induced G₁ Cell Cycle Arrest. *Cell Growth & Differentiation*, Vol. 11, No. 3, pp. 149-156, ISSN 1044-9523
- Gretarsdottir, S.; Thoriacius, S.; Valgardsdottir, R.; Gudlaugsdottir, S.; Sigurdsson S.; Steinarsdottir, M.; Jonansson, J.G; Anamthawat, T. & Jonsson, K. (1998). BRCA2 and p53 Mutations in Primary Breast Cancer in Relation to Genetic Instability. *Cancer Research*, Vol. 58, No. 5, pp. 859-862, ISSN 0008-5472
- Guo, R.; Chen, J.; Mitchell, D.L. & Johnson, D.G. (2011). GCN5 and E2F1 Stimulate Nucleotide Excision Repair by Promoting H3K9 Acetylation at Sites of Damage. *Nucleic Acids research*, Vol. 39, No. 4, pp. 1390-1397, ISSN 0305-1048
- Gupta, R.C.; Reddy, M.V. & Randerath, K. (1982). 32P Postlabeling Analysis of Non-radioactive Aromatic Carcinogen-DNA Adducts. *Carcinogenesis*, Vol. 3, No. 9, pp. 1081-1092, ISSN 0143-3334
- Gupta, S.; Husser, R.C.; Geske, R.S.; Welty, S.E. & Smith, C.V. (2000). Sex Differences in Diquat-induced Hepatic Necrosis and DNA Fragmentation in Fischer 344 Rats. *Toxicological Sciences*, Vol. 54, No. 1, pp. 203-211, ISSN 1096-6080
- Hagmar, L.; Brogger, A.; Hansteen, I.-L.; Heim, S.; Hagstadt, B.; Knudsen, L.; Lambert, B.; Linnainmaa, K.; Mitelman, F.; Nordenson, I.; Reuterwall, C.; Salomaa, S.; Skerfving, S. & Sorsa, M. (1994). Cancer Risk in Humans Predicted by Increased Levels of Chromosome Aberrations in Lymphocytes: Nordic Study Group on the Health Risk of Chromosome Damage. *Cancer Research*, Vol. 54, pp. 2919-2922, ISSN 0008-5472
- Hagmar, L.; Stromberg, U.; Tinnerberg, H. & Mikoczy, Z. (2004). *Epidemiological Evaluation of Cytogenetic Biomarkers as Potential Surrogate End-points for Cancer*, Vol. 157, pp. 207-215, IARC Sci. Publ., ISBN 9283221575,
- Hannan, M.A.; Hellan, A.; M.Al-Khodairy, F.; Kunhi, M.; Siddiqui, Y.; Al-Yussef, N.; Pangué-Cruz, N.; Siewertsen, M.; N.AL-Adhal, M. & Aboussekhra, A. (2002). Deficiency in the Repair of UV-induced DNA Damage in Human Skin Fibroblasts Compromised for the ATM Gene. *Carcinogenesis*, Vol. 23, No. 10, pp. 1617-1624, ISSN 0143-3334
- Harriott-Smith, T.G. & Halliday, W.J. (1988). Suppression of Contact Hypersensitivity by Short-Term Ultraviolet Irradiation: II. The Role of Urocanic Acid, *Clinical and Experimental Immunology*, Vol. 72, pp. 174-177, ISSN 1365-2249
- Hartwig, A. (2010). The Role of DNA Repair in Benzene-Induced Carcinogenesis. *Chemico-Biological Interactions*, Vol. 184, No. 1-2, pp. 269-272. ISSN 0009-2797
- Hayes, R.B. (1992). Biomarkers in Occupational Cancer Epidemiology: Consideration in Study Design. *Environmental Health Perspectives*, Vol. 98, pp.149-154, ISSN 0091-6765
- Heflich, R.H. (1991). Chemical Mutagens, In: *Genetic Toxicology*, Li A. P. & Heflich R. H., (Eds.), CRC Press, ISBN 0849388155, Boca Raton
- Hennekens, C.H.; Buring, J.E.; Manson, J.E.; Stampfer, M.; Rosner, B.; Cook, N.R.; Belanger, C.; Lamotte, F.; Gaziano, J.M.; Ridker, P.M.; Willett, W. & Peto, R. (1996). Lack of Effect of Long-term Supplementation with Beta Carotene on the Incidence of Malignant Neoplasms and Cardiovascular Disease. *The New England Journal of Medicine*, Vol. 334, No. 18, pp. 1145-1149, ISSN 0028-4793

- Hercberg, S. (2005). Antioxidant Vitamin and Mineral Supplementation and Prostate Cancer Prevention in the SU.VI.MAX Trial. *International Journal of Cancer*, Vol. 116, pp. 182-186, ISSN 0020-7136
- Herr, I. & Debatin, M. (2001). Cellular Stress Response and Apoptosis in Cancer Therapy. *Blood*, Vol. 98, No. 9, pp. 2603-2614, ISSN 0006-4971
- Hill, R.; Bodzak, E.; Blough, M.D. & Lee, P.W.K. (2008). p53 Binding to The p21 Promoter is Dependent on the Nature of DNA Damage. *Cell Cycle*, Vol. 7, No. 16, pp. 2535-2543, ISSN 1538-4101
- Hussain, S.P. & Harris, C.C. (1998). Molecular Epidemiology of Human Cancer: Contribution of Mutation Spectra Studies of Tumor Suppressor Genes. *Cancer Research*, Vol. 58, pp. 4023-4037, ISSN 0008-5472
- Jakab, M.G.; Major, J. & Tompa, A. (2001). Follow-Up Genotoxicological Monitoring of Nurses Handling Antineoplastic Drugs. *Journal of Toxicology and Environmental Health, Part A*, Vol. 62, No. 5, pp. 307-318. ISSN 1528-7394
- Jakab, M.G.; Klupp, T.; Besenyei, K.; Biró, A.; Major, J. & Tompa, A. (2010). Formaldehyde-Induced Chromosomal Aberrations and Apoptosis in Peripheral Blood Lymphocytes of Personnel Working in Pathology Departments. *Mutation Research*, Vol. 698. No. 1-2. pp. 11-17, ISSN 0921-8262
- Ji, K.; Kogame, T.; Chio, K.; Wang, X.; Lee, J.; Taniguchi, Y. & Takeda, S. (2009). A Novel Approach Using DNA-repair-deficient Chicken DT40 Cell Lines for Screening and Characterizing the Genotoxicity of Environmental Contaminants. *Environmental Health Perspectives*, Vol. 117, No. 11, pp. 1737-1744, ISSN 0091-6765
- Kaufman, W.K. (1995). Cell Cycle Checkpoints and DNA-Repair Preserve the Stability of the Human Genome. *Cancer and Metastasis Reviews.*, Vol. 14, pp. 31-41, ISSN 0167-7659
- Keretetse, G.S.; Laubscher, P.J.; Du Plessis, J.L.; Pretorius, P.J.; van der Westhuizen, F.H.; van Devanter, E.; van dyk, E.; Eloff, F.C.; van Ardee, M.N. & Du Plessis, L. H. (2008). DNA Damage and Repair Detected by the Comet Assay in Lymphocytes of African Petrol Attendants: A Pilot Study. *Annals of Occupational Hygiene*, Vol. 52, No. 7, pp. 653-662. ISSN 0003-4878
- Klaunig, J.E.; Xu, Y.; Isenberg, J.S., Bachowski, S.; Kolaja, K.L.; Jiang, J.Z.; Stevenson, D.E. & Walborg, E.F. (1998) The Role of Oxidative Stress in Chemical Carcinogenesis. *Environmental Health Perspectives*, Vol. 106, Suppl. 1., pp. 289-295, ISSN 0091-6765
- Klein, E. & Thompson, I.M. (2004). Update on Chemoprevention of Prostate Cancer. *Current Opinion in Urology*, Vol. 14, No. 3, pp. 143-149, ISSN 0963-0643
- Kopanja, D.; Stoyanova, T.; Okur, N.M.; Huang, E.; Bagchi, S. & Raychaudhuri, P. (2009). Proliferation Defects and Genome Instability in Cells Lacking Cul4A. *Oncogene*, Vol. 28, No. 26, pp. 2456-2465, ISSN 0950-9232
- Kriek, E.; Rojas, M.; Alexandrov, K. & Bartsch, H. (1998). Polycyclic Aromatic Hydrocarbon-DNA Adducts in Humans: Relevance as Biomarkers for Exposure and Cancer Risk. *Mutation Research*, Vol. 400, pp. 215-231, ISSN 0027-5107
- Laffon, B.; Teixeira, J.P.; Silva, S.; Loureiro, J.; Torres, J.; Pásaro, E.; Méndez, J. & Mayan, O. (2005). Genotoxic Effects in a Population of Nurses Handling Antineoplastic Drugs, and Relationship with Genetic Polymorphism in DNA Repair Enzymes. *American Journal of Industrial Medicine*, Vol. 48. No. 2. pp. 128-136. ISSN 0271-3586

- Lamprecht, S. & Lipkin, M. (2003). Chemoprevention of Colon Cancer by Calcium, Vitamin D and Folate: Molecular Mechanisms. *Nature Reviews Cancer*, Vol. 3, pp. 601-614, ISSN 1474-175X
- Lans, H.; Marteijn, J.A.; Schumacher, B.; Hoeijmakers, J.H.J.; Jansen, G. & Vermeulen, G. (2010). Involvement of Global Genome Repair, Transcription Coupled Repair, and Chromatin Remodeling in UV DNA Damage Response Changes during Development. *Plos Genetics*, Vol. 6, No. 5, Art. No. e1000941, ISSN 1553-7390
- Legrand, M.; Chan, C.L.; Jauert, P.A. & Kirkpatrick, D.T. (2008). Analysis of Base Excision and Nucleotide Excision Repair in *Candida Albicans*. *Microbiology*, Vol. 154, Part: past 8, pp. 2446-2456, ISSN 1350-0872
- Loeb, L.A. (1991). Mutator Phenotype May be Required for Multi-stage Carcinogenesis. *Cancer Research*, Vol. 51, pp. 3075-3079, ISSN 0008-5472
- Lowe, S.W. & Lin, A.W. (2000). Apoptosis in Cancer. *Carcinogenesis*, Vol. 21, No. 3, pp. 485-495, ISSN 0143-3334
- Major, J.; Szende, B.; Lapis, K. & Thész, Z. (1985). Increased SCE Inducibility by Low Doses of Methylcholanthrene in Lymphocytes Obtained from Patients with Down's Disease. *Mutation Research*, Vol. 149, pp. 51-55, ISSN 0921-8262
- Major, J.; Jakab, M.G. & Tompa A. (1996). Genotoxicological Investigation of Hospital Nurses Occupationally Exposed to Ethylene-Oxide: I. Chromosome Aberrations, Sister-Chromatid Exchanges, Cell Cycle Kinetics, and UV-Induced DNA Synthesis in Peripheral Blood Lymphocytes. *Environmental and Molecular Mutagenesis*, Vol. 27, No. 2, pp. 84-92. ISSN 0893-6692
- Major, J.; Jakab, M.G. & Tompa A. (1999). The Frequency of Induced Premature Centromere Division in Human Populations Occupationally Exposed to Genotoxic Chemicals. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, Vol. 445, pp. 241-249, ISSN 1383-5718
- Méhes, K. & Bajnóczky, K. (1981). Non-random Centromere Division: Analysis of G-banded Human Chromosomes. *Acta Biologica Academiae Scientiarum Hungariae*, Vol. 32, pp. 55-59, ISSN 0001-5288
- Méhes, K. (1978). Non-random Centromere Division: a Mechanism of Non-disjunction Causing Aneuploidy? *Human Heredity*, Vol. 28, pp. 255-260, ISSN 0001-5652
- Meydani, S.N.; Wu, D. & Hayek, M.G. (1995). Antioxidant and Immune Response in Aged Persons: Overview of Present Evidence. *American Journal of Clinical Nutrition*, Vol. 62, (suppl), pp. 1462 S-1476 S, ISSN 0002-9165
- Moorhead, P.S.; Nowell, P.C.; Mellman, W.J.; Battips, D.M. & Hungerford, D.A. (1960). Chromosome Preparation of Leukocytes Cultured from Human Peripheral Blood. *Experimental Cell Research*, Vol. 20, pp. 613-616, ISSN 0014-4827
- Nair, J.; Ohshima, H.; Nair, U.J. & Bartsch, H.: (1996). Endogenous Formation of Nitrosamines and Oxidative DNA Damaging Agents in Tobacco Users. *Critical Reviews in Toxicology*, Vol. 26, pp. 149-161, ISSN 1040-8444
- Newby, J.A. & Howard, M.B. (2005). Environmental Influences in Cancer Aetiology. *Journal of Nutritional & Environmental Medicine*, Vol. 15, No 2/3, pp. 56-114, ISSN 1359-0847
- Norppa, H. (1997). Cytogenetic Markers of Susceptibility: Influence of Polymorphic Carcinogen-metabolizing Enzymes. *Environmental Health Perspectives*, Vol. 105, (Suppl. 4), pp. 829-835, ISSN 0091-6765

- Ohshima, H. & Bartsch, H. (1994). Chronic Infections and Inflammatory Processes as Cancer Risk Factors: Possible Role of Nitric Oxide in Carcinogenesis. *Mutation Research*, Vol. 305, pp. 253–264, ISSN 0921-8262
- Okada, T.; Sonoda, E.; Yoshimura, M.; Kawano, Y.; Saya, H.; Kohzaki, M. & Takeda, S. (2005). Multiple Roles of Vertebrate REV Genes in DNA Repair and Recombination. *Molecular and Cellular Biology*, Vol. 25, No. 14, pp. 6103-6111, ISSN 0270-7306
- Omenn, G.S.; Goodman, G.E.; Thornquist, M.D.; Balmes, J.; Cullen, M.R.; Glass, A.; Keogh, J.P.; Meyskens, F.L.; Valanis, B.; Williams, J.H.; Barnhart, S. & Hammar, S. (1996). Effects of a Combination of Beta Carotene and Vitamin A on Lung Cancer and Cardiovascular Disease. *New England Journal of Medicine*, Vol. 334, pp. 1150-1155, ISSN 0028-4793
- Orelli, B.; McClendon, T.B.; Tsodikov, O.V.; Ellenberger, T.; Neidernhofer, L.J. & Schäfer, O.D. (2010). The XPA-binding Domain of ERCC1 is Required for Nucleotide Excision Repair but not other DNA Repair Pathways. *The Journal of Biological Chemistry*, Vol. 285, No. 6, pp. 3705-3712, ISSN 0021-9258
- Pavanello, S.; Pulliero, A.; Siwinska, E., Mielzynska, D. & Clonfero, E. (2005). Reduced Nucleotide Excision Repair and *GSTM1*-Null Genotypes Influence *Anti-B[A]P*-DNA Adduct Levels in Mononuclear White Blood Cells of Highly PAH-Exposed Coke Oven Workers. *Carcinogenesis*, Vol. 26, No. 1, pp. 169-175. ISSN 0143-3334
- Parry, J.M.; Jenkins, G.J.; Haddad, F.; Bourner, R. & Parry, E.M. (2000). In Vitro and in Vivo Extrapolations of Genotoxin Exposures: Consideration of Factors Which Influence Dose-response Thresholds. *Mutation Research*, Vol. 464, pp. 53–63, ISSN 1383-5718
- Perry, P. & Wolff. S. (1974). New Giemsa Method for the Differential Staining of Sister Chromatids. *Nature*, Vol. 251, pp. 156-158, ISSN 0028-0836
- Peto, R.; Doll, R.; Buckley, J.D. & Sporn, M.B. (1981). Can Dietary Beta-carotene Materially Reduce Human Cancer Rates? *Nature*, Vol. 290, pp. 201-209, ISSN 0028-0836
- Poulsen, H.E.; Prieme, H. & Loft, S. (1998). Role of Oxidative DNA Damage in Cancer Initiation and Promotion. *European Journal of Cancer Prevention*, Vol. 7, pp. 9-16, ISSN 0959-8278
- Prasad, S.; Phromnoi, K.; Yadav, V.R.; Chaturvedi, M.M.; Aggarwal, B.B. (2010). Targeting Inflammatory Pathways by Flavonoids for Prevention and Treatment of Cancer. *Planta Medica*; Vol. 76, No 11, pp. 1044-1063, ISSN 0032-0943
- Preston, R.J. & Williams, G.M. (2005). DNA-reactive Carcinogens: Mode of Action and Human Cancer Hazard. *Critical Reviews in Toxicology*, Vol. 35, pp. 673–683, ISSN 1040-8444
- Rappaport, S.M.; Waidyanatha, S.; Qu, Q.; Shore, R.; Jin, X.; Cohen, B.; Chen, L.C.; Melikian, A.A.; Li, G.; Yin, S.; Yan, H.; Xu, B.; Mu, R.; Li, Y.; Zhang, X. & Li, K. (2002). Albumin Adducts of Benzene Oxide and 1,4-benzoquinone as Measures of Human Benzene. *Cancer Research*, Vol. 62, pp. 1330–1337, ISSN 0008-5472
- Rastogi, R.P.; Richa; Kumar, A.; Tyagi, M.B. & Sinha, R.P. (2010). Molecular Mechanisms of Ultraviolet Radiation-induced DNA-damage and Repair. *Journal of Nucleic Acids*, Vol. 2010, Article ID 592980, 32 pages, doi:10.4061/2010/592980
- Reg. 440/2008/EC: Council Regulation (EC) No 440/2008 of 30 May 2008 Laying down Test Methods Pursuant to Regulation (EC) No. 1907/2006 of the European

- Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). *Official Journal L 142*, 31/05/2008 P. 0001 – 0739.
- Rothfus, A.; Schütz, P.; Bochum, S.; Volm, T.; Eberharat, E.; Kreienberg, R.; Vogel, W. & Speit, G. (2000). Induced Micronucleus Frequencies in Peripheral Blood Lymphocytes as a Screening Test for Carriers of a BRCA1 Mutation in Breast Cancer Families. *Cancer Research*, Vol. 60, pp. 390–394, ISSN 0008-5472
- Ruchirawat, M.; Navasumrit, P. & Settachan, D. (2009). Exposure to Benzene in Various Susceptible Populations: Co-Exposures to 1,3-butadiene and PAHs and Implications for Carcinogenic Risk, *Chemico-Biological Interactions*, Vol. 184, No. 1-2, pp. 67-76. ISSN 0009-2797
- Shi, Q.; Wang, L-E.; Bondy, M.; Brewster, A.; Singletary, S.E. & Wei, Q. (2004). Reduced DNA Repair of Benzo[a]pyrene Diol Epoxide-Induced Adducts and Common XPD Polymorphisms in Breast Cancer Patients. *Carcinogenesis*, Vol. 25, No. 9, pp. 1695-1700 ISSN 0143-3334
- Shishodia, S.; Amin, H. M.; Lai, R. & Aggarwal, B.B. (2005). Curcumin (Diferuloylmethane) Inhibits Constitutive NF- κ B Activation, Induces G1/S Arrest, Suppresses Proliferation, and Induces Apoptosis in Mantle Cell Lymphoma. *Biochemical Pharmacology*, Vol. 70, pp. 700–713, ISSN 0006-2952
- Solomon, E.; Borrow, J. & Goddard, A. D. (1991). Chromosome Aberrations and Cancer. *Science*, Vol. 254, pp. 1153–1160, ISSN 0036-8075
- Song, J.; Han, H.; Sabapathy, K.; Lee, B.; Yu, E. & Choi, J. (2010). Expression of Homeostatic Regulator, Wip1 (Wild-type p53-induced Phosphatase), is Temporally Induced by c-Jun and p53 in Response to UV Irradiation. *The Journal of Biological Chemistry*, Vol. 285, No. 12, pp. 9067-9076, ISSN 0021-9258
- Soria, G.; Speroni, J.; Podhajcer, O.P.; Prives, C. & Gottifredi, V. (2008). p21 Differentially Regulates DNA Replication and DNA-repair-associated Processes after UV Irradiation. *Journal of Cell Science*, Vol. 121, pp. 3271-3282, ISSN 0021-9533
- Sorsa, M. (1984). Monitoring of Sister Chromatid Exchanges and Micronuclei as Biological Endpoints, In: *Monitoring Human Exposure to Carcinogenic and Mutagenic Agents*, A. Berlin, M. Draper, K. Hemminki, & H. Vainio, (eds.), No. 59, pp. 339-349, IARC Scientific Publications, ISBN 9283211569, Lyon: International Agency for Research on Cancer
- Sorsa, M. & Anderson, D. (1996). Monitoring of Occupational Exposure to Cytostatic Anticancer Drugs, *Mutation Research*, Vol. 355. No. 1-2. pp. 253-261, ISSN 0921-8262
- Sørensen, M.; Autrup, H.; Møller, P.; Hertel, O.; Jensen, S.S.; Vinzents, P.; Knudsen, L.E. & Loft, S. (2003). Linking Exposure to Environmental Pollutants with Biological Effects. *Mutation Research*, Vol. 544, No. 2-3, pp. 255-271, ISSN 0921-8262
- Speit, G.; Schütz, P. & Merk, O. (2000). Induction and Repair of Formaldehyde-induced DNA-Protein Crosslinks in Repair Deficient Human Cell Lines, *Mutagenesis*, Vol. 15. No. 1. pp. 85-90. ISSN 0267-8357
- Squires, S.; Coates, J.A.; Goldberg, M.; Toji, L.H.; Jackson, S.P.; Clarke, D.J. & Johnson, R.T. (2004). p53 Prevents the Accumulation of Double-Strand DNA Breaks at Stalled-replication Forks Induced by UV in Human Cells. *Cell Cycle*, Vol. 3, No. 12., pp. 1543-1557, ISSN 1538-4101

- Sreevidya, C.S.; Fukunaga, A.; Khaskhely, N.M.; Masaki, T.; Ono, R.; Nishigori, C. & Ullrich, S.E. (2010). Agents That Reverse UV-Induced Immune Suppression and Photocarcinogenesis Affect DNA Repair. *Journal of Investigative Dermatology*, Vol. 130, No. 5, pp. 1428-1437, ISSN 0022-202X
- Tompa, A. & Sápi, E. (1989). Detection of 6-thioguanine Resistance in Human Peripheral Blood Lymphocytes /PBL/ of Industrial Workers and Lung Cancer Patients. *Mutation Research*, Vol. 210, pp. 345-351, ISSN 0921-8262
- Tompa A.; Major J. & Jakab, M. (1994). Monitoring of Benzene-exposed Workers for Genotoxic Effects of Benzene: Improved-working-condition-related Decrease in the Frequencies of Chromosomal Aberrations in Peripheral Blood Lymphocytes. *Mutation Research*, Vol. 304, pp. 159-165, ISSN 0921-8262
- Tompa, A.; Jakab, M.G. & Major J. (2005). Risk Management among Benzene-exposed Oil Refinery Workers. *International Journal of Hygiene and Environmental Health*, Vol. 208, No.6, pp. 509-516, ISSN 1438-4639
- Tompa, A.; Major, J. & Jakab, M.G. (1999). Is Breast Cancer Cluster Influenced by Environmental and Occupational Factors among Hospital Nurses in Hungary? *Pathology and Oncology Research*, Vol. 5. No. 2. pp. 117-121, ISSN 1219-4956
- Tompa, A.; Jakab, M.; Biró, A.; Magyar, B.; Fodor, Z.; Klupp, T. & Major J. (2006). Chemical Safety and Health Conditions among Hungarian Hospital Nurses. *Annals of the New York Academy of Sciences*, Vol. 1076. pp. 635-648. ISSN 0077-8923
- Tompa, A.; Jakab, M.G. & Major, J. (2010). Cancer Risk Assessment, Primary Prevention and Chemoprevention in Occupational Health Using Chromosomal Aberration and Sister Chromatid Exchange (SCE) as Biomarkers. *European Journal of Oncology*, Vol. 15, pp. 149-156, ISSN 1128-6598
- Trichopoulou, A.; Lagiou, P.; Kuper, H.; Trichopoulos, D. (2000). Cancer and Mediterranean Dietary Traditions. *Cancer Epidemiology, Biomarkers & Prevention*, Vol. 9, pp. 869-873, ISSN 1055-9965
- Tsuda; Matsumoto, H.K.; Ogino, H.; Ito, M; Hirono, I.; Nagao, M.; Sato, R.; Cabral R & Bartsch, H. (1993). Demonstration of Initiation Potential of Carcinogens by Induction of Preneoplastic Glutathione S-transferase P-form-positive Liver Cell Foci: Possible in Vivo Assay System for Environmental Carcinogens. *Japanese Journal of Cancer Research*, Vol. 84, pp. 230-236, ISSN 0910-5050
- Tucker, J.D.; Eastmond, D.A. & Littlefield, L.G. (1997). *Cytogenetic End-points as Biological Dosimeters and Predictors of Risk in Epidemiological Studies*, Vol. 142, pp. 185-200, IARC Sci. Publ., ISBN 928322-1427
- Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J. & Telser, J. (2004). Role of Oxygen Radicals in DNA Damage and Cancer Incidence. *Molecular and Cellular Biochemistry*, Vol. 266, pp. 37-56, ISSN 0300-8177
- van Erp, P.E.J.; Brons, P.P.T.; Boezeman, J.B.M.; de Jongh, G.J. & Bauer, F.W. (1988). A Rapid Flow Cytometric Method for Bivariate Bromodeoxyuridine/DNA Analysis Using Simultaneous Proteolytic Enzyme Digestion and Acid Denaturation. *Cytometry*, Vol. 9, pp. 627-630, ISSN 0196-4763
- Vig, B.K. (1981). Sequence of Centromere Separation: an Analysis of Mitotic Chromosomes in Man. *Human Genetics*, Vol. 57, pp. 247-252, ISSN 0340-6717
- Vineis, P. (1997). Molecular Epidemiology: Low-dose Carcinogens and Genetic Susceptibility. *International Journal of Cancer*, Vol. 71, pp. 1-3, ISSN 0020-7136

- Vineis, P.; Talaska, G.; Malaveille, C.; Bartsch, H.; Martone, T.; Sithisarankul, P. & Strickland, P. (1996). DNA Adducts in Urothelial Cells: Relationship with Biomarkers of Exposure to Arylamines and Polycyclic Aromatic Hydrocarbons from Tobacco Smoke. *International Journal of Cancer*, Vol. 65, pp. 314-316, ISSN 0020-7136
- Vogelstein, B. & Kinzler, K.W. (1998). in *The Genetic Basis of Human Cancer*, Kinzler, K.W. & Vogelstein, B., (eds.), McGraw-Hill, ISBN 0070675961
- Walterscheid, J.P.; Nghiem, D.X.; Kazimi, N.; Nutt, L.K.; McConkey, D.J.; Norval M, & Ulrich, S.E. (2006). *Cis-Urocanic Acid*, a Sunlight-induced Immunosuppressive Factor, Activates Immune Suppression via the 5-HT_{2A} Receptor. *Proceedings of the National Academy of Sciences U S A*, Vol. 103, pp. 17420-17425, ISSN
- Ward, E. (1995). Overview of Preventable Industrial Causes of Occupational Cancer, *Environmental Health Perspectives*, Vol. 103, Suppl. 8., pp. 197-203, ISSN 0090-6765
- Wei, Q.; Lee, J.E.; Gershenwald, J.E.; Ross, M.I.; Mansfield, P.F.; Strom, S.S.; Wang, L.; Guo, Z.; Qiao, Y.; Amos, C.I.; Spitz, M.R. & Duvic, M. (2003). Repair of UV Light-induced DNA Damage and Risk of Cutaneous Malignant Melanoma. *Journal of the National Cancer Institute*, Vol. 95, No. 4, pp. 308-315, ISSN 0027-8874
- Weitsman, G.E.; Ravid, A.; Liberman, U.; Koren, R.; (2003). Vitamin D Enhances Caspase-dependent and -independent TNF α -induced Breast Cancer Cell death: the Role of Reactive Oxygen Species and Mitochondria. *International Journal Cancer*, Vol. 106, pp. 178-186, ISSN 0077-8923
- Wogan, G.N.; (1992). Molecular Epidemiology in Cancer Risk Assessment and Prevention: Recent Progress and Avenues for Future Research. *Environmental Health Perspectives*, Vol. 98, pp. 167-178, ISSN 0091-6765
- World Cancer Research Fund/American Institute of Cancer Research (2007). Food, Nutrition, Physical Activity and the prevention of Cancer: A Global Perspective, American Institute of Cancer Research, Washington DC.
- Xu, X.; Kelsey, K.T.; Wiencke, J.K.; Wain, J.C. & Christiani, D.C.; (1996). Cytochrome 450 CYP1A1 MSPI Polymorphism and Lung Cancer Susceptibility. *Cancer Epidemiology, Biomarkers & Prevention*, Vol. 5, pp. 687-692, ISSN 1055-9965
- Yang, G.; Liao, J.; Kim, K.; Yurkow, E.J. & Yang, C.S. (1998). Inhibition of Growth and Induction of Apoptosis in Human Cancer Cell Lines by Tea Polyphenols. *Carcinogenesis*, Vol. 19, No. 4, pp. 611-616, ISSN 0143-3334
- Young-In Kim (2004). Will Mandatory Folic Acid Fortification Prevent or Promote Cancer? *American Journal of Clinical Nutrition*, Vol. 80, No. 5, pp. 1123-1128, ISSN 0002-9165
- Zampetti-Bosseler, F. & Scott, D. (1981). Cell Death, Chromosome Damage and Mitotic Delay in Normal Human, Ataxia Teleangiectasia and Retinoblastoma Fibroblasts after X-ray Irradiation. *International Journal of Radiation Biology*, Vol. 39, pp. 547-558, ISSN 0020-7616
- Zatonski, W.; Ohshima, H.; Przewozniak, K.; Drosik, K.; Mierzwinska, J.; Krygier, M.; Chmielarczyk W. & Bartsch, H. (1989). Urinary Excretion of N-nitrosamino Acids and Nitrate by Inhabitants of High- and Low-risk Areas for Stomach Cancer in Poland. *International Journal of Cancer*, Vol. 44, pp. 823-827, ISSN 0020-7136

- Zhang, Y.; Rohde, L.H. & Wu, H. (2009). Involvement of Nucleotide Excision and Mismatch Repair Mechanisms in Double Strand Break Repair. *Current Genomics*, Vol. 10, No. 4, pp. 250-258, ISSN 1389-2029
- Zhong, Q.; Amin, S.; Lazarus, P. & Spratt, T.E. (2010). Differential Repair of Polycyclic Aromatic Hydrocarbon DNA Adducts from an Actively Transcribed Gene. *DNA Repair (Amsterdam)*, Vol. 9, No. 9, pp. 1011-1016. ISSN 1568-7864

Hypoxia Inhibits DNA Repair to Promote Malignant Progression

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1. Introduction

DNA repair is essential to genomic integrity of the cell. Numerous studies have provided compelling evidence that hereditary defects in DNA repair are linked with predispositions to cancer, shedding light on the mechanism of DNA repair and its importance in cancer biology. The majority of human cancers however rarely undergo somatic mutations in DNA repair genes, yet they acquire genetic alterations as they progress. Little is known about the mechanism of genetic alteration during malignant progression. We and others have shown recently that hypoxia—oxygen deficiency—plays an important role in inhibiting DNA repair. Tumor hypoxia gives rise to the over-expression and activation of hypoxia-inducible factor 1 α (HIF-1 α), an oxygen-sensitive transcription factor commonly over-expressed in human cancers. Although HIF-1 α is best known for its transcriptional up-regulation of a host of target genes responsible for tumor growth and survival, in this chapter we describe a novel mechanism by which HIF-1 α inhibits DNA repair, thereby promoting genetic alterations and malignant progression.

2. Tumor hypoxia and hypoxic response

2.1 Role of hypoxia in tumor progression

In mammals, molecular oxygen is required for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor. Although the oxygen concentration in ambient air is approximately 21%, oxygen levels in mammalian tissues are generally maintained at 2–9%. Tissues become hypoxic when oxygen levels drop below 2% and severely hypoxic or anoxic when they are less than 0.02% (Bertout et al, 2008). Whereas mild hypoxia is part of normal development and physiology, as well as pathophysiology, severe hypoxia occurs mostly in pathophysiological conditions.

Human cancers frequently experience mild to severe hypoxia arising from rapid cell proliferation that outstrips oxygen supply and aberrant angiogenic processes that yield vascular anomaly, such as vessel tortuosity, arteriovenous shunts, and blind ends. Consequently, despite rampant angiogenic response, necrotic lesions become conspicuous

as a result of severe hypoxia or anoxia, especially in advanced human cancers accompanying cell proliferation. By contrast, benign tumors are less hypoxic, with cells proliferating at a pace compatible with vasculature development. Therefore, the degree of malignancy appears to be associated with the severity of hypoxia.

Many studies have indicated that hypoxia promotes tumor metabolism, angiogenesis, and metastasis (Bertout et al, 2008; Giaccia et al, 2003; Harris, 2002; Semenza, 2003). Solid tumors manifest enhanced glucose consumption resulting from hypoxic switching to anaerobic glycolysis from the tricarboxylic acid cycle for bioenergetics. In advanced human gliomas, tumor angiogenesis is rampant and frequently accompanied by rapid cell proliferation. Hypoxia confers metastatic potential by altering extracellular matrix function and increasing the motility of tumor cells.

Moreover, it has long been recognized that tumor hypoxia is tightly linked with DNA damage, mutation, and over-replication (Bindra & Glazer, 2005; Bristow & Hill, 2008; Huang et al, 2007). It is proposed that hypoxia provides a physiological selective pressure in tumors for the expansion of apoptosis-resistant variants by acquiring p53 mutations (Graeber et al, 1996). Hypoxia also induces genomic rearrangement through activation of fragile sites, thereby triggering breakage–fusion–bridge cycles and, in turn, gene amplification (Coquelle et al, 1998). Furthermore, hypoxia is known to contribute to the development of resistance to radiation therapy and chemotherapy by, for example, up-regulating the expression of the multidrug resistance gene *ABCB1*, also known as *MDR1*.

2.2 Hypoxic response

Through evolution, mammals have developed a coordinated physiological system to cope with hypoxic stress (Bunn & Poyton, 1996; Semenza, 1999). Respiratory, circulatory, and hematopoietic systems are developed at the systemic level for oxygen uptake, transport, and delivery. Oxygen-sensing and responsive mechanisms are widely present in mammalian cells to maintain oxygen homeostasis by altering DNA replication, gene expression, and metabolism. In particular, the identification of HIF-1 α (Wang & Semenza, 1993; Wang & Semenza, 1995) has immensely advanced the understanding of hypoxic response at the molecular level.

HIF-1 α is an oxygen-sensitive transcription factor that belongs to the PAS (Per-ARNT-Sim) superfamily (Wang et al, 1995) (Fig. 1). Upon dimerization with ARNT (arylhydrocarbon receptor nuclear translocator; also known as HIF-1 β), HIF-1 α recognizes the hypoxia-responsive element (5'-RCGTG-3') in the promoter and/or enhancer of its target genes via a basic helix–loop–helix domain at the amino terminus (Semenza, 1999). HIF-1 α heterodimerization depends on both the helix–loop–helix domain and the downstream PAS domain, which can be divided into two subdomains: PAS-A and PAS-B. Whereas PAS-A has been implicated in the heterodimerization and interaction with other proteins such as the heat shock protein 90 (Gradin et al, 1996; Semenza, 1999), the role of PAS-B remains less understood. HIF-1 α harbors an oxygen-dependent degradation (ODD) domain that mediates proteolysis by the ubiquitin–proteasome pathway (Huang et al, 1998; Pugh et al, 1997). Hypoxia-stabilized HIF-1 α recruits the transcription coactivator p300/CBP for transcriptional activation of its target genes via a potent transactivation domain at the carboxyl terminus (Arany et al, 1996; Gu et al, 2001; Kallio et al, 1998). Likewise, HIF-2 α (also known as EPAS1) (Tian et al, 1997) shares similar structural domains with HIF-1 α and binds to ARNT.

HIF-1 α is regulated primarily at the post-translational level (Huang & Bunn, 2003). Whereas both HIF-1 α and ARNT are constitutively expressed at mRNA and protein levels, HIF-1 α is



Fig. 1. Schematic representation of HIF-1 α , ARNT, and HIF-2 α . HIF-1 α possesses a basic helix–loop–helix (*bHLH*) domain, a Per–ARNT–Sim (*PAS*) domain that can be divided into PAS-A and PAS-B, and a transactivation (*TA*) domain. Although similar domains can be found in ARNT based on sequence homology, an oxygen-dependent degradation (*ODD*) domain is uniquely present in HIF-1 α . HIF-2 α shares strong sequence homology with HIF-1 α .

extremely sensitive to proteolysis under oxygenated conditions (Gradin et al, 1996; Huang et al, 1996; Pugh et al, 1997). The ODD domain is crucial for HIF-1 α proteolysis and is targeted for polyubiquitination by the E3 ubiquitin ligase containing the von Hippel-Lindau protein (pVHL) (Cockman et al, 2000; Ohh et al, 2000; Tanimoto et al, 2000). pVHL binding requires hydroxylation of two proline residues (Pro-402 and Pro-564 within the ODD domain) (Ivan et al, 2001; Jaakkola et al, 2001). Prolyl hydroxylation is catalyzed by a family of prolyl-4-hydroxylases (EglN1, EglN2, and EglN3, which are widely known as PHD2, PHD1, and PHD3, respectively) that belong to the 2-oxoglutarate-dependent oxygenase superfamily (Bruick & McKnight, 2001; Epstein et al, 2001; Kaelin & Ratcliffe, 2008). Accordingly, these prolyl-4-hydroxylases sense and transduce oxygen signals through hydroxylation, resulting in polyubiquitination and degradation of HIF-1 α . By contrast, hypoxia inhibits the oxygen-dependent enzymatic activity, thereby preventing HIF-1 α degradation. Consequently, a stabilized HIF-1 α exerts its transcriptional function through heterodimerization, DNA binding, and recruitment of p300/CBP for transactivation (Fig. 2).

Given the widespread over-expression of HIF-1 α and/or HIF-2 α in solid cancers, this canonical mechanism of HIF-1 α has been the guiding light for elucidating many of the pathological processes crucial for tumor growth and progression through the identification of numerous target genes of HIF-1 α and HIF-2 α (Bertout et al, 2008; Giaccia et al, 2003; Harris, 2002; Semenza, 2003). In particular, HIF-1 α and HIF-2 α have been shown to contribute to tumorigenesis, angiogenesis, metastasis, metabolism, and stem cell maintenance (Bertout et al, 2008; Majmundar et al, 2010). Despite these remarkable advancements in understanding the molecular basis of tumor hypoxia, the key question—whether HIF-1 α and HIF-2 α are responsible for genetic alterations that drive malignant progression—remains unanswered.

3. Regulation of DNA repair by hypoxia

3.1 DNA repair and human cancer

Hereditary defects in DNA repair have been linked with genetic instability and predispositions to cancer (Vogelstein & Kinzler, 2004). Genetic mutations in a host of DNA repair genes have been attributed to the development of a variety of human cancers including breast, colon, brain, and skin cancers, as well as leukemias and lymphomas. These genes participate in different pathways of DNA repair, such as mismatch

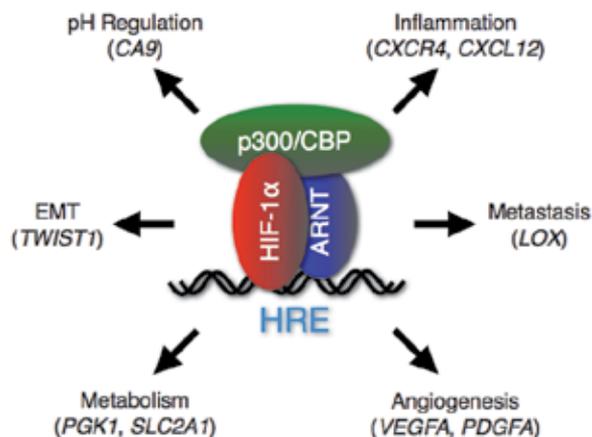


Fig. 2. The canonical mechanism of HIF-1 α is responsible for the expression of various relevant genes and pathological processes in tumor development and progression. *HRE*, hypoxia-responsive element; *EMT*, epithelial-mesenchymal transition.

repair (*MSH2*, *MSH6*), base excision repair (*MUTYH*), nucleotide excision repair (*XPA*), and double-strand break repair (*NBS1*, *BRCA1*). A wealth of knowledge has been gained that significantly advances our understanding of the biology of DNA repair and the mechanism of cancer development, and yet the role of DNA repair in the majority of cancers remains obscure because somatic mutations are rarely detected in DNA repair genes of sporadic cancers.

3.2 Functional impairment of DNA repair by hypoxia

To account for genetic alterations in sporadic cancers, functional impairment rather than genetic defect in DNA repair has been proposed on the basis of the evidence that hypoxia inhibits the expression of genes involved in DNA mismatch repair and double-strand break repair (Bindra & Glazer, 2005; Bristow & Hill, 2008; Huang et al, 2007). Indeed, multiple studies have shown hypoxic suppression of DNA repair genes including *MLH1* (Mihaylova et al, 2003), *MSH2* and *MSH6* (Koshiji et al, 2005), *RAD51* (Bindra et al, 2004), *BRCA1* (Bindra et al, 2005), and *NBS1* (To et al, 2006). Furthermore, it appears that hypoxia specifically inhibits homologous recombination but not non-homologous end joining, thereby inappropriately shunting double-strand break repair from high-fidelity homologous repair to error-prone non-homologous end joining. Moreover, hypoxic down-regulation of DNA repair genes results in DNA damage and genetic alteration, further supporting the notion that functional impairment of DNA repair induced by hypoxia contributes to genetic alterations in cancer. A challenging puzzle, however, is identifying the underlying mechanism by which hypoxia inhibits DNA repair. Some studies suggest that hypoxic down-regulation of *RAD51* and *BRCA1* involves formation of the E2F4/p130 repressive complex that occupies E2F sites in the target promoter (Bindra et al, 2005; Bindra & Glazer, 2007). It remains unclear, however, how hypoxia signals the E2F4/p130 complex and whether this mechanism of genetic alteration is relevant *in vivo* to cancer biology.

3.3 Mechanism of hypoxic inhibition of DNA repair

In the process of understanding how hypoxia inhibits DNA repair, we made the following observations (Koshiji et al, 2005): *i*) HIF-1 α but not HIF-2 α was required for hypoxic

induction of microsatellite instability and down-regulation of *MSH2* and *MSH6* in *MSH2*-proficient colon cancer cells; *ii*) ectopic expression of HIF-1 α but not HIF-2 α was sufficient to recapitulate the hypoxic effects; *iii*) whereas the transcriptional activator c-Myc was required for maintaining *MSH2* and *MSH6* expression, HIF-1 α competed with c-Myc for occupying the target gene promoter via the DNA-binding factor Sp1; and *iv*) HIF-1 α DNA-binding and transactivation domains were dispensable for *MSH2* and *MSH6* down-regulation. Hence, these findings have led us to propose a distinct mechanism by which HIF-1 α inhibits DNA repair (Fig. 3), which is referred to hereafter as the HIF-1 α -c-Myc pathway, in contrast to the aforementioned canonical mechanism as the HIF-1 α -ARNT pathway.

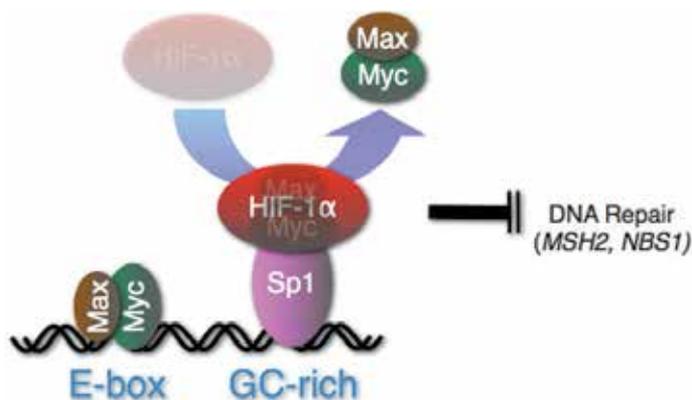


Fig. 3. A schematic representation of the HIF-1 α -c-Myc pathway, in which HIF-1 α competes with c-Myc for Sp1 binding to the promoter of DNA repair genes, resulting in c-Myc displacement and therefore inhibition of DNA repair.

In keeping with the finding that hypoxia inhibits DNA mismatch repair, we showed that the HIF-1 α -c-Myc pathway also accounted for hypoxic inhibition of *NBS1* and induction of DNA double-strand break (To et al, 2006). We not only identified HIF-1 α PAS-B as necessary and sufficient to mediate the HIF-1 α -c-Myc pathway, but also elucidated the molecular distinction between HIF-1 α and HIF-2 α that engenders functional difference in DNA repair. We demonstrated that despite strong sequence homology in the PAS-B subdomain, subtle differences between HIF-1 α Val-317, Ala-321, and Thr-327 and the corresponding amino acid residues of HIF-2 α essentially enabled HIF-1 α to inhibit DNA repair (To et al, 2006). This important finding has prompted us to test the hypothesis that the HIF-1 α -c-Myc pathway is crucial to malignant progression.

4. A mechanism of genetic alteration that drives malignant progression

Although we provided a molecular mechanism by which HIF-1 α but not HIF-2 α mediates hypoxic induction of genetic alteration by inhibiting DNA repair, whether such mechanism is relevant to cancer biology had yet to be demonstrated. Given the close association of malignant progression with genetic alteration, we sought to test our hypothesis that the HIF-1 α -c-Myc pathway is crucial to malignant progression by inducing genetic alterations. To isolate the HIF-1 α -c-Myc pathway from the HIF-1 α -ARNT pathway, we elected to uncouple the two pathways by inactivating each individually with site-directed

mutagenesis. Mutations were carried out in the context of a stabilized form of HIF-1 α , designated as HIF1 α (PP) (Kageyama et al, 2004), in which the prolyl hydroxylation sites Pro-402 and Pro-564 had been destroyed to prevent oxygen-dependent proteolysis (Fig. 4). Subsequently, the HIF-1 α -c-Myc pathway was inactivated by substituting the aforementioned Val, Ala, Thr residues in the PAS-B domain with those of HIF-2 α , yielding the mutant HIF1 α (PP)+VAT (Yoo et al, 2011). Likewise, the HIF-1 α -ARNT pathway was disabled by mutating residues critical for DNA binding and transactivation (Gu et al, 2001; Koshiji et al, 2004; Land & Tee, 2007) to create the mutant HIF1 α (PP)+RFC. Our results showed that the two pathways are functionally independent because HIF1 α (PP)+VAT was functional only in the HIF-1 α -ARNT pathway, while HIF1 α (PP)+RFC was active only in the HIF-1 α -c-Myc pathway (Yoo et al, 2011). In contrast, HIF1 α (PP) was active in both pathways.

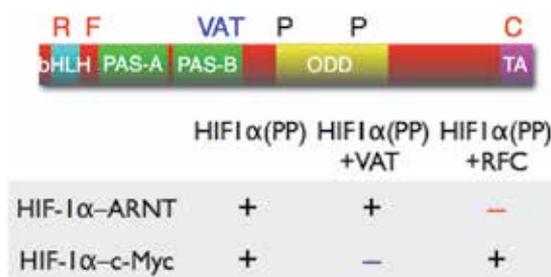


Fig. 4. The HIF-1 α -ARNT pathway and the HIF-1 α -c-Myc pathway are functionally independent. Both pathways are inactivated individually by site-directed mutagenesis with substitutions of specific amino acid residues, as indicated, in the functional domains.

After retroviral transduction with HIF1 α (PP) and HIF1 α (PP)+RFC, tumor cells exhibited conspicuous DNA double-strand breaks as indicated by a sharp increase of γ -H2AX foci in the nucleus and microsatellite instability when analyzed with specific mononucleotide and dinucleotide markers (Yoo et al, 2011) (Fig. 5). Furthermore, there was a loss of the tumor suppressor genes *FHIT* and *WWOX* at the genomic level, indicative of chromosomal fragile site instability (Yoo et al, 2011). However, none of these DNA damage and genetic alterations were detected in cells expressing HIF1 α (PP)+VAT, which was functional only in the HIF-1 α -ARNT pathway.

Importantly, all of these cells that had undergone genetic alterations not only acquired malignant traits including anchorage-independent growth and tumorigenicity but also became malignantly aggressive in mouse xenograft models, as indicated by rapid tumor growth, massive hemorrhagic necrosis, and rampant invasion of neighboring tissues (Yoo et al, 2011; Yoo et al, 2009). Moreover, advanced malignant progression was recapitulated in cells expressing HIF-1 α PAS-B alone but not its VAT mutant. Altogether, these studies strongly indicate that the HIF-1 α -c-Myc pathway is the underlying mechanism of malignant progression by inhibiting DNA repair and inducing genetic alterations.

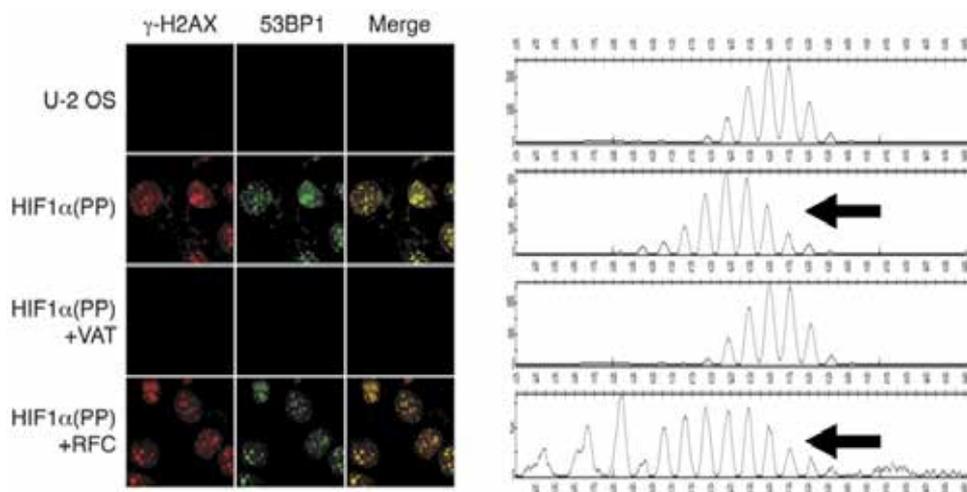


Fig. 5. The HIF-1 α -c-Myc pathway is responsible for DNA damage and genetic alteration. *Left*, Human osteosarcoma U-2 OS cells transduced with HIF-1 α variants as specified were determined with immunofluorescence for colocalization (*Merge*) of the phosphorylated histone variant γ -H2AX foci with those of 53BP1. Both serve as indicators of DNA damage, which were absent in the parental and HIF1 α (PP)+VAT cells. *Right*, The transduced cells were also analyzed for microsatellite instability, as indicated by the shortening (shift to the left) of genomic DNA fragments amplified with the mononucleotide marker BAT25. (Figure modified with permission from Yoo et al, 2011.)

5. Conclusion

Cancer remains one of the most deadly diseases overall and the leading cause of death in persons under the age of 85 years in the United States (Jemal et al, 2007). Technological advancements in cancer prevention and early detection have greatly reduced cancer incidence and mortality, yet the progressive nature of the disease seems to inevitably drive cancer to the advanced stage, posing an insurmountable challenge to the available treatments. The identification of the mechanism of hypoxia-driven malignant progression at the genetic level may provide a molecular basis for controlling the evolutionary process in tumors, a strategy proposed 35 years ago to combat tumor progression (Nowell, 1976).

6. Acknowledgment

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7. References

- Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, Livingston DM (1996) An essential role for p300/CBP in the cellular response to hypoxia. *Proc Natl Acad Sci U S A*, Vol.93, No.23, pp. 12969-12973.

- Bertout JA, Patel SA, Simon MC (2008) The impact of O₂ availability on human cancer. *Nat Rev Cancer*, Vol.8, No.12, pp. 967-975.
- Bindra RS, Gibson SL, Meng A, Westermarck U, Jasin M, Pierce AJ, Bristow RG, Classon MK, Glazer PM (2005) Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. *Cancer Res*, Vol.65, No.24, pp. 11597-11604.
- Bindra RS, Glazer PM (2005) Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis. *Mutat Res*, Vol.569, No.1-2, pp. 75-85.
- Bindra RS, Glazer PM (2007) Repression of RAD51 gene expression by E2F4/p130 complexes in hypoxia. *Oncogene*, Vol.26, No.14, pp. 2048-2057.
- Bindra RS, Schaffer PJ, Meng A, Woo J, Maseide K, Roth ME, Lizardi P, Hedley DW, Bristow RG, Glazer PM (2004) Down-regulation of Rad51 and decreased homologous recombination in hypoxic cancer cells. *Mol Cell Biol*, Vol.24, No.19, pp. 8504-8518.
- Bristow RG, Hill RP (2008) Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer*, Vol.8, No.3, pp. 180-192.
- Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, Vol.294, No.5545, pp. 1337-1340.
- Bunn HF, Poyton RO (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev*, Vol.76, No.3, pp. 839-885.
- Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH (2000) Hypoxia inducible factor- α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem*, Vol.275, No.33, pp. 25733-25741.
- Coquelle A, Toledo F, Stern S, Bieth A, Debatisse M (1998) A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. *Mol Cell*, Vol.2, No.2, pp. 259-265.
- Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*, Vol.107, No.1, pp. 43-54.
- Giaccia A, Siim BG, Johnson RS (2003) HIF-1 as a target for drug development. *Nat Rev Drug Discov*, Vol.2, No.10, pp. 803-811.
- Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgard R, Tora L, Gassmann M, Poellinger L (1996) Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol*, Vol.16, No.10, pp. 5221-5231.
- Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, Vol.379, No.6560, pp. 88-91.
- Gu J, Milligan J, Huang LE (2001) Molecular mechanism of hypoxia-inducible factor 1 α - p300 interaction. A leucine-rich interface regulated by a single cysteine. *J Biol Chem*, Vol.276, No.5, pp. 3550-3554.
- Harris AL (2002) Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer*, Vol.2, No.1, pp. 38-47.

- Huang LE, Arany Z, Livingston DM, Bunn HF (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem*, Vol.271, No.50, pp. 32253-32259.
- Huang LE, Bindra RS, Glazer PM, Harris AL (2007) Hypoxia-induced genetic instability—a calculated mechanism underlying tumor progression. *J Mol Med*, Vol.85, No.2, pp. 139-148.
- Huang LE, Bunn HF (2003) Hypoxia-inducible factor and its biomedical relevance. *J Biol Chem*, Vol.278, No.22, pp. 19575-19578.
- Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A*, Vol.95, No.14, pp. 7987-7992.
- Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001) HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, Vol.292, No.5516, pp. 464-468.
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-1alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, Vol.292, No.5516, pp. 468-472.
- Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007) Cancer statistics, 2007. *CA Cancer J Clin*, Vol.57, No.1, pp. 43-66.
- Kaelin WG, Jr., Ratcliffe PJ (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*, Vol.30, No.4, pp. 393-402.
- Kageyama Y, Koshiji M, To KK, Tian YM, Ratcliffe PJ, Huang LE (2004) Leu-574 of human HIF-1alpha is a molecular determinant of prolyl hydroxylation. *FASEB J*, Vol.18, No.9, pp. 1028-1030.
- Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, Poellinger L (1998) Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *EMBO J*, Vol.17, No.22, pp. 6573-6586.
- Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE (2004) HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO J*, Vol.23, No.9, pp. 1949-1956.
- Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P, Huang LE (2005) HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. *Mol Cell*, Vol.17, No.6, pp. 793-803.
- Land SC, Tee AR (2007) Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J Biol Chem*, Vol.282, No.28, pp. 20534-20543.
- Majmundar AJ, Wong WJ, Simon MC (2010) Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*, Vol.40, No.2, pp. 294-309.
- Mihaylova VT, Bindra RS, Yuan J, Campisi D, Narayanan L, Jensen R, Giordano F, Johnson RS, Rockwell S, Glazer PM (2003) Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. *Mol Cell Biol*, Vol.23, No.9, pp. 3265-3273.
- Nowell PC (1976) The clonal evolution of tumor cell populations. *Science*, Vol.194, No.4260, pp. 23-28.

- Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol*, Vol.2, No.7, pp. 423-427.
- Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, Ratcliffe PJ (1997) Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. *J Biol Chem*, Vol.272, No.17, pp. 11205-11214.
- Semenza GL (1999) Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol*, Vol.15, pp. 551-578.
- Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, Vol.3, No.10, pp. 721-732.
- Tanimoto K, Makino Y, Pereira T, Poellinger L (2000) Mechanism of regulation of the hypoxia-inducible factor-1alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J*, Vol.19, No.16, pp. 4298-4309.
- Tian H, McKnight SL, Russell DW (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev*, Vol.11, No.1, pp. 72-82.
- To KK, Sedelnikova OA, Samons M, Bonner WM, Huang LE (2006) The phosphorylation status of PAS-B distinguishes HIF-1alpha from HIF-2alpha in NBS1 repression. *EMBO J*, Vol.25, No.20, pp. 4784-4794.
- Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. *Nat Med*, Vol.10, No.8, pp. 789-799.
- Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A*, Vol.92, No.12, pp. 5510-5514.
- Wang GL, Semenza GL (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*, Vol.90, No.9, pp. 4304-4308.
- Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem*, Vol.270, No.3, pp. 1230-1237.
- Yoo YG, Christensen J, Huang LE (2011) HIF-1 α confers aggressive malignant traits on human tumor cells independent of its canonical transcriptional function. *Cancer Res*, Vol.71, No.4, pp. 1244-1252.
- Yoo YG, Hayashi M, Christensen J, Huang LE (2009) An essential role of the HIF-1alpha-c-Myc axis in malignant progression. *Ann N Y Acad Sci*, Vol.1177, pp. 198-204.

From the Molecular Biology to the Gene Therapy of a DNA Repair Syndrome: Fanconi Anemia

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1. Introduction

Fanconi anemia (FA) was first described in 1927 by the Swiss pediatrician Guido Fanconi in a family with five children, three of which had various physical abnormalities and hematological defects, in a condition that resembled pernicious anemia [1]. In all the three children the disease was manifested between ages of five to seven years old and had fatal consequences. Studies in peripheral blood cells from these and other patients with the same symptoms made Guido Fanconi to realize that the disorder affected all the hematopoietic lineages, not only erythropoiesis, and that these alterations were usually the main cause of mortality (See review in [2]). From the beginning of his studies, he thought that this disease was too complex to be caused by mutations in one single gene, but neither he, nor the rest of the research community could imagine at that time that mutations in at least fourteen different genes, currently known as FA genes, could account for the same disease. The description of the chromosomal instability of FA cells as a hallmark of the disease in 1964[3] focused the etiology of the disease as a DNA repair failure, but it was almost forty years later when this idea was confirmed.

In this chapter we will make an overview of the implications of the FA pathway in DNA repair and cell survival, and discuss the advances, limitations and perspectives of the therapeutic approaches used for the treatment of the most severe problem that takes place in FA patients, the bone marrow failure (BMF).

2. Fanconi anemia proteins form a complex pathway involved in the repair of DNA inter-strand cross-links

Mutations in at least 14 genes have been associated with FA. Patients with biallelic mutations in any of these FA genes (except in *FANCB*, which is X-linked) are assigned to different complementation groups (Table 1). The identification of the first FA complementation group was conducted by the fusion of cell lines generated from different FA patients[4]. The first FA gene, *FANCC*, was then identified by the transfection of cells from a FA patient with a cDNA expression library, followed by their exposure to mitomycin

C (MMC), a DNA cross-linking drug that is extremely toxic and generates specific chromosomal instability in FA cells[5]. Only those cells complemented with *FANCC* grew after MMC exposure, allowing the identification of the defective gene in these patients. Similar approaches, together with positional cloning and linkage analysis, allowed the identification of other protein members of the so called "FA core complex", which included *FANCA*, *FANCG*, *FANCF* and *FANCE*[6-10]. Although the description of *FANCD2*[11] and its activation by monoubiquitination after DNA damage linked FA with DNA repair, the confirmation of the involvement of the FA pathway in DNA repair and its link to homologous recombination occurred in 2002, when *BRCA2* was identified as the *FANCD1* gene[12]. After the discovery of *BRCA2* as a FA gene, several other FA genes were described, initially *FANCL*, containing the ubiquitin ligase activity of the complex [13] and later on, *FANCB*; the only FA gene linked to the X chromosome[14]. The description of *FANCI* (*BRIP1*)[15-17], *PALB2*[18] and *RAD51C* (not formally assigned yet as a FA gene)[19, 20] together with the previously described *BRCA2*, definitively linked the FA/*BRCA* pathway with increased cancer susceptibility[19, 21-24]. In subsequent studies *FANCI* [25-27] was found to be the partner of *FANCD2*. Additionally, the finding that *FANCM*[28] can interact with DNA, and the observation that *FANCP* (*SLX4*) had endonuclease activity, is allowing to unravel the role of the FA/*BRCA* pathway in the repair of DNA interstrand cross-links (ICLs) during replication [29](Table 1).

Interstrand cross-linking drugs covalently bind both strands of the DNA helix, blocking the DNA replication and transcription. As a consequence of stalled replication produced by ICLs or at S-phase entry, the FA pathway is activated (See review in [29]). Although the exact role of FA proteins in the repair of ICLs is not clear yet, it is known that they work together in a complex network, where the key event is the monoubiquitination of *FANCD2*[30] and *FANCI* [25-27] (D2-I complex). This monoubiquitination requires the presence of the FA core complex, currently known to be formed by *FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL* and *FANCM*[31], together with other FA associated proteins (FAAP; see Figure 1). The description of the last members of the FA/*BRCA* pathway, particularly *FANCM*, *FAN1* (associated nuclease, with no mutations still found in FA patients)[32-35] and *FANCP* (*SLX4*)[36, 37] has added new critical elements in this intriguing pathway.

After the generation of an ICL in the DNA, the progression of the DNA replication fork is stalled, and this activates the FA core complex and ATR. The first protein from FA pathway that seems to initiate DNA repair is *FANCM*. This protein, thanks to its translocase activity [28], together with FAAP24 and MHF1/2[29], moves along the DNA to sense the stalled replication fork[38], facilitating the translocation and anchoring the rest of the FA core complex to damaged DNA. In this way, the FA core complex can monoubiquitinate *FANCD2/I*, inducing the replication fork pause and the incision/unhooking of the ICL, facilitating the translesion synthesis and finally the HR machinery required for the repair of the DNA damage [39, 40]. Once the D2-I complex is monoubiquitinated, another FA protein - *FAN1* - is also recruited to sites of DNA damage, where all these proteins form large nuclear foci. The endonuclease activity of *FAN1* suggests that this protein could also pair with MUS81-EME1 to unhook the ICL[32, 35, 40] necessary for DNA repair. In this step, the endonuclease XPF-ERCC1, whose activity is coordinated by the last described member of the FA family *SLX4* (*FANCP*[36, 37]), probably also plays an important role in the repair of the DNA damage (See Figure 1).

C. group	FA genes	Prevalence	Chromosomal location	Protein size (kDa)	Protein characteristics	Required for Ub-FANCD2/I	RAD51 foci
FA-A	<i>FANCA</i>	66%	16q24.3	163	Member of the core complex. Phosphorylated by ATR kinase. Two NLS	Yes	Normal
FA-B	<i>FANCB</i>	~2%	Xp22.31	95	Member of the core complex. Contains a NLS	Yes	Normal
FA-C	<i>FANCC</i>	10%	9q22.3	63	Localized in the nucleus and cytoplasm. Member of the core complex.	Yes	Normal
FA-D1	<i>BRCA2</i>	~2%	13q12-13	380	Essential in HR by controlling RAD51. Interacts with FANCG, FANCD2 and PALB2. Cancer susceptibility gene.	No	Reduced
FA-D2	<i>FANCD2</i>	~2%	3q25.3	155,162	Monoubiquitinated by FA core complex. Phosphorylated by ATR and probably ATM after DNA damage.	Yes	Normal
FA-E	<i>FANCE</i>	~2%	6p21-22	60	Member of the core complex. Directly binds FANCD2. Contains two NLS.	Yes	Normal
FA-F	<i>FANCF</i>	~2%	11p15	42	Required for the assembly of FA core complex.	Yes	Normal
FA-G	<i>FANCG/XRCC9</i>	9%	9p13	68	Member of the core complex. Interacts with FANCD1, FANCD2 and XRCC3.	Yes	Normal
FA-I	<i>FANCI</i>	~2%	15q25-26	140,147	Monoubiquitinated by FA complex in a FANCD2 dependent manner. It is also phosphorylated after DNA damage.	Yes	Normal
FA-J	<i>FANCL/BRIP1</i>	~0.2%	17q22-24	140	DNA dependent ATPase and a 5'-3' DNA helicase. Binds the BCRT domain of BRCA1. Cancer susceptibility gene.	No	Normal
FA-L	<i>FANCL (PHF9)</i>	~0.2%	2p16.1	43	Core complex member with Ubiquitin ligase activity.	Yes	Normal
FA-M	<i>FANCM/HEF</i>	~0.2%	14q21.3	250	Member of the core complex with translocase activity and endonuclease domain. DNA damage sensor.	Yes/Partially in mice	Normal
FA-N	<i>FANCN/PALB2</i>	~2%	16p12.1	140	Partner and localizer of BRCA2. It links BRCA2 and BRCA1 proteins. Cancer susceptibility gene.	No	Reduced
FA-O	<i>RAD51C*</i>	~0.2%	17q22	43	Participates in several protein complexes involved in HR. Cancer susceptibility gene.	No	Reduced
FA-P	<i>SLX4</i>	~0.2%	16p13.3	200	Scaffold protein for endonucleases MUS81-EME1 and XPF-ERCC1. Involved in resolution of HR intermediates such as Holliday junctions.	No	Normal

* *FANCO/RAD51C* is not formally defined as a FA gene. NLS; nuclear localization signals. HR; Homologous recombination

Table 1. Main characteristics of the Fanconi anemia genes and their proteins.

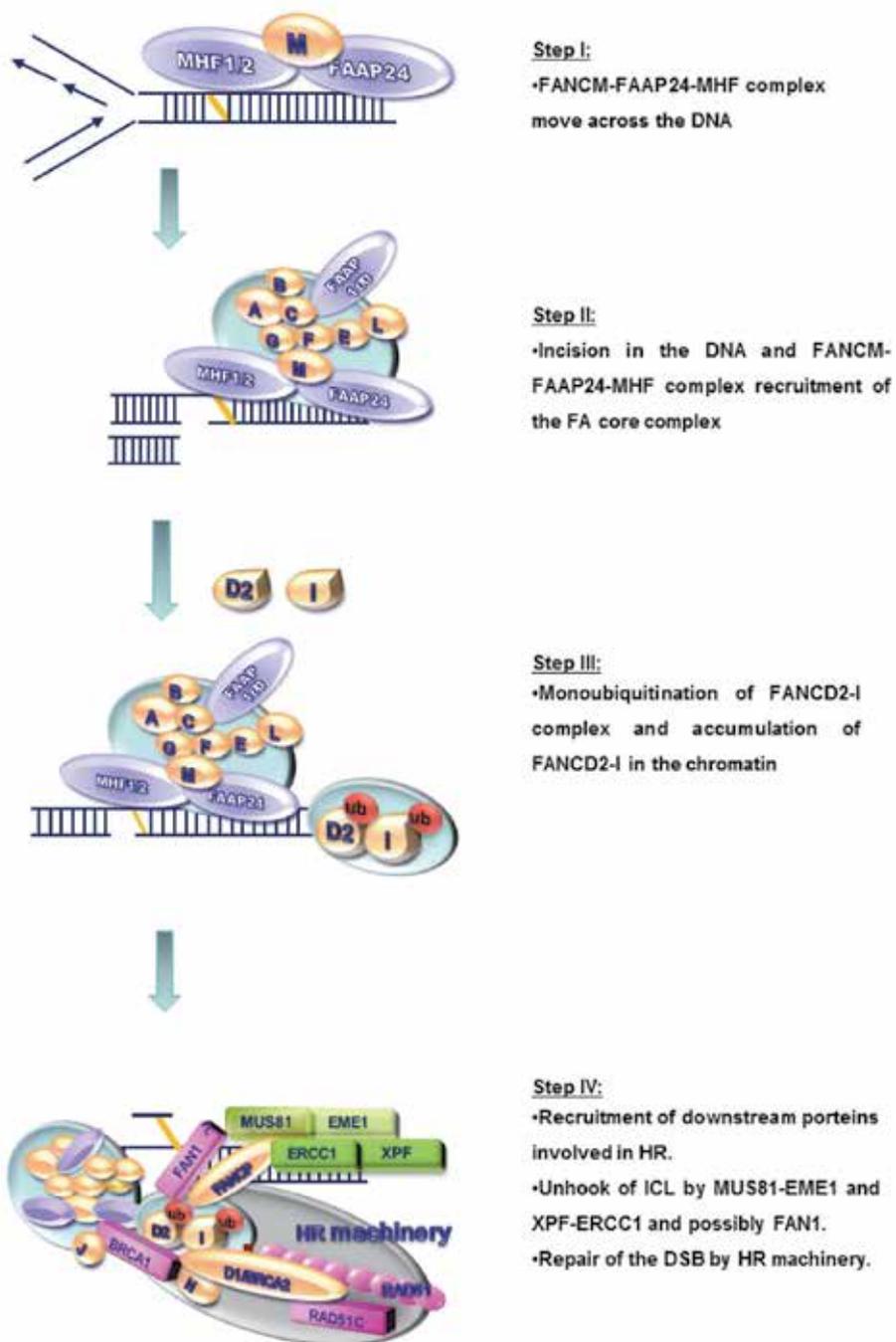


Fig. 1. Description of the four essential steps in the repair of DNA interstrand cross-links of the FA/BRCA pathway (Adapted from Niedernhofer [39], Kee et al.[29], Valeri *et al.* [41] and Cybulski and Howlett[42]).

3. Clinical features of Fanconi anemia

Fanconi anemia is a rare autosomal recessive disease (except for FA-B which is X-linked), with an estimated frequency of 1-5 per 100,000 births. Although the presentation of the disease is very heterogeneous, around 70% of FA patients show physical anomalies, such as skeletal abnormalities, including radial hypoplasia, short stature and microphthalmia. Most FA patients present hypo or hyperpigmentation of the skin, showing the characteristic *café au lait* spots, and with a lower frequency, cardiac, renal, gastrointestinal and other organ malformations [43]. The most common feature of the disease is the bone marrow failure (BMF), which is manifested at a median age of 8 years, being the primary cause of morbidity and mortality in FA patients[44]. Clinical data from the International Fanconi anemia Registry (IFAR) and the German Fanconi anemia Registry (GEFA) have shown that virtually all FA patients would develop BMF by the age of 40 years[45, 46]. At birth, the blood counts are usually normal, macrocytosis usually being the first symptom detected in these patients, followed by thrombocytopenia and neutropenia. Pancytopenia is generally presented between 5 and 10 years of age. The German and American studies showed that the use of a defined abnormality score, based on the analysis of five different congenital abnormalities, can significantly predict the development of the BMF in FA patients[46, 47].

In addition to the BMF, the incidence of cancer in FA patients is also markedly increased compared to the normal population, the incidence of myelodysplasia (MDS) or acute myeloid leukemia (AML) being 33% at the age of 40 years (more than eight hundred fold increased incidence compared to a healthy population). Reports from the IFAR and GEFA have also shown that FA patients are at extraordinary risk of developing specific solid tumors, such as head and neck squamous cell carcinomas (HN-SCCs; with several hundred fold increased frequency), esophagus SCCs (several thousand fold increase) and vulvar cancer in women (also several thousand fold increase)[46, 48, 49].GEFA also observed an increase in breast and brain tumors, while the North American study observed an increased incidence of cervical,osteosarcoma and liver tumors [46].

Myelodysplasia is often presented as a refractory cytopenia with multilineage dysplasia, with or without excess of blasts[49]. Acute myeloid leukemia can be diagnosed primarily or after a MDS phase, with an increasing fraction of blast cells in the BM[50]. The high selective pressure during their teens or early adulthood is probably involved in the development of clonal MDS and AML. The most common abnormalities found in FA patients with MDS/AML are gains of chromosome 1q, monosomy 7, gains of 3q (where *EVI1* is included) [44, 49-51], and abnormalities in *RUNX1/AML1* gene at chromosome 21q[50]. Results by Quentin *et al* also suggest a model of multi-step oncogenesis progression in the BM of FA patients, in which 1q+ (which can be found in the aplastic anemia form of the disease) would possibly constitute the initiating event, while the 3q+, -7/7q and *RUNX1* abnormalities would lead to high grade MDS or AML. In this model 1q+ might clonally rescue the BMF of FA patients, but would not protect against progression towards MDS and leukemia[50].

A remarkable aspect concerning the clinical symptoms of the disease is the observation that some of FA patients can undergo somatic mosaicism by means of a spontaneous reverse mutation or mitotic recombination in one of the FA pathogenic mutations in particular somatic cells[52-54]. If such a reversion occurs in a hematopoietic progenitor or stem cell (HSC), it may confer a proliferation advantage to the reverted cell, leading to the recovery of the BMF in the patient[52-55].

A recent paper described for the first time a characteristic phenotype in a number of FA patients who showed two of the FA hallmark features (high number of chromosomal aberrations after DNA damage and absence of FANCD2 monoubiquitination), compatible with normalized hematological counts[56]. Remarkably, blood cells from these patients did not show the FA characteristic G2/M cell cycle arrest after exposure to DNA cross-linkers[56]. Moreover, in contrast to mosaic FA patients, no reversion in the pathogenic mutations occurred in this new group of FA patients. Based on the above observations, a phenomenon defined as attenuation of the phenotype was described in FA[56]. Attenuation was associated with almost normal blood cell counts, and in some cases with development of MDS or AML. This process was accompanied by clonal hematopoiesis, implying that all peripheral blood cells derive from a single progenitor cell, in which a molecular event reducing CHK1 expression took place, resulting in the attenuated G2 arrest. The conclusions of this work have important implications in the management of FA patients because patients with this phenotype, although having essentially normal blood cell counts, should be followed closely to prevent the development of MDS or AML. Additionally, this study offers new explanations that could account for differences in the severity of the disease between FA siblings with the same mutation. Another important study has recently shown that FA proteins also play a role in mitosis, since FANCD2, FANCI and FANCM are localized to the extremities of ultrafine DNA bridges (UFBs), which link sister chromatids during cell division. FA cells show increase number of UFBs that may inhibit cytokinesis, leading to binucleated or multinucleated cells, a phenomenon that could account for the increased apoptosis and thus, also contribute to BMF [57, 58].

4. Main phenotypic characteristics of Fanconi anemia cells

FA cells are hypersensitive to ICL agents such as mitomycin C (MMC) and diepoxibutane (DEB). Additionally, these drugs specifically induce a high number of chromosomal aberrations in FA cells[59, 60], thus constituting a hallmark for the diagnosis of FA patients[60, 61]. Although normal cells exposed to DNA cross-linkers develop a transient accumulation in the G2/M phase of the cell cycle, the defective ability of FA cells to repair the DNA damage leads to a very significant accumulation of these cells in G2/M after exposure to ICLs[62]. This property has been also used for the diagnosis of FA patients, particularly in skin fibroblasts for the characterization of FA mosaic patients in which no obvious phenotypic markers may be apparent in their peripheral blood cells[63, 64].

FA cells are also characterized by their hypersensitivity to ambient oxygen conditions [65-67], manifested by a poor *ex vivo* growth and clonogenic capacity. This impaired growth properties of FA cells can be significantly restored when the incubation atmosphere is changed to hypoxic conditions (<5% of oxygen)[67], mimicking the low oxygen concentrations present in most tissues, including the BM niche[68, 69].

FA cells also show an increased apoptotic predisposition, something that may account for the BMF of these patients and for the development of malformations during embryonic development. One of the biochemical pathways involved in the increased apoptosis of FA cells is related to the over-production of cytokines such as TNF α and interferon- γ [70, 71]; an observation that has been confirmed in BM from FA patients[72].

FA patient hematopoietic progenitor cells have also shown a defective adhesion and homing activities, associated with an aberrant regulation of Cdc42 activity[73].

5. Identification of complementation groups in Fanconi anemia patients

As described before, fourteen different FA genes have been described so far, whose mutations account for the different FA complementation groups already identified (Table 1). However, there is still a number of patients with clinical symptoms of FA without mutations in any of the 14 FA genes, suggesting that more FA genes will be added to this long list. The identification of the FA complementation group (FA subtyping) in a patient has several advantages in the management of the disease. In this respect FA subtyping 1) confirms the FA diagnosis; 2) facilitates the identification of pathogenic mutations in FA genes, 3) allows to investigate relationships between the phenotype and the genotype in the patients, and 4) it is required for a potential future treatment of the patient by gene therapy.

Once a patient is diagnosed with FA, several alternatives have been used for FA subtyping. Because of the technical difficulties of the cell fusion approaches initially developed by Buschwald[4, 5], Hanenberg *et al.* in 2002 developed a new strategy based on genetic complementation strategies with retroviral vectors harboring the different FA genes[74]. This strategy, combined with Western blot analyses, principally of the monoubiquitinated and non ubiquitinated forms of FANCD2 (to detect if the mutation is upstream or downstream FANCD2 monoubiquitination), and also with the analysis of foci of nuclear proteins, has allowed the assignment of FA patients to the different complementation groups[75]. Sequencing has also been used [76] however, the large number and complexity of some FA genes and their mutations, together with the necessity of verifying the pathogenicity of each new mutation, implies that subtyping of patients with FA by mutational analysis is often time consuming and laborious [75]. Nevertheless, sequencing combined with genetic complementation strategies can be useful to deeply characterize FA patients.

6. Current treatments of the bone marrow failure in Fanconi anemia patients

As discussed above, the BMF is the main cause of mortality in FA patients. Many of the treatments of the BMF are palliative and directed to maintain acceptable numbers of peripheral blood cells. The use of androgens, in some cases combined with corticoids, constitute one of the most common treatments of FA patients in early stages of the disease, when a residual endogenous hematopoiesis remains[43, 77]. However, not all the patients respond to this treatment and, in most cases the response is slow, transient and normally limited to the red blood cells. Although a longer survival has been reported in patients who have been treated with androgens, in comparison with those who have not (20 years *vs* 14 years) [77], it has been reported that their use might constitute an adverse predictor when hematopoietic cell transplantation (HCT) is required [78]. Additionally, side effects such as liver tumors[48, 79] and masculinization may occur when androgens are used[80].

The use of growth factors to activate specific hematopoietic lineages has been also used in FA patients[81, 82], although generally with a limited success due to the transient benefit [82] and risks of leukemia due to the activation of potential pre-leukemic clones already present in the patient[83].

So far, the only curative treatment capable of restoring the hematopoiesis of FA patients in the long-term is allogeneic HCT. Many of the obstacles initially found for the HCT of FA patients have been overcome nowadays. The hypersensitivity of FA patients to conditioning regimens was a limiting factor for the success of the first HCTs in FA patients. However,

conditioning is necessary to eliminate the endogenous hematopoiesis and allow the engraftment of donor cells. In 1984, Gluckman *et al.* [84] developed the first successful conditioning regimen for FA patients consisting of a low dose of cyclophosphamide (CY) and a single dose of total body irradiation (TBI). Since then, many different protocols have been developed aiming to limit the radiation exposure in HCT preparative regimens[85], and thus to minimize risks of malignancies in the long-term[77]. As a result of these improvements, current HCTs from HLA(Human Leukocyte Antigen) identical siblings do not generally include irradiation in the conditioning. The use of mild conditioning regimens and the inclusion of fludarabine (FLU) (an antimetabolite with profound immunosuppressive effects) has, therefore, markedly improved the outcome of HCTs with HLA-matched grafts from related donors.

Although the outcome of transplants from alternative donors in FA has also markedly improved in the last decade, the morbidity and mortality associated to these transplants is still significant[86]. Problems like graft failure, acute and chronic graft versus host disease (GVHD) and opportunistic infections are the major obstacles to address. Again, the inclusion of FLU and the use of T cell depletion (TCD) have significantly improved the efficacy of the transplant of FA patients from unrelated donors[87]. Recent clinical trials conducted in the University of Minnesota have shown the relevance of thymic shielding during irradiation with reduced doses of TBI to limit opportunistic infections, and thus to increase overall survivals after unrelated HCTs in FA patients[88]. Ideally, transplantation should be done previously to the development of a myelodysplastic syndrome or leukemia[88]. The success of HCTs in patients that already have developed any of these pathologies is limited in comparison to those who have not. The main risk associated with the HCT of these patients is that the low doses of radio/chemotherapy that must be used in FA patients might not be enough to destroy the endogenous leukemic cells, thus increasing the risk of future relapses [88].

Traditionally the preferential source for the HCT for FA patients was BM or mobilized progenitors from peripheral blood (mPB). However, umbilical cord blood is nowadays also a good alternative for the transplantation of FA patients. This is not only the case for the HCT from HLA-identical siblings - in some instances derived from *in vitro* fertilization and preimplantation genetic diagnosis - but also from unrelated donors[89].

7. Gene therapy as a new strategy for the treatment of Fanconi anemia

Hematopoietic gene therapy, defined as the HCT of genetically corrected autologous HSCs, is considered a good alternative to allogenic HCT in FA. This strategy would avoid GVHD and limit, at least partially, the side effects associated to severe chemo/radiotherapy and immunosuppression (See review in [90]).

The previous observation that a number of mosaic FA patients (those who have reverted a pathogenic mutation in a HSC) could progressively improve their hematological status[52-55] opened the possibility of rescuing the BMF of FA patients after the infusion of gene-corrected HSCs, even in the absence of conditioning. As it is the case with HSCs that have reverted a pathogenic mutation, it is expected that *ex vivo* corrected FA HSCs may also develop a proliferation advantage over uncorrected cells, thus restoring progressively hematopoietic system of the patient.

To allow the stable integration of the transgene in the HSCs genome, gamma-retroviral vectors (RVs) have been the most frequently used vectors in clinical gene therapy protocols

[90, 91]. The principle of most of these protocols was based on the purification of CD34⁺ cells, either from BM or mPB, followed by the transduction with the therapeutic vector, and the re-infusion of the transduced cells in the patient, either pre-conditioned or not [91-94].

Two different gene therapy trials have been already conducted in FA. The first one was developed by Liu and colleagues in FA-C patients [95] and the second one by Kelly and colleagues in FA-A patients [96]. Both protocols used similar conditions to those previously used for the gene therapy of other monogenic diseases, such as X1-SCID. Essentially, in both protocols *in vitro* pre-stimulated CD34⁺ cells were transduced with RVs for three or four days in culture, and thereafter infused into non conditioned patients. In contrast to the results observed in X1-SCID patients, none of these protocols improved the clinical status of FA patients, indicating the necessity of improving the therapeutic vector and/or the manipulation of the target cells.

8. Towards the development of improved protocols of Fanconi anemia gene therapy

8.1 Lessons from Fanconi anemia mouse models

Mouse models represent an invaluable tool for improving the understanding of the mechanisms responsible of different pathologies, and also for developing new therapies with improved efficacy and reduced side effects. In the case of FA, where intrinsic difficulties exist to engraft immunodeficient mice with BM from FA patients, the relevance of mouse models is even higher.

Different FA mouse models with disruptions in FA genes such as *Fanca* [97, 98], *Fancc* [99, 100], *Fancd1* (*Brca2*) [101], *Fancd2* [102], *Fancg* [103], *Fancm* [104] and more recently *Fancp* (*Slx4*) [105] have been generated (See Table 2). Additionally, a FA mouse model based on the deletion of *Usp1* (the enzyme responsible for *FANCD2* deubiquitination) has been described [106]. This gene, however, is not currently considered a FA gene since no FA patients have been so far identified with *Usp1* mutations.

Although all FA mouse models are characterized by their hypersensitivity to DNA-cross-linking agents and in some instances to cytokines such as TNF α and IFN γ (See Table 2), the severe BMF that takes place in FA patients is far from being reproduced in these models. In fact, only the recently developed mouse model of FA-P (*Btbd12*^{-/-} mice) is prone to develop marked blood cytopenias, reflected by a reduction in white blood cells (WBCs) and platelets in a significant number of animals [105] (Table 2).

Aiming to generate FA mouse models that resemble more closely the disease observed in FA patients, double knock-outs have been also generated. Among these mouse models, only the double knock-out *Fancc*^{-/-}/*Fancg*^{-/-} mice [107] and the *Fancc*^{-/-}/*Sod1*^{-/-} mice showed evidences of BMF [108].

Significantly, although some FA mouse models are prone to develop tumors (i.e. FA-D1, FA-D2, FA-M), AML is not spontaneously generated in the FA mouse models generated so far. Studies conducted in *Fancc*^{-/-} mice have shown, however, that the *ex vivo* culture and/or incubation of *Fancc*^{-/-} BM cells with TNF- α , whose expression is significantly increased in FA patients, induces leukemic clonal evolution after transplantation [109, 110], suggesting that leukemia development in FA patients could be at least partially related to the deregulated expression of this cytokine.

Fanca^{-/-} as well as *Fancc*^{-/-} mice, and also mice with a hypomorphic mutation in *Brca2*/*Fancd1* (FA-D1 mice) constitute the FA mouse models more frequently used both to understand the

role of FA genes in HSCs functionality, and also to evaluate the preclinical efficacy of new therapies in FA. Defects in the HSCs have been observed in all tested FA mouse models, not only in terms of clonogenic potential, but also regarding the engrafting ability and repopulating properties of these cells (see Table 2). Although no aplastic anemia was observed in FA-D1 mice, this mouse model showed a more severe hematopoietic phenotype compared to other models with mutations in FA genes upstream in the FA/BRCA pathway [111]. In this respect, results from our laboratory showed a defective function in the repopulating potential of endogenous FA-D1 HSCs in their own natural microenvironment. This was demonstrated by the observation that BM cells from WT animals could repopulate in the long-term the hematopoietic tissues of FA-D1 unconditioned recipients [111]. This contrasts with studies conducted in other FA mouse models where only after the treatment with IFN γ [112, 113] or DNA damaging drugs, wild-type BM cells could be engrafted in FA recipients (Table 2).

In the field of gene therapy, it was shown for the first time in *Fancc*^{-/-} mice that the retroviral-mediated expression of *Fancc* corrects the defective repopulation ability of FA HSCs [114]. Similar conclusions were obtained in different FA mouse models using RVs, Lentiviral and also Foamyviral vectors (LVs and FVs; Table 1). Significantly, Li *et al.* showed that the *ex vivo* culture of *Fancc*^{-/-} HSCs increases apoptosis and promotes the development of clonal aberrations [109]. Studies conducted with *Fanca*^{-/-} and *Fancc*^{-/-} mice showed, on the other hand, that rapid transductions with LVs or FVs markedly improved the repopulating properties of the HSCs (Table 2). Taken together, these studies suggest the convenience of using similar short-transduction protocols in human FA gene therapy.

Working with the FA-D1 mouse model, our group showed that the infusion of LV-transduced cells in mice pre-treated with a mild conditioning results in a progressive increase in the proportion of genetically-corrected cells, in the absence of any selection treatment. This is in contrast to data obtained in other FA mouse models, where exposures to cytokines or DNA damaging agents were required (Table 2). Moreover, our data showed that in the long term after transplantation, most of the hematopoietic cells of recipient FA-D1 mice were resistant to otherwise cytotoxic doses of MMC and became genetically stable [115], suggesting that a similar proliferation advantage of *ex vivo* corrected HSCs may occur in FA clinical trials.

8.2 Lessons from *in vitro* studies conducted with bone marrow samples from FA patients

Based on the current knowledge on the biology of FA cells, it is now clear that marked differences distinguish FA HSCs from HSCs successfully treated with gene therapy (i.e. X1-SCID HSCs). In this respect, it has been already shown that in the case of FA HSCs, *in vitro* incubation induces apoptosis and genomic instability [109, 116, 135]. Therefore, it is now considered that short transduction strategies would improve the possibilities of engrafting FA patients with genetically corrected cells, as it has been already shown in FA mouse models [119, 125].

Because of the limited number of hematopoietic progenitors and HSCs present in the BM of FA patients [136], we hypothesized that these precursor cells would be actively cycling in the patient and therefore, directly susceptible to transduction with RVs without further *in vitro* stimulation. Consistent with this hypothesis, our studies showed that hematopoietic progenitors from FA patients can be efficiently transduced by Gibbon Ape Leukemia Virus

(GALV)-packaged RVs in protocols that lasted only 12-24 h [136]. Whether or not the most primitive HSCs were also efficiently transduced in these protocols is, however, unknown due to current limitations to engraft immunodeficient animals with BM from FA patients.

DEFECTIVE GENE	HYPERSENSITIVITY		HEMATOPOIESIS		GENETHERAPY	
	MMC	CTKs	BMF	HSC Defects	<i>In vivo</i> efficacy	<i>In vivo</i> proliferation advantage
<i>Fanca</i>	[97, 116]	IFN-γ [113]	Mild thrombocyt [97,116]	CRA and HSC Mobilization[117]	LVs [118] LVs [119]	+ MMC [118] + Chemo [119]
<i>Fancc</i>	[99],[130]	TNFα: - In vitro [110, 121] - In vivo [113] IFN-γ: - In vitro [100, 109] - In vivo [112, 113] - Not sensitive [122]		CRA [123] HSC Growth [124] CRA [125] HSC Engraftment [117]	RVs [114, 126] [127][116][128] LVs [129] FVs [125]	+MMC [130] +CPA/ IR [120] + MMC [129]
<i>Fancl/Birc2</i>	[111]			CRA [111]	[115]	No Treatment [115]
<i>Fand2</i>	[102],[106],[130]			CRA [130]		
<i>Fang</i>	[103],[131]	IFN-γ [113]		Microenvironment defects [132]		
<i>Fancm</i>	[104]					
<i>Fanp/Btbl2</i>	[105]		Mild cytopenias [105]			
<i>Usp1</i>	[106]			CRA [130]		
<i>Fancc/Fancc</i>	[133]					
<i>Fancc/Fang</i>	[107]		Mild cytopenias [107]	CRA [107]		
<i>Fancc/SOD</i>			Anemia and leukop. [108]	CFC defects [108]		
<i>Fand2/Mth1</i>	[134]					

BMF: Bone marrow failure; CTK: Cytokines; CFC: colony forming cell; Chemo: chemotherapy; CPA: cyclophosphamide; CRA: Competitive repopulation ability; FV: foamyviral vector; HSC: hematopoietic stem cell; IR: Ionizing radiation; LV: lentiviral vector; MMC: Mitomycin C; RV: Retroviral vector

Table 2. Principal characteristics of the hematopoietic system of FA mouse models and results from *ex vivo* HSC gene therapy.

Experimental studies [137, 138] and more recently also human trials [139, 140] have demonstrated that LVs currently constitute the most efficient clinical vectors for stably transducing HSCs after very short transduction periods. Additional studies have shown that these vectors facilitate the stable expression of the transgene *in vivo*, while transgene inactivation has been frequently reported in RVs [137]. Finally, but not less importantly, LVs have shown improved safety properties, compared to the RVs already used in human gene therapy [141-145].

Concerning the level of expression that is required for inducing a therapeutic effect in FA cells, we have recently shown in FA-A and FA-D2 cells that a weak expression of FA genes, at least *FANCA* and *FANCD2*, is sufficient to revert the FA phenotype of hematopoietic cells ([146],[147] and unpublished data). Because FA-A is the most frequent FA complementation group [75, 148] our group [147], as well as Hans-Peter Kiem group [149] have proposed independently the same LV construct for the gene therapy of FA patients. In this vector *FANCA* expression is driven by the phosphoglycerate kinase (PGK) promoter and stabilized by a mutated version of the posttranscriptional regulatory element (WPRE) [147, 149] (see Figure 2).

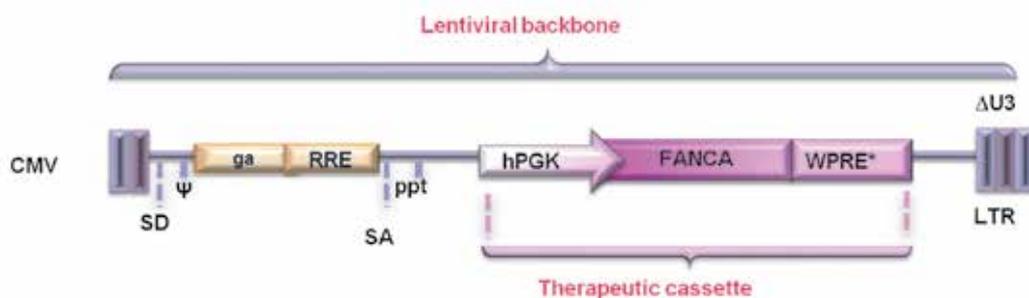


Fig. 2. Illustration of the FANCA-LV proposed independently by González-Murillo *et al* [147] and Becker *et al* [149] for the gene therapy of FA-A patients.

Studies conducted with BM samples from FA patients have also shown that FA progenitor cells are highly sensitive to cytokines such as TNF- α or IFN- γ and also to reactive oxygen species (ROS) [121, 150-154]. Thereafter it was shown that the use of *ex vivo* manipulation conditions that limit the oxidative damage (hypoxia or inclusion of N-acetyl-cysteine) improved the growth of FA progenitor cells [149, 155]. Similar observations were obtained when TNF- α was specifically inhibited with antibodies [72, 136]. Based on these observations and with the aim of improving the repopulation potential of genetically corrected HSCs, many of the experimental protocols aiming the genetic correction of FA HSCs use hypoxia and antibody-mediated inhibition of TNF- α [149, 155].

Concerning the ideal target population to be transduced in FA gene therapy trials, we reasoned that, if possible, the transduction of total BM would be the preferential option [155]. This suggestion derives from the fact that in FA every type of HSC, and also of accessory BM cell, would be directly affected by the genetic defect. Therefore, the genetic correction of each of these populations might be useful for the engraftment of the patient. Using GALV-TR (modified GALV envelope) packaged LVs carrying *FANCA* and/or *EGFP*, we demonstrated the possibility of efficiently transducing hematopoietic and mesenchymal progenitor cells in FA BM samples subjected to a very simple erythrocyte's depletion [155]. Although our data showed that, in contrast to LVs packaged with the G-protein from Vesicular Stomatitis Virus (VSV-G), GALV-TR packaged LVs can efficiently transduce FA BM samples at low multiplicities of infection (around 1-3 infective units/cell), there are still limitations in the production of GMP GALV-TR LVs at high titers. Because high MOIs (Multiplicity of infection) of VSV-G LVs produced in conditions approved for clinical use (GMP) are required to achieve efficient transductions, purified CD34⁺ or CD133⁺ cells currently constitute the preferential populations to be used in the next FA gene therapy protocols [156].

9. Perspectives for the future gene therapy of Fanconi anemia patients

The discovery by S. Yamanaka that the transfer of a few transcription factors can reprogram adult somatic cells and generate induced pluripotent cells (iPS cells) [157] has opened new perspectives for the cell and gene therapy of different diseases, particularly in FA. The collaborative study conducted between our group and J.C. Izpisua-Belmonte and J. Surralles groups demonstrated for the first time the possibility of generating disease-free hematopoietic progenitors from genetically corrected fibroblasts from patients with a monogenic disease,

specifically FA patients[158, 159]. Although the technology related to iPS cell generation should be further improved both in terms of efficiency and safety, these strategies have opened an unpredicted applicability in the management of genetic diseases like FA.

10. Concluding remarks

Since the description of the first FA patient by Guido Fanconi in 1927, an extraordinary advance in the understanding of the mechanisms accounting for the disease has occurred. Although more work is still required to elucidate the interactions between the FA/BRCA pathway with the different mechanisms of DNA repair, the relevance of this pathway in the repair of different insults to the DNA is now clear, accounting for the involvement of the FA/BRCA pathway in hereditary and also acquired cancer. Significant advances in the management of the hematopoietic syndromes that FA patients suffer have been produced within the last years, particularly in the field of hematopoietic transplantation. Thanks to these advantages, FA is nowadays not considered a restricted pediatric disease. New challenges have therefore emerged, particularly due to the necessity of developing improved therapies for syndromes that appear in adult FA patients, such as squamous cell carcinomas.

As happened in other diseases, gene therapy was soon considered a good option for the treatment of the BMF in FA patients. Although intrinsic difficulties in the manipulation of FA HSCs have limited the success of FA gene therapy, new vectors and improved FA HSC manipulations have emerged from studies conducted with FA mouse models and with samples from FA patients. All these technical advances have opened new hopes for the application of gene therapy in FA.

Finally, as it was the case with the clinical application of cord blood cells [160] or cells derived from siblings selected by pre-implantation genetic diagnosis[161, 162], FA is the first genetic disease where disease-free blood cells from non hematopoietic tissues, have been generated [158]. It is our hope that all these advances may have a translational clinical impact in our patients in the near future.

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12. References

- [1] Fanconi G. Familiäre infantile perniziosaartige Anämie (perniciziöses Blutbild und Konstitution). *Jahrbuch für Kinderheilkunde und Erziehung* (Wien). 1927;117:257-80.

- [2] Lobitz S, Velleuer E. Guido Fanconi (1892-1979): a jack of all trades. *Nat Rev Cancer*. 2006 Nov;6(11):893-8.
- [3] Schroeder T, Anschutz F, Knopp A. Spontaneous Chromosome aberrations in Familial Panmyelopathy. *Human Genetic*. 1964;1:194-6.
- [4] Duckworth-Rysiecki G, Cornish K, Clarke C, Buchwald M. Identification of two complementation groups in Fanconi anemia. *Somat Cell Mol Genet*. 1985;11::35-41.
- [5] Strathdee CA, Gavish H, Shannon WR, Buchwald M. Cloning of cDNAs for Fanconi's anaemia by functional complementation. *Nature*. 1992 Apr 30;356(6372):763-7.
- [6] consortium FaBc. Positional cloning of the Fanconi anaemia group A gene. *Nat Genet*. 1996 Nov;14(3):324-8.
- [7] de Winter JP, Leveille F, van Berkel CG, Rooimans MA, van Der Weel L, Steltenpool J, et al. Isolation of a cDNA representing the Fanconi anemia complementation group E gene. *American journal of human genetics*. 2000 Nov;67(5):1306-8.
- [8] de Winter JP, Rooimans MA, van Der Weel L, van Berkel CG, Alon N, Bosnoyan-Collins L, et al. The Fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. *Nat Genet*. 2000 Jan;24(1):15-6.
- [9] de Winter JP, Waisfisz Q, Rooimans MA, van Berkel CG, Bosnoyan-Collins L, Alon N, et al. The Fanconi anaemia group G gene FANCG is identical with XRCC9. *Nat Genet*. 1998 Nov;20(3):281-3.
- [10] Lo Ten Foe JR, Rooimans MA, Bosnoyan-Collins L, Alon N, Wijker M, Parker L, et al. Expression cloning of a cDNA for the major Fanconi anaemia gene, FAA. *Nat Genet*. 1996 Nov;14(3):320-3.
- [11] Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, et al. Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol Cell*. 2001 Feb;7(2):241-8.
- [12] Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*. 2002 Jul 26;297(5581):606-9.
- [13] Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, van de Vrugt HJ, et al. A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat Genet*. 2003 Oct;35(2):165-70.
- [14] Meetei AR, Levitus M, Xue Y, Medhurst AL, Zwaan M, Ling C, et al. X-linked inheritance of Fanconi anemia complementation group B. *Nat Genet*. 2004 Nov;36(11):1219-24.
- [15] Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, Wiegant WW, et al. The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. *Nat Genet*. 2005 Sep;37(9):934-5.
- [16] Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, Rio P, et al. The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat Genet*. 2005 Sep;37(9):931-3.
- [17] Litman R, Peng M, Jin Z, Zhang F, Zhang J, Powell S, et al. BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCF. *Cancer Cell*. 2005 Sep;8(3):255-65.
- [18] Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, Kalb R, et al. Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer. *Nat Genet*. 2006 Dec 31.

- [19] Meindl A, Hellebrand H, Wiek C, Erven V, Wappenschmidt B, Niederacher D, et al. Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat Genet.* 2010 May;42(5):410-4.
- [20] Vaz F, Hanenberg H, Schuster B, Barker K, Wiek C, Erven V, et al. Mutation of the RAD51C gene in a Fanconi anemia-like disorder. *Nat Genet.* 2010 May;42(5):406-9.
- [21] Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, et al. Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet.* 2006 Nov;38(11):1239-41.
- [22] Garcia MJ, Benitez J. The Fanconi anaemia/BRCA pathway and cancer susceptibility. Searching for new therapeutic targets. *Clin Transl Oncol.* 2008 Feb;10(2):78-84.
- [23] Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet.* 2006 Dec 31.
- [24] Wong MW, Nordfors C, Mossman D, Pecenpetelovska G, Avery-Kiejda KA, Talseth-Palmer B, et al. BRIP1, PALB2, and RAD51C mutation analysis reveals their relative importance as genetic susceptibility factors for breast cancer. *Breast cancer research and treatment.* 2011 Mar 16.
- [25] Dorsman JC, Levitus M, Rockx D, Rooimans MA, Oostra AB, Haitjema A, et al. Identification of the Fanconi anemia complementation group I gene, FANCI. *Cell Oncol.* 2007;29(3):211-8.
- [26] Sims AE, Spiteri E, Sims RJ, 3rd, Arita AG, Lach FP, Landers T, et al. FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. *Nat Struct Mol Biol.* 2007 Jun;14(6):564-7.
- [27] Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER, 3rd, Hurov KE, Luo J, et al. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell.* 2007 Apr 20;129(2):289-301.
- [28] Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, Bier P, et al. A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. *Nat Genet.* 2005 Sep;37(9):958-63.
- [29] Kee Y, D'Andrea AD. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev.* 2010 Aug 15;24(16):1680-94.
- [30] Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell.* 2001 Feb;7(2):249-62.
- [31] Wang W. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat Rev Genet.* 2007 Oct;8(10):735-48.
- [32] Kratz K, Schopf B, Kaden S, Sendoel A, Eberhard R, Lademann C, et al. Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. *Cell.* 2010 Jul 9;142(1):77-88.
- [33] Carmona R, Cano E, Grueso E, Ruiz-Villalba A, Bera T, Gaztambide J, et al. Peritoneal repairing cells: A type of bone marrow-derived progenitor cells involved in mesothelial regeneration. *J Cell Mol Med.* 2010 May 14.
- [34] MacKay C, Declais AC, Lundin C, Agostinho A, Deans AJ, MacArtney TJ, et al. Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell.* 2010 Jul 9;142(1):65-76.

- [35] Smogorzewska A, Desetty R, Saito TT, Schlabach M, Lach FP, Sowa ME, et al. A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. *Mol Cell*. 2010 Jul 9;39(1):36-47.
- [36] Kim Y, Lach FP, Desetty R, Hanenberg H, Auerbach AD, Smogorzewska A. Mutations of the SLX4 gene in Fanconi anemia. *Nat Genet*. 2011 Jan 16.
- [37] Stoepker C, Hain K, Schuster B, Hilhorst-Hofstee Y, Rooimans MA, Steltenpool J, et al. SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. *Nat Genet*. 2011 Jan 16.
- [38] Ciccia A, Ling C, Coulthard R, Yan Z, Xue Y, Meetei AR, et al. Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Mol Cell*. 2007 Feb 9;25(3):331-43.
- [39] Niedernhofer LJ. The Fanconi anemia signalosome anchor. *Mol Cell*. 2007 Feb 23;25(4):487-90.
- [40] O'Donnell L, Durocher D. DNA repair has a new FAN1 club. *Mol Cell*. 2010 Jul 30;39(2):167-9.
- [41] Valeri A, Martinez S, Casado JA, Bueren JA. Fanconi anaemia: from a monogenic disease to sporadic cancer. *Clin Transl Oncol*. 2011 Apr;13(4):215-21.
- [42] Cybulski KE, Howlett NG. FANCP/SLX4: A Swiss Army knife of DNA interstrand crosslink repair. *Cell Cycle*. 2011 Jun 1;10(11).
- [43] Dokal I. Fanconi's anaemia and related bone marrow failure syndromes. *Br Med Bull*. 2006;77-78:37-53.
- [44] Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD. Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. *Blood*. 1994 Sep 1;84(5):1650-5.
- [45] Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, et al. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). *Blood*. 2003 Feb 15;101(4):1249-56.
- [46] Rosenberg PS, Alter BP, Ebell W. Cancer risks in Fanconi anemia: findings from the German Fanconi Anemia Registry. *Haematologica*. 2008 Mar 5.
- [47] Rosenberg PS, Huang Y, Alter BP. Individualized risks of first adverse events in patients with Fanconi anemia. *Blood*. 2004 Jul 15;104(2):350-5.
- [48] Alter BP, Greene MH, Velazquez I, Rosenberg PS. Cancer in Fanconi anemia. *Blood*. 2003 Mar 1;101(5):2072.
- [49] Cioc AM, Wagner JE, MacMillan ML, DeFor T, Hirsch B. Diagnosis of myelodysplastic syndrome among a cohort of 119 patients with fanconi anemia: morphologic and cytogenetic characteristics. *American journal of clinical pathology*. 2010 Jan;133(1):92-100.
- [50] Quentin S, Cuccuini W, Ceccaldi R, Nibourel O, Pondarre C, Pages MP, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. *Blood*. 2011 Mar 3.
- [51] Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, Neitzel H. Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. *Blood*. 2003 May 15;101(10):3872-4.

- [52] Gregory JJ, Jr., Wagner JE, Verlander PC, Levran O, Batish SD, Eide CR, et al. Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. *Proc Natl Acad Sci U S A*. 2001 Feb 27;98(5):2532-7.
- [53] Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, Dietrich R, et al. Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. *Cytogenet Genome Res*. 2002;98(2-3):126-35.
- [54] Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, Hoatlin ME, et al. Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. *Nat Genet*. 1999 Aug;22(4):379-83.
- [55] Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, Guardiola P, et al. Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. *Blood*. 2005 Feb 1;105(3):1329-36.
- [56] Ceccaldi R, Briot D, Larghero J, Vasquez N, Dubois d'Enghien C, Chamousset D, et al. Spontaneous abrogation of the G2 DNA damage checkpoint has clinical benefits but promotes leukemogenesis in Fanconi anemia patients. *J Clin Invest*. 2010 Dec 22.
- [57] Mason PJ, Bessler M. Cytokinesis failure and attenuation: new findings in Fanconi anemia. *J Clin Invest*. 2011 Jan 4;121(1):27-30.
- [58] Vinciguerra P, Godinho SA, Parmar K, Pellman D, D'Andrea AD. Cytokinesis failure occurs in Fanconi anemia pathway-deficient murine and human bone marrow hematopoietic cells. *J Clin Invest*. 2010 Nov 1;120(11):3834-42.
- [59] Auerbach AD, Rogatko A, Schroeder-Kurth TM. International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. *Blood*. 1989 Feb;73(2):391-6.
- [60] Castella M, Pujol R, Callen E, Ramirez MJ, Casado JA, Talavera M, et al. Chromosome fragility in patients with Fanconi anaemia: diagnostic implications and clinical impact. *J Med Genet*. 2011 Jan 7.
- [61] Auerbach AD, Wolman SR. Susceptibility of Fanconi's anaemia fibroblasts to chromosome damage by carcinogens. *Nature*. 1976 Jun 10;261(5560):494-6.
- [62] Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, Hentze S, et al. Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. *Blood*. 1995 Apr 15;85(8):2233-7.
- [63] Neveling K, Endt D, Hoehn H, Schindler D. Genotype-phenotype correlations in Fanconi anemia. *Mutat Res*. 2009 Jul 31;668(1-2):73-91.
- [64] Pulsipher M, Kupfer GM, Naf D, Suliman A, Lee JS, Jakobs P, et al. Subtyping analysis of Fanconi anemia by immunoblotting and retroviral gene transfer. *Mol Med*. 1998 Jul;4(7):468-79.
- [65] Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB. Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. *Nature*. 1981 Mar 12;290(5802):142-3.
- [66] Saito H, Hammond AT, Moses RE. Hypersensitivity to oxygen is a uniform and secondary defect in Fanconi anemia cells. *Mutat Res*. 1993 Oct;294(3):255-62.
- [67] Schindler D, Hoehn H. Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. *American journal of human genetics*. 1988 Oct;43(4):429-35.

- [68] Chow DC, Wenning LA, Miller WM, Papoutsakis ET. Modeling pO₂ distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophysical journal*. 2001 Aug;81(2):675-84.
- [69] Parmar K, Mauch P, Vergilio JA, Sackstein R, Down JD. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A*. 2007 Mar 27;104(13):5431-6.
- [70] Rosselli F, Sanceau J, Gluckman E, Wietzerbin J, Moustacchi E. Abnormal lymphokine production: a novel feature of the genetic disease Fanconi anemia. II. In vitro and in vivo spontaneous overproduction of tumor necrosis factor alpha. *Blood*. 1994 Mar 1;83(5):1216-25.
- [71] Schultz JC, Shahidi NT. Tumor necrosis factor-alpha overproduction in Fanconi's anemia. *Am J Hematol*. 1993 Feb;42(2):196-201.
- [72] Dufour C, Corcione A, Svahn J, Haupt R, Poggi V, Beka'ssy AN, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. *Blood*. 2003 Sep 15;102(6):2053-9.
- [73] Zhang X, Shang X, Guo F, Murphy K, Kirby M, Kelly P, et al. Defective homing is associated with altered Cdc42 activity in cells from Fanconi anemia group A patients. *Blood*. 2008 Jun 18.
- [74] Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, et al. Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. *Exp Hematol*. 2002 May;30(5):410-20.
- [75] Casado JA, Callen E, Jacome A, Rio P, Castella M, Lobitz S, et al. A comprehensive strategy for the subtyping of Fanconi Anemia patients: conclusions from the Spanish Fanconi Anemia research network. *J Med Genet*. 2007 Apr;44(4):241-9.
- [76] Ameziane N, Errami A, Leveille F, Fontaine C, de Vries Y, van Spaendonk RM, et al. Genetic subtyping of Fanconi anemia by comprehensive mutation screening. *Hum Mutat*. 2008 Jan;29(1):159-66.
- [77] Dufour C, Svahn J. Fanconi anaemia: new strategies. *Bone Marrow Transplant*. 2008 Jun;41 Suppl 2:S90-5.
- [78] Guardiola P, Pasquini R, Dokal I, Ortega JJ, van Weel-Sipman M, Marsh JC, et al. Outcome of 69 allogeneic stem cell transplantations for Fanconi anemia using HLA-matched unrelated donors: a study on behalf of the European Group for Blood and Marrow Transplantation. *Blood*. 2000 Jan 15;95(2):422-9.
- [79] Velazquez I, Alter BP. Androgens and liver tumors: Fanconi's anemia and non-Fanconi's conditions. *Am J Hematol*. 2004 Nov;77(3):257-67.
- [80] Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. *Blood Rev*. 2010 May;24(3):101-22.
- [81] Guinan EC, Lopez KD, Huhn RD, Felsner JM, Nathan DG. Evaluation of granulocyte-macrophage colony-stimulating factor for treatment of pancytopenia in children with fanconi anemia. *The Journal of pediatrics*. 1994 Jan;124(1):144-50.
- [82] Rackoff WR, Orazi A, Robinson CA, Cooper RJ, Alter BP, Freedman MH, et al. Prolonged administration of granulocyte colony-stimulating factor (filgrastim) to patients with Fanconi anemia: a pilot study. *Blood*. 1996 Sep 1;88(5):1588-93.
- [83] Tischkowitz M, Dokal I. Fanconi anaemia and leukaemia - clinical and molecular aspects. *Br J Haematol*. 2004 Jul;126(2):176-91.

- [84] Gluckman E, Berger R, Dutreix J. Bone marrow transplantation for Fanconi anemia. *Seminars in hematology*. 1984 Jan;21(1):20-6.
- [85] Tan PL, Wagner JE, Auerbach AD, Defor TE, Slungaard A, Macmillan ML. Successful engraftment without radiation after fludarabine-based regimen in Fanconi anemia patients undergoing genotypically identical donor hematopoietic cell transplantation. *Pediatr Blood Cancer*. 2006 May 1;46(5):630-6.
- [86] Wagner JE, Eapen M, MacMillan ML, Harris RE, Pasquini R, Boulad F, et al. Unrelated donor bone marrow transplantation for the treatment of Fanconi anemia. *Blood*. 2007 Mar 1;109(5):2256-62.
- [87] MacMillan ML, Hughes MR, Agarwal S, Daley GQ. Cellular therapy for fanconi anemia: the past, present, and future. *Biol Blood Marrow Transplant*. 2011 Jan;17(1 Suppl):S109-14.
- [88] Macmillan ML, Wagner JE. Haematopoietic cell transplantation for Fanconi anaemia - when and how? *Br J Haematol*. 2010 Feb 5;Epub 2010 Feb 5.
- [89] Gluckman E, Rocha V, Ionescu I, Bierings M, Harris RE, Wagner J, et al. Results of unrelated cord blood transplant in fanconi anemia patients: risk factor analysis for engraftment and survival. *Biol Blood Marrow Transplant*. 2007 Sep;13(9):1073-82.
- [90] Muller LU, Williams DA. Finding the needle in the hay stack: hematopoietic stem cells in Fanconi anemia. *Mutat Res*. 2009 Jul 31;668(1-2):141-9.
- [91] Naldini L. Ex vivo gene transfer and correction for cell-based therapies. *Nat Rev Genet*. 2011 May;12(5):301-15.
- [92] Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science*. 2002;296(5577):2410-3.
- [93] Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 2000;288(5466):669-72.
- [94] Gaspar HB, Parsley KL, Howe S, King D, Gilmour KC, Sinclair J, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004 Dec 18-31;364(9452):2181-7.
- [95] Liu JM, Kim S, Read EJ, Futaki M, Dokal I, Carter CS, et al. Engraftment of hematopoietic progenitor cells transduced with the Fanconi anemia group C gene (FANCC). *Hum Gene Ther*. 1999 Sep 20;10(14):2337-46.
- [96] Kelly PF, Radtke S, Kalle C, Balcik B, Bohn K, Mueller R, et al. Stem cell collection and gene transfer in fanconi anemia. *Mol Ther*. 2007 Jan;15(1):211-9.
- [97] Cheng NC, van de Vrugt HJ, van der Valk MA, Oostra AB, Krimpenfort P, de Vries Y, et al. Mice with a targeted disruption of the Fanconi anemia homolog Fanca. *Hum Mol Genet*. 2000 Jul 22;9(12):1805-11.
- [98] Wong JC, Alon N, McKerlie C, Huang JR, Meyn MS, Buchwald M. Targeted disruption of exons 1 to 6 of the Fanconi Anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia. *Hum Mol Genet*. 2003 Aug 15;12(16):2063-76.
- [99] Chen M, Tomkins DJ, Auerbach W, McKerlie C, Youssoufian H, Liu L, et al. Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat Genet*. 1996 Apr;12(4):448-51.

- [100] Whitney MA, Royle G, Low MJ, Kelly MA, Axthelm MK, Reifsteck C, et al. Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood*. 1996 Jul 1;88(1):49-58.
- [101] McAllister KA, Bennett LM, Houle CD, Ward T, Malphurs J, Collins NK, et al. Cancer susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the Brca2 gene. *Cancer Res*. 2002 Feb 15;62(4):990-4.
- [102] Houghtaling S, Timmers C, Noll M, Finegold MJ, Jones SN, Meyn MS, et al. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev*. 2003 Aug 15;17(16):2021-35.
- [103] Yang Y, Kuang Y, De Oca RM, Hays T, Moreau L, Lu N, et al. Targeted disruption of the murine Fanconi anemia gene, Fancg/Xrcc9. *Blood*. 2001 Dec 1;98(12):3435-40.
- [104] Bakker ST, van de Vrugt HJ, Rooimans MA, Oostra AB, Steltenpool J, Delzenne-Goette E, et al. Fancm-deficient mice reveal unique features of Fanconi anemia complementation group M. *Hum Mol Genet*. 2009 Sep 15;18(18):3484-95.
- [105] Crossan GP, van der Weyden L, Rosado IV, Langevin F, Gaillard PH, McIntyre RE, et al. Disruption of mouse Slx4, a regulator of structure-specific nucleases, phenocopies Fanconi anemia. *Nat Genet*. 2011 Feb;43(2):147-52.
- [106] Kim JM, Parmar K, Huang M, Weinstock DM, Ruit CA, Kutok JL, et al. Inactivation of murine Usp1 results in genomic instability and a Fanconi anemia phenotype. *Dev Cell*. 2009 Feb;16(2):314-20.
- [107] Pulliam-Leath AC, Ciccone SL, Nalepa G, Li X, Si Y, Miravalle L, et al. Genetic disruption of both Fanc and Fancg in mice recapitulates the hematopoietic manifestations of Fanconi anemia. *Blood*. 2010 Oct 21;116(16):2915-20.
- [108] Hadjur S, Ung K, Wadsworth L, Dimmick J, Rajcan-Separovic E, Scott RW, et al. Defective hematopoiesis and hepatic steatosis in mice with combined deficiencies of the genes encoding Fanc and Cu/Zn superoxide dismutase. *Blood*. 2001 Aug 15;98(4):1003-11.
- [109] Li X, Le Beau MM, Ciccone S, Yang FC, Freie B, Chen S, et al. Ex vivo culture of Fanc -/- stem/progenitor cells predisposes cells to undergo apoptosis and surviving stem/progenitor cells display cytogenetic abnormalities and an increased risk of malignancy. *Blood*. 2005 Jan 11;105(9):3465-71.
- [110] Li J, Sejas DP, Zhang X, Qiu Y, Nattamai KJ, Rani R, et al. TNF-alpha induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. *J Clin Invest*. 2007 Nov;117(11):3283-95.
- [111] Navarro S, Meza NW, Quintana-Bustamante O, Casado JA, Jacome A, McAllister K, et al. Hematopoietic dysfunction in a mouse model for Fanconi anemia group D1. *Mol Ther*. 2006 Oct;14(4):525-35.
- [112] Li X, Yang Y, Yuan J, Hong P, Freie B, Orazi A, et al. Continuous in vivo infusion of Interferon-gamma (IFN- γ) preferentially reduces myeloid progenitor numbers and enhances engraftment of syngeneic wildtype cells in Fanc-/- mice. *Blood*. 2004 Apr 27.
- [113] Si Y, Ciccone S, Yang FC, Yuan J, Zeng D, Chen S, et al. Continuous in vivo infusion of interferon-gamma (IFN- γ) enhances engraftment of syngeneic wild-type cells in Fanc-/- and Fancg-/- mice. *Blood*. 2006 Dec 15;108(13):4283-7.
- [114] Gush KA, Fu KL, Grompe M, Walsh CE. Phenotypic correction of Fanconi anemia group C knockout mice. *Blood*. 2000 Jan 15;95(2):700-4.

- [115] Rio P, Meza NW, Gonzalez-Murillo A, Navarro S, Alvarez L, Surralles J, et al. In vivo proliferation advantage of genetically corrected hematopoietic stem cells in a mouse model of Fanconi anemia FA-D1. *Blood*. 2008 Sep 23;112:4853-61.
- [116] Rio P, Segovia JC, Hanenberg H, Casado JA, Martinez J, Gottsche K, et al. In vitro phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice. *Blood*. 2002 Sep 15;100(6):2032-9.
- [117] Milsom MD, Schiedlmeier B, Bailey J, Kim MO, Li D, Jansen M, et al. Ectopic HOXB4 overcomes the inhibitory effect of tumor necrosis factor- α on Fanconi anemia hematopoietic stem and progenitor cells. *Blood*. 2009 Mar 6.
- [118] Yamada K, Ramezani A, Hawley RG, Ebell W, Arwert F, Arnold LW, et al. Phenotype correction of Fanconi anemia group A hematopoietic stem cells using lentiviral vector. *Mol Ther*. 2003 Oct;8(4):600-10.
- [119] Muller LU, Milsom MD, Kim MO, Schambach A, Schuesler T, Williams DA. Rapid Lentiviral Transduction Preserves the Engraftment Potential of Fanca(-/-) Hematopoietic Stem Cells. *Mol Ther*. 2008 June;16((6):):1154-60.
- [120] Carreau M, Gan OI, Liu L, Doedens M, McKerlie C, Dick JE, et al. Bone marrow failure in the Fanconi anemia group C mouse model after DNA damage. *Blood*. 1998 Apr 15;91(8):2737-44.
- [121] Otsuki T, Nagakura S, Wang J, Bloom M, Grompe M, Liu JM. Tumor necrosis factor- α and CD95 ligation suppress erythropoiesis in Fanconi anemia C gene knockout mice. *J Cell Physiol*. 1999 Apr;179(1):79-86.
- [122] Kurre P, Anandakumar P, Grompe M, Kiem HP. In vivo administration of interferon gamma does not cause marrow aplasia in mice with a targeted disruption of FANCC. *Exp Hematol*. 2002 Nov;30(11):1257-62.
- [123] Haneline LS, Gobbett TA, Ramani R, Carreau M, Buchwald M, Yoder MC, et al. Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. *Blood*. 1999 Jul 1;94(1):1-8.
- [124] Aube M, Lafrance M, Charbonneau C, Goulet I, Carreau M. Hematopoietic stem cells from fanc(-/-) mice have lower growth and differentiation potential in response to growth factors. *Stem Cells*. 2002;20(5):438-47.
- [125] Si Y, Pulliam AC, Linka Y, Ciccone S, Leurs C, Yuan J, et al. Overnight transduction with foamyviral vectors restores the long-term repopulating activity of Fanc(-/-) stem cells. *Blood*. 2008 Aug 6.
- [126] Gush KA, Fu KL, Grompe M, Walsh CE. Phenotypic correction of Fanconi anemia group C knockout mice. *Blood*. 2000;95(2):700-4.
- [127] Haneline LS, Li X, Ciccone SL, Hong P, Yang Y, Broxmeyer HE, et al. Retroviral-mediated expression of recombinant FancC enhances the repopulating ability of Fanc(-/-) hematopoietic stem cells and decreases the risk of clonal evolution. *Blood*. 2003 Feb 15;101(4):1299-307.
- [128] Noll M, Bateman RL, D'Andrea AD, Grompe M. Preclinical protocol for in vivo selection of hematopoietic stem cells corrected by gene therapy in Fanconi anemia group C. *Mol Ther*. 2001;3(1):14-23.
- [129] Galimi F, Noll M, Kanazawa Y, Lax T, Chen C, Grompe M, et al. Gene therapy of Fanconi anemia: preclinical efficacy using lentiviral vectors. *Blood*. 2002 Oct 15;100(8):2732-6.

- [130] Parmar K, Kim J, Sykes SM, Shimamura A, Stuckert P, Zhu K, et al. Hematopoietic stem cell defects in mice with deficiency of *Fancd2* or *Usp1*. *Stem Cells*. 2010 Jul;28(7):1186-95.
- [131] Koomen M, Cheng NC, van De Vrugt HJ, Godthelp BC, van Der Valk MA, Oostra AB, et al. Reduced fertility and hypersensitivity to mitomycin C characterize *Fancg/Xrcc9* null mice. *Hum Mol Genet*. 2002;11(3):273-81.
- [132] Li Y, Chen S, Yuan J, Yang Y, Li J, Ma J, et al. Mesenchymal stem/progenitor cells promote the reconstitution of exogenous hematopoietic stem cells in *Fancg*^{-/-} mice in vivo. *Blood*. 2009 Jan 7.
- [133] Noll M, Battaile KP, Bateman R, Lax TP, Rathbun K, Reifsteck C, et al. Fanconi anemia group A and C double-mutant mice: functional evidence for a multi-protein Fanconi anemia complex. *Exp Hematol*. 2002 Jul;30(7):679-88.
- [134] van de Vrugt HJ, Eaton L, Hanlon Newell A, Al-Dhalimy M, Liskay RM, Olson SB, et al. Embryonic lethality after combined inactivation of *Fancd2* and *Mlh1* in mice. *Cancer Res*. 2009 Dec 15;69(24):9431-8.
- [135] Cumming RC, Lightfoot J, Beard K, Youssoufian H, O'Brien PJ, Buchwald M. Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. *Nat Med*. 2001 Jul;7(7):814-20.
- [136] Jacome A, Navarro S, Casado JA, Rio P, Madero L, Estella J, et al. A simplified approach to improve the efficiency and safety of ex vivo hematopoietic gene therapy in fanconi anemia patients. *Hum Gene Ther*. 2006 Feb;17(2):245-50.
- [137] Guenechea G, Gan OI, Inamitsu T, Dorrell C, Pereira DS, Kelly M, et al. Transduction of human CD34⁺ CD38⁻ bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. *Mol Ther*. 2000;1(6):566-73.
- [138] Naldini L, Blomer U, Gally P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996;272(5259):263-7.
- [139] Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009 Nov 6;326(5954):818-23.
- [140] Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature*. 2011 Sep 16;467(7313):318-22.
- [141] Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, et al. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol*. 2004 Aug;2(8):E234.
- [142] Modlich U, Navarro S, Zychlinski D, Maetzig T, Knoess S, Brugman MH, et al. Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther*. 2009 Nov;17(11):1919-28.
- [143] Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, et al. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol*. 2006 Jun;24(6):687-96.
- [144] Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 2003 Jun 13;300(5626):1749-51.

- [145] Biffi A, Bartolomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, et al. Lentiviral-vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*. 2011 Mar 14.
- [146] Almarza E, Rio P, Meza NW, Aldea M, Agirre X, Guenechea G, et al. Characteristics of lentiviral vectors harboring the proximal promoter of the *vav* proto-oncogene: a weak and efficient promoter for gene therapy. *Mol Ther*. 2007 Aug;15(8):1487-94.
- [147] Gonzalez-Murillo A, Lozano ML, Alvarez L, Jacome A, Almarza E, Navarro S, et al. Development of lentiviral vectors with optimized transcriptional activity for the gene therapy of patients with Fanconi anemia. *Hum Gene Ther*. 2010 May;21(5):623-30.
- [148] Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. *Blood*. 2006 Jun 1;107(11):4223-33.
- [149] Becker PS, Taylor JA, Trobridge GD, Zhao X, Beard BC, Chien S, et al. Preclinical correction of human Fanconi anemia complementation group A bone marrow cells using a safety-modified lentiviral vector. *Gene Ther*. 2010 May 20;Epub ahead of print.
- [150] Haneline LS, Broxmeyer HE, Cooper S, Hangoc G, Carreau M, Buchwald M, et al. Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from *Fac*^{-/-} mice. *Blood*. 1998 Jun 1;91(11):4092-8.
- [151] Rathbun RK, Christianson TA, Faulkner GR, Jones G, Keeble W, O'Dwyer M, et al. Interferon-gamma-induced apoptotic responses of Fanconi anemia group C hematopoietic progenitor cells involve caspase 8-dependent activation of caspase 3 family members. *Blood*. 2000;96(13):4204-11.
- [152] Zhang X, Li J, Sejas DP, Rathbun KR, Bagby GC, Pang Q. The Fanconi anemia proteins functionally interact with the protein kinase regulated by RNA (PKR). *J Biol Chem*. 2004 Oct 15;279(42):43910-9.
- [153] Sejas DP, Rani R, Qiu Y, Zhang X, Fagerlie SR, Nakano H, et al. Inflammatory reactive oxygen species-mediated hemopoietic suppression in *Fancc*-deficient mice. *J Immunol*. 2007 Apr 15;178(8):5277-87.
- [154] Vanderwerf SM, Svahn J, Olson S, Rathbun RK, Harrington C, Yates J, et al. TLR8-dependent TNF-(alpha) overexpression in Fanconi anemia group C cells. *Blood*. 2009 Dec 17;114(26):5290-8.
- [155] Jacome A, Navarro S, Rio P, Yanez RM, Gonzalez-Murillo A, Lozano ML, et al. Lentiviral-mediated genetic correction of hematopoietic and mesenchymal progenitor cells from Fanconi anemia patients. *Mol Ther*. 2009 Jun;17(6):1083-92.
- [156] Tolar J, Adair JE, Antoniou M, Bartholomae CC, Becker PS, Blazar BR, et al. Stem Cell Gene Therapy for Fanconi Anemia: Report from the 1st International Fanconi Anemia Gene Therapy Working Group Meeting. *Mol Ther*. May 3.
- [157] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006 Aug 25;126(4):663-76.
- [158] Raya A, Rodriguez-Piza I, Guenechea G, Vassena R, Navarro S, Barrero MJ, et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature*. 2009 Jul 2;460(7251):53-9.
- [159] Raya A, Rodriguez-Piza I, Navarro S, Richaud-Patin Y, Guenechea G, Sanchez-Danes A, et al. A protocol describing the genetic correction of somatic human cells and subsequent generation of iPS cells. *Nat Protoc*. 2010 Apr;5(4):647-60.

- [160] Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med.* 1989 Oct 26;321(17):1174-8.
- [161] Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE. Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. *Blood.* 2004 Feb 1;103(3):1147-51.
- [162] Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Designer babies - are they a reality yet? Case report: simultaneous preimplantation genetic diagnosis for Fanconi anaemia and HLA typing for cord blood transplantation. *Reproductive biomedicine online.* 2000;1(2):31.

Fanconi Anemia/Brca Pathway and Head and Neck Squamous Cell Carcinomas

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1. Introduction

DNA repair defect is one of the hallmarks of tumorigenesis, and is intimately linked to various human cancers, both inherited and sporadic (1). The two best examples are perhaps the DNA mismatch repair pathway in colorectal cancer, and the Fanconi Anemia/Brca (Fanc/Brca) pathway in head and neck squamous cell carcinomas (HNSCCs) (1). In this book chapter, I will review the updated knowledge of Fanc/Brca pathway in human cancers particularly in HNSCCs.

Fanconi anemia is a rare autosomal recessive or X-linked chromosomal instability disorder, with incidence of 1 to 5 cases per millions. The affected children have multiple congenital defects, and typically develop bone marrow failure during the first decade of life. They are at the risk for developing hematological cancers with the acute myelogenous leukemia (AML) the most frequent (2). Recently studies also showed a predisposition of Fanconi anemia patients to multiple solid tumors (3, 4), particularly to HNSCCs (5). Other tumors include gynecologic SCCs, and tumors of esophagus, liver, and skin (3, 4). Since Fanconi anemia is characterized by spontaneous chromosome breakage and cellular hypersensitivity to DNA cross-linking agents, such as mitomycin C, or diepoxybutane (DEB), the DEB-induced chromosome-breakage assay is widely used as a diagnostic test for Fanconi anemia patients, and the complementation test is used to define the Fanconi anemia subtypes. Androgens, hematopoietic growth factors, or stem-cell transplantation is currently used for treating bone marrow failure in Fanconi anemia patients(2).

The Fanconi anemia pathway is complex and interacts with other DNA repair pathways (6, 7). The pathway itself is regulated by so far thirteen Fanconi anemia proteins (FANCA, B, C, D1, D2, E, F, G, I, J, L, M and N). Among those proteins, eight are assembled in a nuclear ubiquitin E3 ligase complex (FANCA/B/C/E/F/G/L/M), known as the Fanconi anemia core complex, which mono-ubiquitinates FANCD2 and FANCI. The mono-ubiquitinated FANCD2/FANCI complex is targeted to chromatin, where it interacts, either directly or indirectly, with additional downstream Fanconi anemia proteins (FANCD1, FANCN, and FANCF) (6, 7). The first evidence of the convergence of Fanconi anemia pathway with

the Breast cancer (Brca) pathway came from the finding that the breast cancer susceptibility gene, Brca2 is actually identical to a Fanconi anemia gene, FANCD1 (8). Later studies showed that Fanconi anemia proteins form foci with Brca1, another major breast cancer susceptibility gene, and Rad51 for DNA repair (9). In addition, Brca1 and Brca2 also interact with another Fanconi anemia protein, FANCN (10, 11). Thus the Fanconi anemia and Brca pathways are intimately connected, and are summarized as Fanc/Brca pathway [Figure 1].

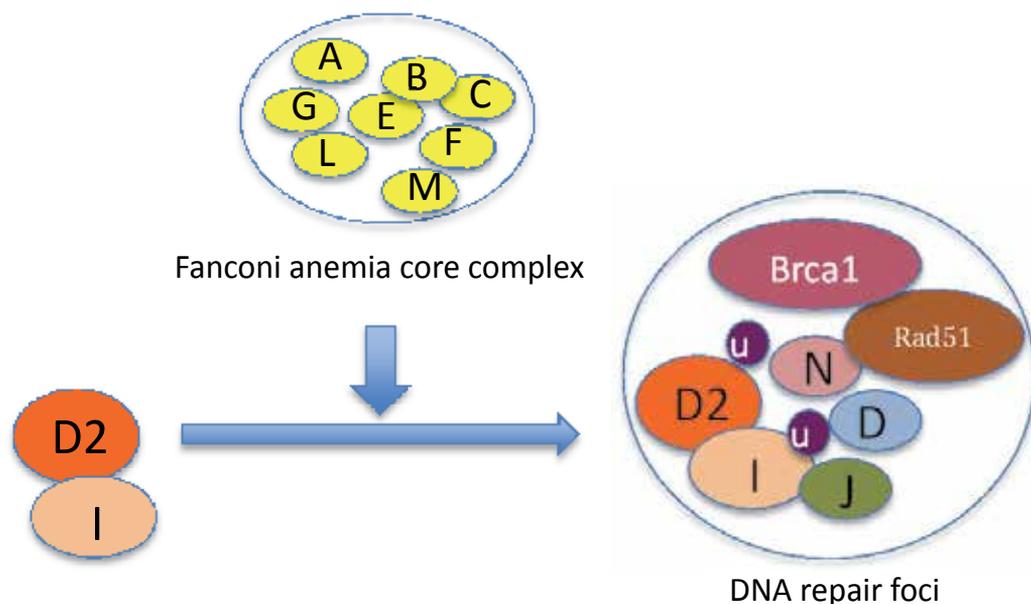


Fig. 1. Schematic of Fanc/Brca pathway in DNA repair. A-D1, D2, E-G, I-J, N stands for Fanconi anemia proteins FancA-D1, FancD2, FancE-G, FancI-J, and FancN.

2. Fanc/Brca pathway and human cancers

As briefly mentioned in the introduction section, the correlations of Fanc/Brca pathway and human cancers are well demonstrated by at least three lines of evidence: I). Susceptibility to various human cancers, including both hematologic and solid tumors in homozygous Fanconi anemia patients (3, 4). About one-third of Fanconi anemia patients will develop either hematologic or solid tumors by the age of 40 years. While AML is the predominant among hematologic cancers, squamous cell carcinomas (SCC) are the majority group of solid tumors developed in Fanconi anemia patients (3, 4). SCC of head and neck region is the most common (5), followed by SCC of gynecological system (vulva and cervix), esophagus, and skin (3, 4). II). Increased risk of cancers in heterozygous carriers of gene mutations in the Fanc/Brca pathway. The most common cancers in the heterozygous carriers of the Fanc/Brca gene mutations are breast and ovarian cancers (2, 12). In addition, development of pancreatic cancer was also reported in the heterozygous carriers of Fanc/Brca gene mutations, including FANCC, FANCG, and FANCN/PALB2 (13-15). Other cancers associated with heterozygous Fanc/Brca gene mutations are mainly prostate, lung, gastric cancer, and melanoma (16, 17). III). Molecular alterations of Fanc/Brca pathway genes in

sporadic human cancers. The most common molecular alteration of the Fanc/Brca pathway genes in sporadic human cancers is epigenic silencing of FANCF gene, which is most frequently seen in ovarian (18), cervical cancers (19), non-small-cell lung cancers (20), and HNSCC (20). In addition, methylation of Brca1 has also been reported in breast, ovarian, and non-small-cell lung cancers (20, 21).

3. Fanc/Brca pathway and HNSCCs: Clinical and molecular studies

HNSCC refers to SCCs arising from oral cavity, tongue, pharyngeal and laryngeal regions, and is the 6th most common human cancer worldwide. There are about 600,000 new cases and 350,000 cancer deaths worldwide each year (22, 23). HNSCCs usually occur in relatively late age of life, and higher in male with well known etiological factors of tobacco and/or alcohol (22, 23). However, the incidence of HNSCC is increasing recently in women with relatively young age, and correlates with human papilloma virus (HPV) infection (22, 23). The inherited form of HNSCC is very rare, in comparison with those in colorectal or breast cancers. Thus, the higher incidence of HNSCC developed in Fanconi anemia patients is both interesting and surprising. Thus, the Fanconi anemia patients may represent the first and perhaps the only one example of hereditary cancer syndromes predisposing to HNSCC.

The first evidence of high incidence of HNSCC in the Fanconi anemia patients came from studies of a 20-year perspective on 754 Fanconi anemia patients from the International Fanconi Anemia Registry (3, 5). These studies combined with another study showed that incidence of HNSCC is about 500 to 700 times increased in Fanconi anemia patients than those in general population (3-5). The Fanconi anemia patients ranged from 15 to 49 years of age, and comprised subtypes of FANCA, C, D2, F, G and nontype ones, and patients with HNSCC were found in these subtypes except FANCD2 and FANCF (3, 5). The incidence of HNSCC is even higher in the Fanconi anemia patients after hematopoietic stem cell transplantation (24). Compared to about 3% of HNSCC incidence in the Fanconi anemia patients before bone marrow transplantation, the incidence of HNSCCs increases more than 3 fold to about 10% in the Fanconi anemia patients after bone marrow transplantation (24).

While most of the Fanconi anemia patients develop bone marrow failure before their cancer development, there are about 20% of patients, often with milder physical and hematologic phenotypes, having developed solid tumors before the diagnosis of Fanconi anemia. These so-called adult head and neck cancer and hematopoietic mosaicism have been described in patients as mosaicism of 2 populations of cells in blood, one carrying FA defect, and the other seemingly normal (25). These findings have great impact on understanding the causal pathway of head and neck cancers in general population. Although there are no reports of genetic mutations in Fanc/Brca pathway in HNSCCs, FANCB and FANCF methylation have been described in about 31% and 15% of sporadic HNSCCs, respectively (20, 26). In addition, loss or reduced expression of Fanc/Brca pathway genes, such as FANCB, FANCF, FANCF, FANCM, Brca1, Brca2, FANCD2 and FANCG have been reported in sporadic HNSCCs (27, 28). Interestingly, reduced expression of FANCA and FANCG is more common in young HNSCC patients than older ones, suggesting different molecular mechanisms of HNSCC tumorigenesis between younger and older patients (29).

Given the clinical characteristics of HNSCC in Fanconi anemia patients, it is speculated that the molecular characteristics of HNSCC from Fanconi anemia patients might be different from sporadic HNSCC patients. It was suggested that Fanconi anemia patients have higher susceptibility to HPV-induced HNSCC (30). However, separate studies failed to show the

link between Fanconi Anemia and HPV-associated HNSCC (31), and molecular characteristics of HNSCC from Fanconi Anemia patients are not significantly different from sporadic HNSCC, except for the sensitivity to the chemotherapy drug, cisplatin (32). Interestingly, a study showed that cigarette smoke, one of the major etiological factors in sporadic HNSCC, induces genetic instability by suppressing FANCD2 expression (33), suggesting the molecular similarities shared between HNSCC from Fanconi Anemia patients and sporadic HNSCC.

4. Fanc/Brca pathway and HNSCCs: Lessons from animal models

Utilizing genetically engineered mouse models of Fanc/Brca pathway provides a powerful platform to study the causal role of Fanc/Brca pathway in human cancer development, including HNSCC (34). The first mouse model demonstrating the role of Fanc/Brca pathway in epithelial cancer development is the FANCD2 knockout mouse (35). FANCD2 is the common downstream effector of the Fanconi anemia nuclear complex, and acts as readout for the Fanc/Brca pathway. In addition, it forms nuclear foci with Rad51 and Brca1 for functional DNA repair (6, 7) [Figure 1]. Similar to human Fanconi anemia patients, FANCD2 knockout mice exhibited sensitivity to DNA interstrand cross-linking agents. Further more, these mice developed epithelial cancers in various organs, including mammary, bronchoalveolar, lung, and ovarian cancers (35). Knockout FANCD1/Brca2 developed breast and ovarian cancers; in addition, high incidence of squamous cell carcinoma of forestomach was seen in these mice (36). Cancer development, progression, and latency of both models were further enhanced by combination with p53 knockout (37, 38). Mice with germline knockout of FANCA and FANCC also developed sarcoma, lymphoma, and adenocarcinomas (34).

Using tissue specific promoters, such as Keratin 5, or 14, which target gene specifically in stratified epithelial cells (39), several studies showed that disruption of Fanc/Brca pathway lead to development of squamous cell carcinoma in multiple organs. For example, tissue specific deletion of Brca1 driven by Keratin 5 in mice developed squamous cell carcinomas in skin, ear canal, oral cavity, esophagus, and forestomach (40). Furthermore, another study, using tissue specific promoter Keratin 14-driven HPV mice crossed with FANCD2 knockout mice, showed an increased susceptibility to HNSCC when treated with a chemical carcinogen, supporting the hypothesis that Fanconi anemia patients have increased susceptibility to HPV-associated HNSCC observed in human samples (41).

Although the germline or tissue specific knockout mouse models of Fanc/Brca pathway suggested a causal role of this pathway in HNSCC tumorigenesis, the various types of cancers developed in multiple organs still hampered the study of this pathway specifically in HNSCC pathogenesis. To overcome this problem, we recently developed an inducible head-and-neck region specific knockout system (42, 43). This system uses the Keratin 5 or Keratin 14 promoter to direct head-and-neck specific expression of CrePR1, a fusion protein comprised of Cre recombinase fused to a truncated progesterone receptor ligand binding domain (Δ PR). In this system, RU486 treatment causes the CrePR1 fusion protein to translocate into the nucleus where it excises DNA sequences that have been flanked by loxP sites ("floxed"). Since the Keratin 5 or 14 promoter targets transgene expression to epithelial stem cells of the basal layer of stratified epithelium, such as head and neck epithelia, once RU486-induced excision occurs in stem cells, the stratified epithelium will eventually be replaced by cells in which the targeted gene is deleted for the lifetime of the mice (42, 43) [Figure 2].

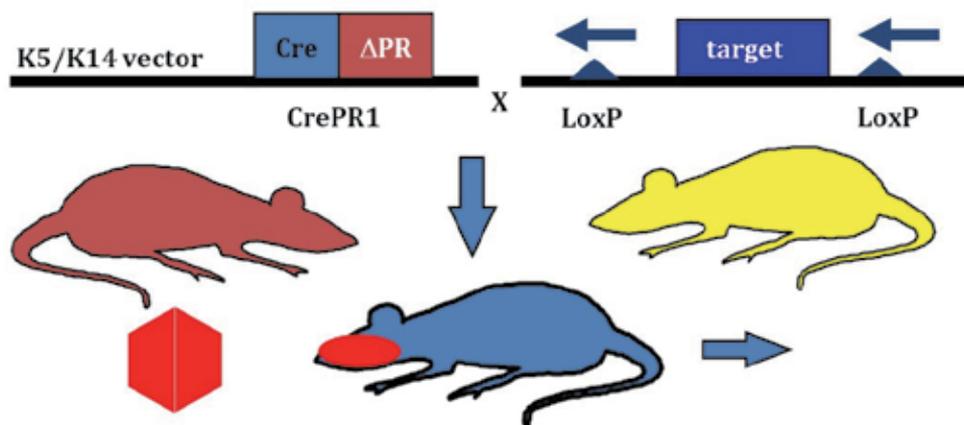


Fig. 2. Inducible head and neck specific gene knockout system.

We have used this system to establish a metastatic HNSCC mouse model in which Smad4, the central signal mediator of transforming growth factor β (TGF β) is specifically deleted in the head and neck epithelia (43). TGF β is a multifunctional cytokine that regulates cell proliferation, apoptosis, tissue remodeling, and angiogenesis. In addition, TGF β is also known to regulate genomic stability (44). The TGF β signaling initiates from ligand binding to heteromers of TGF β type I and type II receptors, and activate intracellular signal mediators Smad2 and Smad3 through phosphorylation. Smad3 binds to the smad-binding element (SBE) of a target gene, and subsequently recruits Smad4 to the same SBE. The Smad complexes then translocate to the nucleus to regulate gene expression of Smad targets involved in a wide variety of cancer-related processes (44) [Figure 3]. When the Smad4 gene is specifically deleted in mouse head and neck epithelia, the mice developed spontaneous HNSCC (43). Interestingly, the Smad4^{-/-} head and neck epithelia and tumors exhibited genomic instability as revealed by abnormal centrosomes, increased genomic aberrations, and increased sensitivity to mitomycin C. Further molecular analysis found that Fanc/Brca pathway gene expression and function correlate with Smad4 expression level. Specific knockdown of Smad4 in normal keratinocytes decreases expression of Fanc/Brca pathway genes, such as FancA, FancD2, Brca1, and Rad51. Restoration of Smad4 in a Smad4-null HNSCC cell line Cal27, increases the expression of Brca1 and Rad51 and the number of DNA repair nuclear foci. Interestingly, SBE sites were found in the promoters of FancA, FancD2, and Brca1 genes, suggesting that these genes may be transcriptional targets of TGF β /Smad4 signaling pathway (43). Thus, the TGF β /Smad4 signaling pathway is directly connected with the Fanc/Brca pathway in HNSCC tumorigenesis [Figure 3].

5. Components of FA/Brca pathway as targets for cancer therapy

DNA repair genes, including Fanc/Brca pathway, critically regulate the cellular response to chemotherapy and radiation therapy (45). The Fanc/Brca pathway regulates genomic stability required for cellular resistance to DNA cross-linking agents, thus the defects of this pathway contribute to chemo-, or radiation sensitivities (46).

The milestone discovery for Fanc/Brca pathway conferring chemosensitivity came from the discovery of epigenic silencing of FANCF in ovarian cancer (18). Ovarian cancer cells

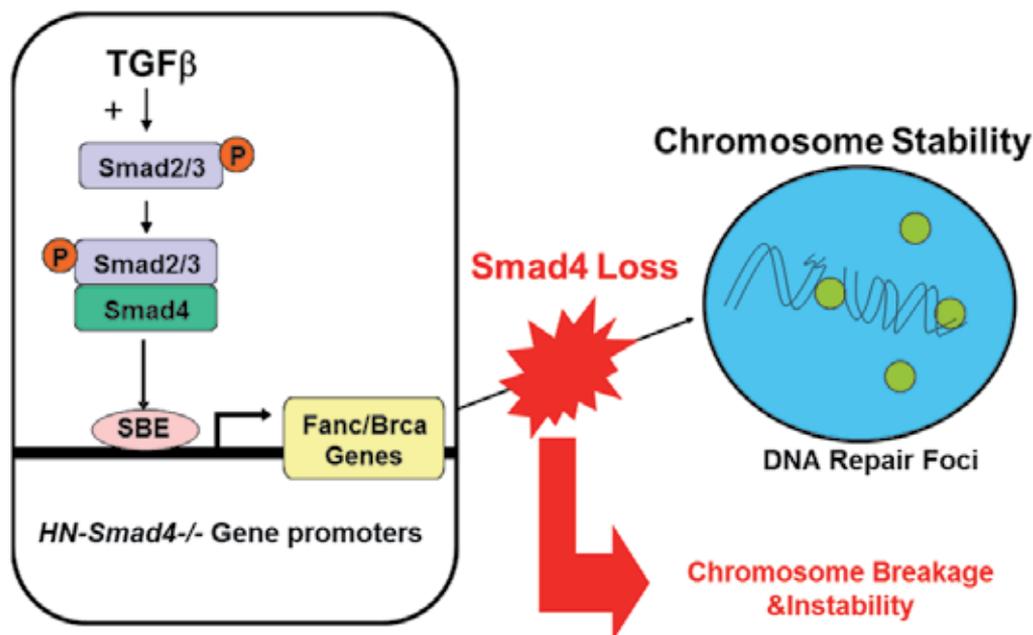


Fig. 3. Potential mechanism for chromosome instability in *HN-Smad4*^{-/-} Lesions. HN: head and neck

are usually hypersensitive to chemotherapeutic drug, such as cisplatin initially, but become resistant to the drug over time. The underlying molecular mechanism was revealed to be due to DNA methylation of the CpG island of FANCF, and the resistance is correlated with demethylation of FANCF (18). This study questioned the application of demethylation agents in treatment of ovarian cancer, and suggested that targeted disruption of Fanc/Brca pathway may be a better therapeutic option for ovarian cancer (18). While similar results of targeting Fanc/Brca pathway in sensitizing chemotherapy were reported in other types of cancers, such as colorectal cancer, peritoneal carcinomas, and multiple myeloma (47-50), the results from HNSCC are still controversial. It was reported that targeting Fanc/Brca pathway by the histone deacetylase inhibitor phenylbutyrate sensitizes human HNSCC cells to cisplatin (51). However, a separate study failed to correlate Fanc/Brca pathway inactivation with cisplatin sensitivity based on lack of evidence of FANCF methylation, and down-regulation of other Fanconi anemia genes (52). Another interesting finding for the mechanisms of cisplatin resistance in ovarian cancer is to identify secondary mutations of Brca2/FANCD1 (53) and Brca1 gene (54). Similar mutations of Brca2/FANCD1 have also been detected in pancreatic cancers (55). All these results highlight the functional importance of Fanc/Brca pathway in modulating sensitivity of cancer chemotherapy (50). Recent studies showed that cancer cells deficient in DNA repair pathways become highly dependent on alternative pathways for survival (45). For example, cancers deficient in Brca1 or Brca2 usually exhibit impaired ability to repair double-stranded DNA breaks via homologous recombination (56, 57). Moreover, in the setting of defective homologous recombination, inhibition of a second DNA repair pathway, such as base excision repair, is often a lethal event (56, 57). This so called "synthetic lethality" has been utilized in designing the ultimate cancer therapy (45). One of the best examples is to apply

poly(ADP-ribose) polymerase 1 (PARP1) inhibitors in breast or ovarian cancer patients with Brca1 or Brca 2 mutation (56-59). PARP1 is a nuclear protein that rapidly binds to DNA single-strand breaks and facilitates DNA repair (60). Use of the PARP1 inhibitor also induced significant sensitization to radiation therapy in HNSCC cells (61). Although alterations of Brca1 and Brca2 are rare in human HNSCC, HNSCC with loss of Smad4 are common and exhibit Fanc/Brca pathway defects in DNA repair as we showed previously (43), thus, providing a promising rationale and biomarker in utilizing PARP1 inhibitor for cancer therapy in HNSCC with Smad4 loss. In addition to the sensitivity to PARP1 inhibition, Fanc/Brca pathway-deficient tumor cells are also hypersensitive to inhibition of ataxia telangiectasia mutated kinase ATM (62), and checkpoint kinase CHK1 (63). With discoveries of more pathways, defects in which confer synthetic lethality with defects in Fanc/Brca pathway, more sophisticated and efficient therapeutic approaches will be designed and tested.

6. Future perspectives

Defect of Fanc/Brca pathway represents by far the only genetic predisposition to HNSCC through clinical genetic studies. Given the complexity of this pathway and its interaction with other DNA repair pathways, there are still lots of unanswered questions about the molecular mechanisms of this pathway in HNSCC tumorigenesis. However, with more biomarkers being identified and utilized to stratify HNSCC patients with particular defects of Fanc/Brca pathway, a personalized therapy with more efficacy and less side effect will ultimately be available, which will have significant impact on HNSCC management.

7. References

- [1] Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11:220-228.
- [2] D'Andrea, A.D. Susceptibility pathways in Fanconi's anemia and breast cancer. *N Engl J Med* 362:1909-1919.
- [3] Kutler, D.I., Singh, B., Satagopan, J., Batish, S.D., Berwick, M., Giampietro, P.F., Hanenberg, H., and Auerbach, A.D. 2003. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). *Blood* 101:1249-1256.
- [4] Rosenberg, P.S., Greene, M.H., and Alter, B.P. 2003. Cancer incidence in persons with Fanconi anemia. *Blood* 101:822-826.
- [5] Kutler, D.I., Auerbach, A.D., Satagopan, J., Giampietro, P.F., Batish, S.D., Huvos, A.G., Goberdhan, A., Shah, J.P., and Singh, B. 2003. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. *Arch Otolaryngol Head Neck Surg* 129:106-112.
- [6] Moldovan, G.L., and D'Andrea, A.D. 2009. How the fanconi anemia pathway guards the genome. *Annu Rev Genet* 43:223-249.
- [7] Kee, Y., and D'Andrea, A.D. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev* 24:1680-1694.

- [8] Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., et al. 2002. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 297:606-609.
- [9] Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A.D. 2001. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* 7:249-262.
- [10] Zhang, F., Ma, J., Wu, J., Ye, L., Cai, H., Xia, B., and Yu, X. 2009. PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr Biol* 19:524-529.
- [11] Sy, S.M., Huen, M.S., and Chen, J. 2009. PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc Natl Acad Sci U S A* 106:7155-7160.
- [12] King, M.C., Marks, J.H., and Mandell, J.B. 2003. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* 302:643-646.
- [13] Hahn, S.A., Greenhalf, B., Ellis, I., Sina-Frey, M., Rieder, H., Korte, B., Gerdes, B., Kress, R., Ziegler, A., Raeburn, J.A., et al. 2003. BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* 95:214-221.
- [14] van der Heijden, M.S., Yeo, C.J., Hruban, R.H., and Kern, S.E. 2003. Fanconi anemia gene mutations in young-onset pancreatic cancer. *Cancer Res* 63:2585-2588.
- [15] Jones, S., Hruban, R.H., Kamiyama, M., Borges, M., Zhang, X., Parsons, D.W., Lin, J.C., Palmisano, E., Brune, K., Jaffee, E.M., et al. 2009. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* 324:217.
- [16] Berwick, M., Satagopan, J.M., Ben-Porat, L., Carlson, A., Mah, K., Henry, R., Diotti, R., Milton, K., Pujara, K., Landers, T., et al. 2007. Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer. *Cancer Res* 67:9591-9596.
- [17] Liede, A., Karlan, B.Y., and Narod, S.A. 2004. Cancer risks for male carriers of germline mutations in BRCA1 or BRCA2: a review of the literature. *J Clin Oncol* 22:735-742.
- [18] Taniguchi, T., Tischkowitz, M., Ameziane, N., Hodgson, S.V., Mathew, C.G., Joenje, H., Mok, S.C., and D'Andrea, A.D. 2003. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. *Nat Med* 9:568-574.
- [19] Narayan, G., Arias-Pulido, H., Nandula, S.V., Basso, K., Sugirtharaj, D.D., Vargas, H., Mansukhani, M., Villeda, J., Meyer, L., Schneider, A., et al. 2004. Promoter hypermethylation of FANCF: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. *Cancer Res* 64:2994-2997.
- [20] Marsit, C.J., Liu, M., Nelson, H.H., Posner, M., Suzuki, M., and Kelsey, K.T. 2004. Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. *Oncogene* 23:1000-1004.
- [21] Esteller, M., Silva, J.M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I.C., Repasky, E.A., et al. 2000. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 92:564-569.
- [22] Argiris, A., Karamouzis, M.V., Raben, D., and Ferris, R.L. 2008. Head and neck cancer. *Lancet* 371:1695-1709.
- [23] Leemans, C.R., Braakhuis, B.J., and Brakenhoff, R.H. The molecular biology of head and neck cancer. *Nat Rev Cancer* 11:9-22.

- [24] Masserot, C., Peffault de Latour, R., Rocha, V., Leblanc, T., Rigolet, A., Pascal, F., Janin, A., Soulier, J., Gluckman, E., and Socie, G. 2008. Head and neck squamous cell carcinoma in 13 patients with Fanconi anemia after hematopoietic stem cell transplantation. *Cancer* 113:3315-3322.
- [25] Alter, B.P., Joenje, H., Oostra, A.B., and Pals, G. 2005. Fanconi anemia: adult head and neck cancer and hematopoietic mosaicism. *Arch Otolaryngol Head Neck Surg* 131:635-639.
- [26] Smith, I.M., Mithani, S.K., Mydlarz, W.K., Chang, S.S., and Califano, J.A. Inactivation of the tumor suppressor genes causing the hereditary syndromes predisposing to head and neck cancer via promoter hypermethylation in sporadic head and neck cancers. *ORL J Otorhinolaryngol Relat Spec* 72:44-50.
- [27] Wreesmann, V.B., Estilo, C., Eisele, D.W., Singh, B., and Wang, S.J. 2007. Downregulation of Fanconi anemia genes in sporadic head and neck squamous cell carcinoma. *ORL J Otorhinolaryngol Relat Spec* 69:218-225.
- [28] Sparano, A., Quesnelle, K.M., Kumar, M.S., Wang, Y., Sylvester, A.J., Feldman, M., Sewell, D.A., Weinstein, G.S., and Brose, M.S. 2006. Genome-wide profiling of oral squamous cell carcinoma by array-based comparative genomic hybridization. *Laryngoscope* 116:735-741.
- [29] Tremblay, S., Pintor Dos Reis, P., Bradley, G., Galloni, N.N., Perez-Ordóñez, B., Freeman, J., Brown, D., Gilbert, R., Gullane, P., Irish, J., et al. 2006. Young patients with oral squamous cell carcinoma: study of the involvement of GSTP1 and deregulation of the Fanconi anemia genes. *Arch Otolaryngol Head Neck Surg* 132:958-966.
- [30] Kutler, D.I., Wreesmann, V.B., Goberdhan, A., Ben-Porat, L., Satagopan, J., Ngai, I., Huvos, A.G., Giampietro, P., Levran, O., Pujara, K., et al. 2003. Human papillomavirus DNA and p53 polymorphisms in squamous cell carcinomas from Fanconi anemia patients. *J Natl Cancer Inst* 95:1718-1721.
- [31] van Zeeburg, H.J., Sniijders, P.J., Wu, T., Gluckman, E., Soulier, J., Surralles, J., Castella, M., van der Wal, J.E., Wennerberg, J., Califano, J., et al. 2008. Clinical and molecular characteristics of squamous cell carcinomas from Fanconi anemia patients. *J Natl Cancer Inst* 100:1649-1653.
- [32] van Zeeburg, H.J., Sniijders, P.J., Pals, G., Hermsen, M.A., Rooimans, M.A., Bagby, G., Soulier, J., Gluckman, E., Wennerberg, J., Leemans, C.R., et al. 2005. Generation and molecular characterization of head and neck squamous cell lines of fanconi anemia patients. *Cancer Res* 65:1271-1276.
- [33] Hays, L.E., Zodrow, D.M., Yates, J.E., Deffebach, M.E., Jacoby, D.B., Olson, S.B., Pankow, J.F., and Bagby, G.C. 2008. Cigarette smoke induces genetic instability in airway epithelial cells by suppressing FANCD2 expression. *Br J Cancer* 98:1653-1661.
- [34] Parmar, K., D'Andrea, A., and Niedernhofer, L.J. 2009. Mouse models of Fanconi anemia. *Mutat Res* 668:133-140.
- [35] Houghtaling, S., Timmers, C., Noll, M., Finegold, M.J., Jones, S.N., Meyn, M.S., and Grompe, M. 2003. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev* 17:2021-2035.
- [36] McAllister, K.A., Bennett, L.M., Houle, C.D., Ward, T., Malphurs, J., Collins, N.K., Cachafeiro, C., Haseman, J., Goulding, E.H., Bunch, D., et al. 2002. Cancer

- susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the Brca2 gene. *Cancer Res* 62:990-994.
- [37] Houghtaling, S., Granville, L., Akkari, Y., Torimaru, Y., Olson, S., Finegold, M., and Grompe, M. 2005. Heterozygosity for p53 (Trp53+/-) accelerates epithelial tumor formation in fanconi anemia complementation group D2 (Fancd2) knockout mice. *Cancer Res* 65:85-91.
- [38] McAllister, K.A., Houle, C.D., Malphurs, J., Ward, T., Collins, N.K., Gersch, W., Wharey, L., Seely, J.C., Betz, L., Bennett, L.M., et al. 2006. Spontaneous and irradiation-induced tumor susceptibility in BRCA2 germline mutant mice and cooperative effects with a p53 germline mutation. *Toxicol Pathol* 34:187-198.
- [39] Lu, S.L., Herrington, H., and Wang, X.J. 2006. Mouse models for human head and neck squamous cell carcinomas. *Head Neck* 28:945-954.
- [40] Berton, T.R., Matsumoto, T., Page, A., Conti, C.J., Deng, C.X., Jorcano, J.L., and Johnson, D.G. 2003. Tumor formation in mice with conditional inactivation of Brca1 in epithelial tissues. *Oncogene* 22:5415-5426.
- [41] Park, J.W., Pitot, H.C., Strati, K., Spardy, N., Duensing, S., Grompe, M., and Lambert, P.F. Deficiencies in the Fanconi anemia DNA damage response pathway increase sensitivity to HPV-associated head and neck cancer. *Cancer Res* 70:9959-9968.
- [42] Lu, S.L., Herrington, H., Reh, D., Weber, S., Bornstein, S., Wang, D., Li, A.G., Tang, C.F., Siddiqui, Y., Nord, J., et al. 2006. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev* 20:1331-1342.
- [43] Bornstein, S., White, R., Malkoski, S., Oka, M., Han, G., Cleaver, T., Reh, D., Andersen, P., Gross, N., Olson, S., et al. 2009. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. *J Clin Invest* 119:3408-3419.
- [44] Ikushima, H., and Miyazono, K. TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer* 10:415-424.
- [45] Helleday, T., Petermann, E., Lundin, C., Hodgson, B., and Sharma, R.A. 2008. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* 8:193-204.
- [46] Kennedy, R.D., and D'Andrea, A.D. 2006. DNA repair pathways in clinical practice: lessons from pediatric cancer susceptibility syndromes. *J Clin Oncol* 24:3799-3808.
- [47] Gallmeier, E., Calhoun, E.S., Rago, C., Brody, J.R., Cunningham, S.C., Hucl, T., Gorospe, M., Kohli, M., Lengauer, C., and Kern, S.E. 2006. Targeted disruption of FANCC and FANCG in human cancer provides a preclinical model for specific therapeutic options. *Gastroenterology* 130:2145-2154.
- [48] Swisher, E.M., Gonzalez, R.M., Taniguchi, T., Garcia, R.L., Walsh, T., Goff, B.A., and Welsh, P. 2009. Methylation and protein expression of DNA repair genes: association with chemotherapy exposure and survival in sporadic ovarian and peritoneal carcinomas. *Mol Cancer* 8:48.
- [49] Yarde, D.N., Oliveira, V., Mathews, L., Wang, X., Villagra, A., Boulware, D., Shain, K.H., Hazlehurst, L.A., Alsina, M., Chen, D.T., et al. 2009. Targeting the Fanconi anemia/BRCA pathway circumvents drug resistance in multiple myeloma. *Cancer Res* 69:9367-9375.

- [50] Gallmeier, E., and Kern, S.E. 2007. Targeting Fanconi anemia/BRCA2 pathway defects in cancer: the significance of preclinical pharmacogenomic models. *Clin Cancer Res* 13:4-10.
- [51] Burkitt, K., and Ljungman, M. 2008. Phenylbutyrate interferes with the Fanconi anemia and BRCA pathway and sensitizes head and neck cancer cells to cisplatin. *Mol Cancer* 7:24.
- [52] Snyder, E.R., Ricker, J.L., Chen, Z., and Waes, C.V. 2007. Variation in cisplatin sensitivity is not associated with Fanconi Anemia/BRCA pathway inactivation in head and neck squamous cell carcinoma cell lines. *Cancer Lett* 245:75-80.
- [53] Sakai, W., Swisher, E.M., Karlan, B.Y., Agarwal, M.K., Higgins, J., Friedman, C., Villegas, E., Jacquemont, C., Farrugia, D.J., Couch, F.J., et al. 2008. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 451:1116-1120.
- [54] Swisher, E.M., Sakai, W., Karlan, B.Y., Wurz, K., Urban, N., and Taniguchi, T. 2008. Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. *Cancer Res* 68:2581-2586.
- [55] Edwards, S.L., Brough, R., Lord, C.J., Natrajan, R., Vatcheva, R., Levine, D.A., Boyd, J., Reis-Filho, J.S., and Ashworth, A. 2008. Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* 451:1111-1115.
- [56] Konstantinopoulos, P.A., Spentzos, D., Karlan, B.Y., Taniguchi, T., Fountzilas, E., Francoeur, N., Levine, D.A., and Cannistra, S.A. Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. *J Clin Oncol* 28:3555-3561.
- [57] Fong, P.C., Boss, D.S., Yap, T.A., Tutt, A., Wu, P., Mergui-Roelvink, M., Mortimer, P., Swaisland, H., Lau, A., O'Connor, M.J., et al. 2009. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 361:123-134.
- [58] Tutt, A., Robson, M., Garber, J.E., Domchek, S.M., Audeh, M.W., Weitzel, J.N., Friedlander, M., Arun, B., Loman, N., Schmutzler, R.K., et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet* 376:235-244.
- [59] Audeh, M.W., Carmichael, J., Penson, R.T., Friedlander, M., Powell, B., Bell-McGuinn, K.M., Scott, C., Weitzel, J.N., Oaknin, A., Loman, N., et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. *Lancet* 376:245-251.
- [60] Ratnam, K., and Low, J.A. 2007. Current development of clinical inhibitors of poly(ADP-ribose) polymerase in oncology. *Clin Cancer Res* 13:1383-1388.
- [61] Khan, K., Araki, K., Wang, D., Li, G., Li, X., Zhang, J., Xu, W., Hoover, R.K., Lauter, S., O'Malley, B., Jr., et al. Head and neck cancer radiosensitization by the novel poly(ADP-ribose) polymerase inhibitor GPI-15427. *Head Neck* 32:381-391.
- [62] Kennedy, R.D., Chen, C.C., Stuckert, P., Archila, E.M., De la Vega, M.A., Moreau, L.A., Shimamura, A., and D'Andrea, A.D. 2007. Fanconi anemia pathway-deficient tumor cells are hypersensitive to inhibition of ataxia telangiectasia mutated. *J Clin Invest* 117:1440-1449.
- [63] Chen, C.C., Kennedy, R.D., Sidi, S., Look, A.T., and D'Andrea, A. 2009. CHK1 inhibition as a strategy for targeting Fanconi Anemia (FA) DNA repair pathway deficient tumors. *Mol Cancer* 8:24.

- [64] Hoeijmakers, J.H. 2001. Genome maintenance mechanisms for preventing cancer.
Nature 411:366-374.

DNA Repair Deficiency Associated with Hematological Neoplasms

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1. Introduction

The genome of living organisms is constantly subjected to conditions that induce damage to DNA. A wide variety of DNA lesions are produced either as the result of normal metabolic processes or exogenous sources (ionizing radiation, UV light) (Seviour and Lin 2010). This damage to genomic material represents a persistent threat to genomic stability.

Mammalian cells have developed a range of molecular mechanisms capable of responding to DNA damage. These mechanisms include activation of DNA damage repair pathways or tolerance systems, initiation of complex regulatory networks that coordinate cell cycle progression and repair and induction of apoptosis if errors are detected (Madhusudan and Middleton 2005). At a cellular level, if a DNA lesion is misrepaired, it can lead to genomic instability. It is therefore essential for a cell to efficiently respond to DNA damage. Different DNA Repair Mechanisms exist and play a major role in restoring genomic integrity. These include: The Direct Repair Pathway (DR), Base Excision Repair Mechanism (BER), Nucleotide Excision Repair Pathway (NER), Non-Homologous End Joining (NHEJ), Homologous Recombination (HR) and DNA Mismatch Repair Pathway (MMR). It is quite obvious that any defect in these mechanisms can lead to improperly repaired DNA lesions and genomic abnormalities, which constitute the hallmark of tumorigenesis (Cline and Hanawalt 2003, Hwang et al. 1999). Indeed, mutations and polymorphisms of DNA repair genes have been correlated with different hereditary and sporadic cancer types (Altieri et al. 2008).

Hematological malignancies account for about 8% of all cancers in men and 6% of all cancers in women. There is a considerable body of evidence that suggests a strong association between DNA repair deficiency and hematological malignancies. This article is meant to serve as an overview of the relevant literature focusing on mutations and polymorphisms of DNA repair genes that predispose to certain hematological malignancies. We also discuss how defects in DNA repair affect sensitivity to chemotherapy.

2. DNA repair mechanisms

Below, we briefly summarize our current knowledge regarding DNA Repair Mechanisms.

2.1 Direct Repair (DR)

Direct Repair of DNA damage (DR) is the simplest repair process that copes with the repair of O⁶-alkylguanine adducts in humans. O⁶-alkylguanine transferase (AGT, also known as MGMT) is the key enzyme that reverses O⁶-alkyl-guanine to guanine by transferring the inappropriate methyl group to one of its own cysteine residues, leading to inactivation of itself (Scharer 2003).

2.2 Nucleotide Excision Repair (NER)

The Nucleotide Excision Pathway (NER) processes bulky DNA lesions with potential to block DNA replication or transcription (de Laat et al. 1999, Gillet and Scharer 2006, Lockett et al. 2005, Park and Choi 2006, Sancar 1996, Wood 1997). It mainly corrects products of UV light and chemotherapy (reviewed in (Scharer 2003)). When NER mechanism repairs damaged lesions in non-transcribing genomic DNA, it is called Global Genome NER (GG-NER). In contrast, Transcription-Coupled NER (TC-NER) removes damaged DNA specifically on the RNA polII transcribed strand of transcriptionally active DNA.

2.3 Base Excision Repair (BER)

The Base Excision Repair (BER) is a pathway that removes damaged bases from DNA, but also repairs single-strand breaks. Several variations exist, including short-patch BER (SP-BER) that typically replaces a single nucleotide and long-patch BER (LP-BER) where repair synthesis can extend beyond one nucleotide. The two pathways progress through different processes that initially involve removal of the damaged base by glycolases (Fortini and Dogliotti 2007, Fortini et al. 1999).

2.4 Mismatch Repair (MMR)

The Mismatch Repair Pathway (MMR) recognizes and repairs base-base mismatches and insertion/deletion loops that could arise during DNA replication (Jiricny 2006). Therefore, it contributes to the accuracy of DNA replication process (Jiricny 1998).

Defects in MMR result in Microsatellite Instability (MSI), a form of genetic instability characterized by frequent errors occurring during the replication of short nucleotide repeats (Velangi et al. 2004).

2.5 Double Strand Break (DSB) Repair

DNA double strand breaks (DSBs) occur as the result of two simultaneous nicks in opposite strands of the DNA helix. Although they are formed much less frequently than other forms of DNA damage, the consequences of DSBs can be severe. They are induced by exogenous sources (ionizing radiation, UV light, topoisomerase inhibitors) or arise spontaneously during natural processes (DNA synthesis, V-(D)-(J) recombination) (Karagiannis and El-Osta 2004).

A cell has evolved the DNA damage response (DDR) to initially respond to a DSB. DDR involves the sensing of the damage, followed by transduction of the damage signal to either cell cycle checkpoints and DSB repair or apoptosis. DDR is dependent on transient recruitment of MRN (MRE11/RAD50/NBS1) complex followed by activation of two phosphatidylinositol-3-related kinases, ATM and ATR that phosphorylate a variety of molecules to execute DDR (Figure 1) (Bakkenist and Kastan 2004). The activation of cell cycle checkpoints aims at the growth arrest of damaged cells and allows DSB repair to mend the damage. A DSB can be repaired by two separate mechanisms, either Homologous Recombination (HR) or Non Homologous End Joining (NHEJ) (Kim et al. 2006).

2.6 Homologous Recombination (HR)

Homologous Recombination (HR) is a highly complex pathway that uses an intact homologous template to repair a double strand break (DSB) accurately. It is the predominant pathway in lower eukaryotes, whereas it accounts only for the repair of 10% of DSBs in mammalian cells (reviewed in (Hakem 2008)). HR operates at S and G2 phase of the cell cycle, where a sister chromatid exists, and acts on rapidly dividing cells (Helleday et al. 2007). Furthermore, it fulfills specialized roles; it participates in meiosis and the repair of DNA interstrand crosslinks, which are highly cytotoxic DNA lesions (reviewed in (Scharer 2003)). The whole HR process is summarized in Figure 1.

2.7 Non Homologous End Joining (NHEJ)

Non Homologous End Joining (NHEJ) is the predominant pathway for repair of DSBs in higher eukaryotes (Kanaar et al. 2008). It is a conceptually simple pathway that involves the religation of broken ends and as the DNA ends may be damaged, this mode of repair is not necessarily accurate (Lieberman 2008). NHEJ is more important in quiescent or terminally differentiated cells and in G1 phase of the cell cycle. It also plays a crucial role in the generation of diversity in the immune system in V (D) (J) recombination and in telomere maintenance (reviewed in (Hakem 2008)). The NHEJ mechanism is illustrated in Figure 1.

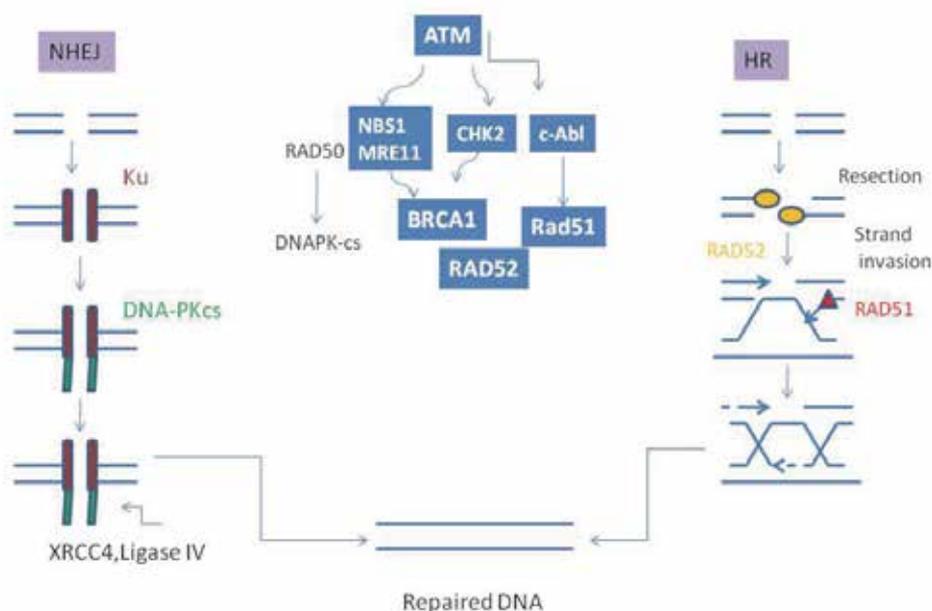


Fig. 1. Model for DSB initial response and repair. Initial recognition of DSB is performed by various sensor proteins (ATM, MRN complex, BRCA1, RAD51, DNA-PKcs). A DSB is repaired either by NHEJ or HR. In NHEJ, the heterodimer Ku70/80 binds to DSB and recruits DNA-PKcs, which promotes ends juxtaposition. Gaps are filled by polymerases μ or λ and nicks are sealed by ligase complex (Ligase IV, XRCC4). In HR, after resection of DSB, Rad51 forms a nucleoprotein filament. Then, a strand-exchange reaction generates a joint molecule between the damaged and the undamaged DNA. Then, repair synthesis is performed by DNA polymerases δ/ϵ .

Design and nomenclature of Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide – A, T, C, or G – in the genome differs between members of a biological species or paired chromosomes in an individual (Stenson et al. 2009). It indicates a non disease-causing change or a change found at a frequency of 1% or higher in the population. SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy (redundancy) of the genetic code (den Dunnen and Antonarakis 2003).

The nomenclature for SNPs can be confusing: several variations can exist for an individual SNP and consensus has not yet been achieved. One approach is to write SNPs with a prefix showing the wild-type and altered nucleotide or amino acid. Variants can be described either at the DNA, RNA or protein level (Ogino et al. 2007).

Descriptions at DNA level refer to a single nucleotide substitution designated by a ">"-character that indicates the change from the wild type to the altered nucleotide. Description is made in relation to a coding or genomic reference sequence. Regarding nucleotide numbering, when a coding reference is used, nucleotide 1 is the A of the ATG-translation initiation codon and the nucleotide 5' of the ATG-translation initiation codon is -1; the previous -2 etc. When a genomic reference is used, nucleotide numbering is purely arbitrary and starts with 1 at the first nucleotide of the database reference file (den Dunnen and Antonarakis 2000, 2003). For example, SNP RAD51 135 G>C, denotes that at nucleotide 135 a G is changed to a C. On the other hand, SNP RAD50 -38C>T denotes a C to T substitution 38 nucleotides 5' of the ATG translation initiation codon.

Descriptions at RNA and protein level are often deduced and not based on experimental evidence. Sequence changes at RNA level are basically described like those at the DNA level, with a few modifications. Description starts with the nucleotide number, followed by the nucleotide (in lower case), for example 78 u>a. In this chapter, none of the SNPs is described at RNA level. Descriptions at protein level start with the amino acid, followed by its number and the altered amino acid (den Dunnen and Antonarakis 2000, Ogino et al. 2007). For example, SNP XRCC1 Arg194Trp indicates a change of amino acid Arginine 194 to Tryptophan.

Finally, frequently SNPs are reported in relation to their reference SNP ID number, which is assigned to them from the Single Nucleotide Polymorphism Database (dbSNP). dbSNP is a free public archive for genetic variation within and across different species developed and hosted by the National Center for Biotechnology Information (NCBI) in collaboration with the National Human Genome Research Institute (NHGRI) (Wheeler et al. 2007). When submitted, SNPs receive a reference SNP ID number ("rs#"; "refSNP cluster"). For example, XRCC4 rs13178127 refers to the SNP of the gene XRCC4 that has received the reference ID number rs13178127.

3. DNA repair deficiency related to hematological malignancies

Below, we summarize the existing data concerning abnormalities of DNA repair pathways in the most common hematological malignancies.

3.1 Acute Myeloid Leukemia (AML)

Acute Myeloid Leukemia (AML) is a group of marrow-based neoplasms that have clinical similarities but distinct morphologic, immunophenotypic and cytogenetic features (Monzo

et al. 2006). Clinical features are similar at any age and are the result of suppression of normal marrow elements by malignant blasts.

Although many environmental and genetic factors have been suggested to play a role in AML pathogenesis, the mechanism still remains uncertain. Through the last years many studies have focused on identifying genetic differences that could contribute to a patient's predisposition to develop AML. These differences could be in part attributed to polymorphisms that lead to increased or decreased activity of the encoded genes; and DNA repair genes are attractive candidates for studies, since their ineffectiveness causes chromosomal instability. This is further supported by the higher incidence of AML in patients with syndromes characterized by defective DNA repair (Schwartz and Cohen 1988). So far, several polymorphisms have been associated with an increased risk of developing AML, such as polymorphisms of Homologous Recombination genes XRCC3 Thr241Met and RAD51 135G>C (Voso et al. 2007). In another study, these two polymorphisms have been reported to have a synergistic effect on AML development (Seedhouse et al. 2004). In addition, XRCC3 Thr241Met has been found to specifically increase the risk of inv(16)/t(16;16) AML and to be an independent poor prognostic factor for this AML subtype, since it reduces disease free survival in patients that achieved complete remission (CR) (Liu L. et al. 2011). This is an interesting finding, since it could help us substratify patients with inv (16)/t (16; 16) AML, which has favorable prognosis. On the other hand, RAD51 135 G>C has also been associated with a decreased risk of AML (Rollinson et al. 2007). In this study, the authors claim that this polymorphism results in overexpression of RAD51 that might modulate HR in favor of the cells, protecting them from aberrant DNA repair events. Among other polymorphisms, XPD 2251A>C has been associated with increased risk of developing AML, mainly attributed to the C allele (Shi et al. 2011).

It is also known that patients with AML have different response rates to the same treatment agents. Although administration of Ara-C, in conjunction with an anthracycline, is the cornerstone of therapy, the question of the best therapy for AML in first remission remains unanswered, and perhaps it could be stratified according to certain features. In this regard, several studies have focused on finding polymorphisms that have impact on the effects of chemotherapy and as a result, clinical outcome. It is known that good response to chemotherapy is usually associated with low DNA repair capacity, whereas chemoresistance is usually associated with a high DNA repair activity. Although there has been much effort on identifying DNA repair polymorphisms that could predict chemotherapy effectiveness in order to allow appropriate selection of drugs in specific patients, the existing results are of no clinical use and are sometimes contradictory. For example, polymorphism XPD Lys751Gln has been correlated with reduced risk of resistant disease (RD) due to an increased chemotherapy response rate (Kuptsova et al. 2007). However, in another study, the same polymorphism has also been associated with poor response to chemotherapy and decreased survival in patients with intermediate risk AML and normal cytogenetics (Strom et al. 2010). Of course, this controversy might be explained by different patient groups involved in these studies and it could be claimed that XPD Lys751Gln is a predictor of poor response to front line chemotherapy only in patients with normal karyotype. Among other polymorphisms, XPD Asp312Asn is reported to have a positive impact on drug effectiveness (Kuptsova et al. 2007) and XPC Ala499Val has been associated with decreased overall survival in AML patients with normal karyotype (Strom et al. 2010). Furthermore, in a study that included 110 AML patients, patients with XPA genotype -4A>G have been shown to have a higher frequency of chemoresistant disease

when compared to wild type carriers (30% vs. 6, 5 % genotype AA) (Monzo et al. 2006). Finally ATM variant 4138C>T has been associated with poor response to chemotherapy in Chinese population (Shi et al. 2011); however this finding is difficult to be confirmed in European studies, due to its low penetrance (Choudhury et al. 2008, Johnson et al. 2007).

Apart from the above polymorphisms, several studies implicate BRCA1 (involved in initial recognition of a double strand break) and BRCA2 gene (involved in Homologous Recombination) deficiencies in AML pathogenesis. Scardocci et al. observed that 32% of primary AML samples present with hypermethylated BRCA1 (Scardocci et al. 2006). Although this finding has not been confirmed in other studies (Bianco et al. 2000, Esteller et al. 2001), a meta-analysis performed in 2007 which reviewed risks for hematological malignancies associated with mutations in BRCA1/2 pathway, showed that defects in this pathway increase the risk for a subset of AML that are related to gene rearrangements (Friedenson 2007). Consequently, it could be hypothesized that BRCA1/2 pathway's proper function is essential to prevent a group of leukemias. Consistent with these data is the observation that Fanconi anemia patients with biallelic mutations in BRCA2 present with a high risk of AML (Wagner et al. 2004).

Recent evidence suggests a role of error-prone Non Homologous End Joining (NHEJ) in AML development. In preclinical studies, sequencing of breakpoint junctions in AML cells has revealed microhomologous sequences and small deletions, indicative of NHEJ repair (Gillert et al. 1999, Olive 1998, Wiemels et al. 2000, Wiemels et al. 2002, Yoshida et al. 1995). This increased NHEJ activity must, however, be confirmed in other studies.

Regarding Mismatch Repair (MMR) Mechanism, published data in AML are somewhat conflicting. Although there are studies demonstrating increased rates of microsatellite instability in certain types of leukemia (mostly therapy-related AML and myelodysplastic syndromes (Ben-Yehuda et al. 1996, Kaneko et al. 1995)), most reports have described little or no evidence of deficient MMR in AML (Boyer et al. 1998, Sill et al. 1996, Tasak et al. 1996). However, while none of these studies focused on MMR deficiency in different stages of AML, in 2008 Mao et al. showed that more than 30% of AML patients presented with mutations in MMR genes and that MMR deficiency was higher in patients with relapsed/refractory AML (Mao et al. 2008). Authors suggest the possibility of leukemia relapse generating from leukemic cells defective in MMR that have survived chemotherapy and present the minimal residual disease (Mao et al. 2008).

In conclusion, many polymorphisms of DNA repair genes have been associated with individual susceptibility to AML, as well as pathogenesis and sensitivity to chemotherapy. The precise functional mechanism of their effect remains to be determined; however results strongly support the importance of considering them as prognostic factors or indicators of treatment strategy. Hopefully, further studies will elucidate their role with the view to use them in clinical setting.

3.2 Acute Lymphoblastic Leukemia (ALL)

Acute Lymphoblastic Leukemia (ALL) is a malignant disease characterized by accumulation of immature lymphoblasts. It is the most common leukemia in children, accounting for 70-80% of all childhood leukemias. In the contrast, ALL accounts for 15-20% of leukemias in adults (Faderl et al. 1998).

There is strong evidence that acquired genetic changes are central to the development of ALL. Polymorphisms of DNA repair genes have been investigated as potential pathogenetic and prognostic factors. The majority of studies are focused on childhood ALL, due to its

high frequency in children. Several polymorphisms have been implicated, but results are somewhat conflicting, probably because of the different ethnic groups involved in each study. Thus, polymorphism XRCC1 Arg399Gln has been associated with increased risk of childhood ALL, in Indians (Joseph et al. 2005), Chinese (Zhu et al. 2005) and Thais (Pakakasama et al. 2007), but no correlation was found in Mexican (Meza-Espinoza et al. 2009), Brazilian (da Silva Silveira et al. 2009) and Turkish children (Batar et al. 2009). Perhaps this polymorphism has a stronger effect or a higher penetrance in Asian populations. In other studies, it has been proposed that it has a synergistic effect with CYP2E1*5B polymorphism of detoxification enzyme CYP2E1 (Tumer et al. 2010), as well as polymorphism OGG1 Ser326Cys (Stanczyk et al. 2011) in ALL development. Similarly, polymorphism XRCC1 Arg194Trp, has been found to have a protective effect in Thais (Pakakasama et al. 2007), while opposite results have been found in another study (Joseph et al. 2005). Moreover, several XRCC1 haplotypes have been related to ALL; haplotype B, as well as C, have been associated with an increased ALL risk (Meza-Espinoza et al. 2009, Pakakasama et al. 2007).

On the other hand, polymorphisms of Nucleotide Excision Repair genes have also been investigated in ALL in a few studies. XPD Asp312Asn and Lys751Gln polymorphisms, which have been previously associated with AML, have not been found to be correlated with ALL risk (Batar et al. 2009, da Silva Silveira et al. 2009). On the contrary, CC genotype of ERCC18092C>A polymorphism has been suggested to increase susceptibility to ALL in Chinese children (Wang et al. 2006). In the same study, ERCC1 19007G>A was not found to be associated with ALL (Wang et al. 2006).

Regarding Mismatch Repair (MMR) mechanism, most of the existing studies have failed to show an association between MMR deficiency and ALL development (Molenaar et al. 1998, Takeuchi et al. 1997), although preclinical studies have demonstrated MSI in ALL cell lines (Gu et al. 2002, Hangaishi et al. 1997). In addition, no relationship was found between polymorphisms in MMR genes MLH1 and MSH3 and childhood ALL (Mathonnet et al. 2003), although a combined effect was demonstrated with CYP2A1*2A and CYP2E1*5 polymorphisms of detoxification enzymes, suggesting a possible interaction between DNA repair enzymes and detoxification enzymes in childhood ALL. In addition, in another study that evaluated the risk of childhood ALL and the presence of variants in DNA repair enzymes in children exposed to diagnostic X-rays, hMLH1 was found to protect against X-ray exposure (Infante-Rivard et al. 2000).

Finally, it is worth mentioning that a positive correlation has been found between polymorphism 8360G>C in NBS1 gene (component of MRN complex) and ALL. It is interesting that this study included ALL patients from different ages, but results are potentially questionable, since the study was limited to a small size group of Chinese population (Jiang et al. 2011).

In summary, although many polymorphisms have been associated with ALL, investigation remains still at an early stage. Further studies are needed in order to be able to draw definite conclusions for the potential use of polymorphisms for the stratification of patients in different prognostic and treatment groups.

3.3 Myelodysplastic Syndromes (MDS)

Myelodysplastic syndromes (MDS) represent a spectrum of stem cell malignancies that manifest dysplastic and ineffective hematopoiesis, which is associated with a variable risk of transformation to AML. They usually arise de novo with the risk of developing MDS increasing proportionally to age (Doll and List 1989, Noel and Solberg 1992).

It is known that genetic lesions in hematopoietic progenitor cells play a major role to their malignant transformation and there is accumulating evidence that genetic factors modify a person's cumulative risk to MDS (Janssen et al. 1989). It has also been suggested that disease progression of MDS to AML is attributed to accumulation of mutations that cause defects in DNA repair (Rowley 1999); as a consequence, most of the existing studies are interested in identifying whether DNA repair polymorphisms implicated in AML are also involved in MDS. However, only one study found a positive correlation between polymorphism RAD51 135G>C and MDS risk; and it is difficult to interpret this result, since the study is limited to a Chinese population (Li et al. 2010). All other studies did not show any association between DNA repair polymorphisms and MDS (Baumann Kreuziger and Steensma 2008, Fabiani et al. 2009). This contradiction might be interpreted by the fact that these polymorphisms have been associated with *de novo* AML, whose pathogenesis is a different molecular procedure compared to MDS.

Finally, in an effort to examine a possible role of Non Homologous End Joining (NHEJ) repair mechanism in MDS, we have previously tested the expression of NHEJ protein components in 48 cases of adult *de novo* MDS (Economopoulou et al. 2009). We reported a significantly lower Ligase IV expression level in MDS patients compared to controls and an association of Ku70 expression with more aggressive disease, suggesting a potential role of these two molecules in MDS pathogenesis and clinical presentation. However, a larger number of cases need to be examined in order to confirm these findings.

3.4 Chronic Myeloid Leukemia (CML)

Chronic Myeloid Leukemia (CML) is a clonal stem cell disorder characterized by increased proliferation of myeloid elements at all stages of differentiation (Champlin and Golde 1985). It is caused by the Bcr-Abl oncoprotein, the product of the t (9;22) chromosomal translocation that generates the Philadelphia chromosome. CML is a multistep disease, which involves a chronic phase (CP) that finally progresses to a blast crisis (BC).

Although imatinib, a selective inhibitor of the ABL kinase, has revolutionized the treatment of CML (Druker et al. 1996), it has not completely eradicated it, and has a poorer response rate in patients with CML and blast crisis (BC) (Oehler et al. 2007). Progression to BC has been associated with the appearance and accumulation of new cytogenetic abnormalities; therefore, there are many studies focusing on transformation to BC pathogenesis. It has been recently found that BCR-ABL transformed cells have an increased level of Reactive Oxygen Species (ROS) that induces numerous double strand breaks (DSBs) (Nowicki et al. 2004). It was also shown that BCR-ABL upregulates error-prone DSB repair pathways (Non Homologous End Joining-NHEJ) rather than the high-fidelity mechanism of Homologous Recombination, promoting mutagenic DSB repair (Nowicki et al. 2004, Salles et al. 2011). Therefore, it could be suggested that mutagenic DSB repair might provoke chromosomal abnormalities found in the progression to BC (Nowicki et al. 2004). Furthermore, it was recently demonstrated that BCR-ABL increases expression and activation of WRN (involved in NHEJ), which promotes unfaithful repair and protects leukemia cells from apoptosis, resulting in genomic instability (Slupianek et al. 2011).

Moreover, several DNA Repair proteins have been found to be associated with CML and have been suggested to play a potential role in CML-BC pathogenesis. DNA-PKcs has been found to be decreased in BCR-ABL transfected cells, as well as CD34+ cells from CML patients (Deutsch et al. 2001). This reduced expression of DNA-PKcs was correlated with DNA repair deficiency, that might contribute to genetic instability found in BC (Deutsch et

al. 2001). It could be therefore suggested that low expression of DNA-PKcs is involved in progression to CML-BC. Among other proteins, XPB has been isolated from BCR domain and it has been shown that continuous interaction with BCR-ABL provokes XPB deficiency and chromosomal instability (Takeda et al. 1999). Finally, hMSH3 Mismatch Repair protein has been found to be reduced in CML patients (Inokuchi et al. 1995). However, we can't ignore that the percentage of CML patients with reduced hMSH3 expression in that study was very high (90%), but the number of CML samples examined was small (10). Given the fact that the finding has not been confirmed in other studies, its reliability is questionable. Abnormalities of DNA repair components and their impact on myeloid malignancies are summarized in Table 1.

3.5 Non Hodgkin's Lymphoma (NHL)

Non Hodgkin's Lymphoma (NHL) includes multiple neoplastic disorders of the lymphoid system with overlapping features. They most commonly derive from mature B-cells of germinal center origin. Clinical presentation is highly variable and depends on a number of factors; however NHL share a number of common clinical and pathologic features.

DNA repair has a pivotal role in NHL development. Defects in DNA repair can lead to the development of chromosomal aberrations, a hallmark of lymphoma (Palitti 2004). This is further supported by the fact that several hereditary syndromes, including Ataxia telangiectasia and Nijmegen breakage syndrome are characterized by defective DNA repair and high occurrence of lymphoma. Among studies focused on DNA repair polymorphisms and their impact on NHL, three parallel studies conducted in the US and Australia have reported different or contradictory results. In the first one, DNA ligase IV (Lig4) 9 I variant allele has been found with lower frequency in NHL patients, including follicular lymphoma (FL) and Diffuse Large B-Cell Lymphoma (DLBCL) patients, compared to controls (Hill et al. 2006). This finding is in accordance with another case control study, where LIG4 9 I/I homozygotes had a 3 fold reduced NHL risk (Roddam et al. 2002). Among other polymorphisms examined, RAG1 K820R variant allele (involved in V (D) J recombination), BRCA2 Arg372His genotype and XRCC1 Arg194Trp were associated with an increased risk of NHL (Hill et al. 2006). However, the latter has not been confirmed in other studies that evaluated the impact of XRCC1 polymorphisms in NHL (Liu J. et al. 2009, Smedby et al. 2006). Finally, WRN V114I variant was found to be associated with a decreased risk of NHL (Hill et al. 2006). In contrast, in the second study conducted in Connecticut, these results were not confirmed. However, a negative association was demonstrated between NHL and another WRN polymorphism, Cys1367Arg, suggesting a protective role of WRN in NHL (Shen et al. 2006). On the other hand, polymorphism XPG Asp1104His has been for the first time correlated with elevated NHL risk, including DLBCL and T-cell lymphomas (Shen et al. 2006). Finally, the third study which included patients from Australia, found no association between the above polymorphisms and NHL (Shen et al. 2007). However, it has been found that MGMT SNPs Ala143Val and Lys178Arg are associated with elevated NHL risk, including FL, DLBCL and MALT lymphoma (Shen et al. 2007).

Interestingly, a pooled investigation of genetic variation in 27 DNA repair genes based on the three above studies, revealed different results (Shen et al. 2010). Among the examined SNPs, BLM rs441399 was significantly associated with follicular lymphoma and XRCC4 rs13178127 was correlated with NHL overall (Shen et al. 2010). Of note, products of BLM and XRCC4 genes are both involved in DSB repair and they both interact with Ligase IV.

Moreover, in the same study, ERCC3 rs4150506 was associated with reduced risk for marginal zone lymphoma (MZL) (Shen et al. 2010).

Similarly, Worrillow et al. (Worrillow et al. 2009) analyzed polymorphisms in XPD gene, in a UK population study that involved 747 NHL cases. This is the largest study assessing the impact of XPD polymorphisms on NHL. There were no statistically significant case control differences in the distribution of XPD polymorphisms. However, a decreased risk of diffuse large B-cell lymphoma was observed in variants for XPD Lys751Gln. This finding has been previously reported (Shen et al. 2006). Furthermore, XPD 156 A>C was found to be associated with an increased risk of follicular lymphoma (Worrillow et al. 2009).

Similarly, Rollinson et al. (Rollinson et al. 2006) studied the haplotypic variation of tri-complex MRE11-RAD50-NBS1 and its relationship with NHL risk. The main findings of the study were a protective effect for MRE11 rs601341 variant in follicular lymphoma and for the MRE11 GCTCA haplotype in diffuse large B-cell lymphoma (DLBCL), suggesting a possible role of protein MRE11 in DLBCL pathogenesis. No altered NHL risk associated with haplotypes of NBS1 and RAD50 was observed in this study. Another similar study was performed by Schuetz et al (Schuetz et al. 2009). The authors suggest an association of a RAD50 SNP -38C>T with Mantle Cell Lymphoma (MCL), but the low incidence of both the SNP and MCL make these data questionable. No association was observed between NBS1 haplotypes and NHL (Schuetz et al. 2009). In contrast to the previous study, this one failed to show any correlation of MRE11 rs601341 variant with NHL, but the authors propose that this disparity is due to the fact that the part of intron where rs601341 was located was not sequenced.

Additionally, ATM protein, also involved in double strand break response, has been associated with NHL. Schaffner et al. (Schaffner et al. 2000) reported inactivation of both alleles of ATM gene in a high proportion of patients with Mantle Cell Lymphoma (MCL), suggesting that ATM functions as a tumor suppressor gene in MCL. This finding has been confirmed in subsequent studies that provide evidence for somatic mutations in ATM in MCL (Fang et al. 2003, Taylor et al. 1996). On the other hand, ATM has been associated with NHL of B-origin (B-NHL) clinical outcome, since deletion of one ATM allele has been found to strongly influence B-NHL survival (Vorechovsky et al. 1997). However, another study reported contradictory results claiming that common variants in ATM do not influence NHL susceptibility (Sipahimalani et al. 2007). The authors suggest that individuals with common ATM variants are phenotypically normal and that only rare variants could possibly influence NHL susceptibility. The role of rare ATM variants in NHL remains to be elucidated.

Among other DNA repair proteins, it is worth mentioning the role of Nucleotide Excision Repair component hHR23B as an apoptosis-associated protein in Burkitt Lymphoma (Vogel et al. 2005).

Finally, the role of Mismatch Repair (MMR) pathway in NHL has also been investigated, although limited published data exist. Kotoula et al. (Kotoula et al. 2002) described a low expression of proteins hMLH1 and hPMS1 in B-cell lymphomas and in Mantle Cell Lymphoma (MCL) of blastoid type compared to normal controls, although in B-Chronic Lymphocytic Leukemia and Mantle cell Lymphoma of centrocyte-like type the expression of MMR genes remained intact. The small sample size and the heterogeneity of the specimens, along with the lack of additional published data that could support the findings, make these data questionable. Furthermore, Morimoto et al. (Morimoto et al. 2005) observed a reduced expression of MMR genes in patients with adult T-cell Leukemia (ATL), supporting a role of

MMR gene inactivation in disease progression and development of ATL. Although the number of patients included in the study is small, the results are promising and give insight for further investigation.

3.6 Hodgkin's Lymphoma (HL)

Hodgkin's lymphoma (HL) is a hematolymphoid neoplasm, primarily of B-cell lineage, with unique molecular, histologic, immunophenotypic and clinical features. The diagnosis of classical HL requires the recognition of Reed-Steinberg cells in an appropriate cellular milieu (Liu A. et al. 2008).

To date, the effect of DNA repair polymorphisms on HL has not been yet thoroughly investigated. However, few studies have tried to assess their role on HL development. In a large study that involved 200 newly diagnosed adult HL patients, a positive association was demonstrated between XRCC1 Arg399Gln and risk of HL (El-Zein et al. 2009). Interestingly, the authors observed that combined analysis of XRCC1/XRCC3 and XRCC1/XPC polymorphisms revealed significant association with increase in HL risk. For example, XRCC3 Thr241Met was associated with an OR of 2.38 when combined with homozygous XRCC1 Arg/Arg genotype. XRCC1 Gln/Gln along with XRCC3 variant led to a 3 fold increased HL risk. Similarly, XRCC1 Arg399Gln together with XPC Lys/Lys was found to significantly increase HL risk. Similar findings were reported in a recent study that evaluated potential gene-gene interaction between DNA repair genes and their contribution to individual susceptibility to HL (Monroy et al. 2011). Polymorphism XRCC1 Arg399Gln was again associated with increased HL risk, as well as polymorphisms XPC Ala499Val, XRCC3 Thr241Met and XRCC1 Arg194Trp. Most importantly, it was observed that HL risk increases as the number of adverse alleles in Base Excision Repair (BER) and repair of double strand breaks (DSBR) increases. Taken together these two studies suggest a potential cooperation between BER and DSBR mechanisms in HL development; however the exact procedure remains to be elucidated.

Furthermore, there is strong evidence that ATM gene is involved in the pathogenesis of HL. The existing data refers to identification of four SNPs of ATM gene with a defective protein activity (Takagi et al. 2004). Consequently, it could be proposed that in the presence of genotoxic stress, these defects might be amplified and lead to accumulation of mutations that may contribute to a malignant phenotype. Similarly, Liberzon et al. showed that variants of ATM were associated with aggressive disease in children (Liberzon et al. 2004). The main limitation of both these studies is the small sample size that consisted only of children. Further investigation is necessary in order to draw safe conclusions.

Finally, it is worth mentioning that a relationship was recently found between telomere shortening and development of second cancers in HL patients (M'Kacher et al. 2007). Telomere dysfunction is linked to DNA repair and probably to decreased Ku70 expression, although further studies are required to elucidate the mechanism of telomere shortening in HL patients.

3.7 Chronic Lymphocytic Leukemia (CLL)

Chronic Lymphocytic Leukemia (CLL) is the most common form of adult leukemia in the Western hemisphere (Ganster et al. 2009). It is a neoplastic disorder of mature B lymphocytes that is determined by genetic, molecular and environmental factors. The clinical course of CLL is highly variable. There are two groups of patients with diverse

clinical outcomes that are separated by the mutational status of immunoglobulin variable region (IgV) gene (Hamblin et al. 1999).

Although mutation status of IgV gene, as well as acquired chromosomal abnormalities [del(13q), del(11q), del(17p), trisomy 12] have been identified as prognostic factors in CLL, discovery of further prognostic factors would help to predict disease progression among different patient groups. Recently, polymorphism XPD Lys751Gln not only has been found to be associated with CLL development but it also occurred significantly more frequently in high risk patients. In the same study, polymorphisms XRCC1 Arg399Gln and XPF Arg415Gln were similarly positively correlated with cytogenetically high risk CLL patients. In contrast, polymorphism RAD51 135 G>C was associated with low risk CLL. The most important aspect of this study is that it examines the effect of SNPs on cytogenetic aberrations in CLL and suggests that polymorphisms XPD Lys751Gln and XRCC1 Arg399Gln might predict the unfavorable clinical outcome of CLL. Notably, polymorphism XPD Lys751Gln has been previously described as a negative prognostic factor in acute myeloid leukemia. However, contradictory results were produced in another study, where XPD Lys751Gln was associated with a decreased risk of developing CLL; there was no distribution among different cytogenetic risk subgroups though (Enjuanes et al. 2008). Similarly, XRCC1 Arg399Gln has not been correlated with CLL in previous studies, although there was again no distinction between different subgroups (Enjuanes et al. 2008, Sellick et al. 2008). These data suggest that individual genetic polymorphisms may predict the clinical outcome of CLL; however, future studies should focus on confirming the results in a larger group of patients, with the view for clinical application.

It is known that one of the therapies used in CLL is treatment with alkylating agents, which is associated with low rates of complete remission. The ability of CLL cells to efficiently repair alkylator-induced DNA damage might explain lack of response to treatment. Therefore, DNA repair may be linked to chemoresistance in CLL (Sampath and Plunkett 2007). There is strong evidence that both HR and NHEJ play an important role in CLL resistance to chemotherapy. For instance, there is evidence that CLL cells exposed to chlorambucil demonstrate an increase in RAD51 foci (Christodouloupoulos et al. 1999). In addition, resistance to alkylating agents has been associated with enhanced HR repair in CLL cells (Aloyz et al. 2004, Bello et al. 2002, Xu et al. 2005). Similarly, a high level of XRCC3 was associated with lack of response of CLL cells to nitrogen mustards (Bello et al. 2002). Furthermore, it has been reported that low levels of Non Homologous End Joining protein component DNA-PKcs is strongly associated with drug sensitivity in CLL cells, whereas increases in DNA-PK activity resulted in increased resistance to neocarzinostatin and chlorambucil (Amrein et al. 2007, Austen et al. 2005). Inhibition of DNA-PKcs (Amrein et al. 2007), as well as dual inhibition of both RAD51 and DNA-PK (Amrein et al. 2011) has been found to induce drug sensitivity in CLL cells. Recently, the effect of DNA-PK inhibitors in CLL cells was investigated in combination with mitoxantrone. Interestingly, DNA-PK inhibitors provoked mitoxantrone cytotoxicity in CLL cells, especially cells with unfavorable cytogenetic anomalies [del(17p) and del(13q)] (Elliott et al. 2011). These findings could be of vital importance regarding CLL treatment, since DNA repair inhibitors could be used as potential therapeutic targets in combination with alkylating agents for a better clinical outcome, especially in high risk CLL.

Finally, association of DNA repair enzymes' abnormalities with CLL prognosis has been the subject of investigation of several studies. ATM has been implicated in CLL, since it has been shown that approximately 14% of patients with untreated CLL harbor deletions of the

long arm of chromosome 11 at the 11q22-23 (ATM) locus, which is associated with poor survival. An explanation could be that since ATM is an upstream regulator of p53, defects in ATM lead to attenuation of p53-dependent apoptosis, which results in a chemo-refractory phenotype. Furthermore, a correlation was described between protein DNA-PKcs component of Non Homologous End Joining and poor prognosis in CLL in terms of enzyme over-expression in del (17p) and del (11q) unfavorable CLL cases (Willmore et al. 2008). In the same study, high DNA-PKcs levels predicted for reduced treatment-free survival. These findings suggest that DNA-PKcs might contribute to disease progression in CLL, possibly by enhancing error-prone Non Homologous End Joining activity.

3.8 Multiple Myeloma (MM)

The diagnosis of multiple myeloma (MM) requires 10% or more plasma cells on bone marrow examination, M protein in the serum and/or urine (except in patients with true non-secretory myeloma) and evidence of end-organ damage (hypercalcaemia, renal insufficiency and anemia or bone lesions) secondary to the underlying plasma cell disorder. Almost all patients are thought to evolve from an asymptomatic premalignant stage termed monoclonal gammopathy of undetermined significance (MGUS) (Kyle and Rajkumar 2004). Genetic instability is a prominent feature of MM, since malignant plasma cells display aneuploidy and complex cytogenetics associated with poor prognosis (Calasanz et al. 1997, Rajkumar et al. 1999, Smadja et al. 2001). Two pathways are considered important for disease progression; hyperploid MM involves trisomies of several chromosomes, whereas non-hyperploid MM is characterized by translocations in immunoglobulin heavy chain locus at 14q32 (Bergsagel and Kuehl 2005, Fonseca et al. 2004, Ho et al. 2001). The latter events represent aberrant class switch recombination (CSR), a process that normally alters immunoglobulin isotype with the maturation of immune response (Fenton et al. 2002, Liebisch and Dohner 2006).

Given the fact that CSR requires formation of double strand breaks, the role of Homologous Recombination (HR) and Non Homologous End Joining (NHEJ) in MM pathogenesis has been investigated in many studies. Among DNA repair polymorphisms, XRCC4 rs963248 and rs1051685 Ku80 have been found in a higher frequency in MM patients compared to controls (Hayden et al. 2007). At a protein level, XRCC4 was also recently found to be overexpressed in MM (Roddam et al. 2010) Furthermore, recently, protein Ku80 has been associated again with MM, since it was demonstrated that Ku80/Ku70 are translocated to human MM cells surface and that this localization has functional implications which could contribute to MM pathogenesis (Tai et al. 2002).

Homologous Recombination (HR) has also been investigated in MM. Rad50 has been recently found to be overexpressed in MM patients (Roddam et al. 2010). In another study by Shammas et al. model MM cell lines were used in order to evaluate the molecular mechanisms of genetic instability and progression in malignancies (Shammas et al. 2009). An elevated HR activity is reported in MM cells, which leads to mutations. The authors show that inhibition of HR activity by siRNAs leads to reduction of genetic changes. Therefore, we could presume that HR can be targeted not only for prevention of disease progression, but also for therapy improvement. In another study focused on assessing the role of NHEJ in MM phenotype, authors observed that DSBs, when induced in MM cell lines, exhibited corrupt NHEJ (Yang et al. 2009). Interestingly, with the use of DNA-PK inhibitor was used, NHEJ process was suppressed and DNA ends were processed by intact

HR. This finding suggests that deregulated DSB repair in MM might contribute to complex chromosomal aberrations that, as mentioned above, is typical feature of the disease.

Apart from DSB repair, Mismatch Repair deficiency in MM has been investigated in several studies. Microsatellite instability has been reported in MM patients (Timuragaoglu et al. 2009, Velangi et al. 2004) and has been associated with more aggressive forms of the disease (e.g. plasma cell leukemia-PCL) (Velangi et al. 2004). On the other hand, Martin et al. (Martin et al. 2006) examined the methylation status and expression of enzymes MGMT and hMLH1 in 44 cases of plasma cells disorders [(Multiple Myeloma, Monoclonal Gammopathy of Undetermined Significance (MGUS), Plasma Cell Leukemia (PCL)]. Protein hMLH1 was found to be underexpressed in 50% of cases of MM, but not in patients with MGUS, whereas hMLH1 gene promoter was hypermethylated in 10% patients with MM but not in cases with MGUS. This finding suggests that inactivation of hMLH1 could be implicated in the evolution of MGUS to MM. On the contrary, 33% of MGUS and 44% MM patients had lost the MGMT expression, suggesting that MGMT deficiency is an initial alteration in plasma cell disorders.

Additionally, Nucleotide Excision Repair (NER) enzymes and have been associated with MM. Peng et al. reported epigenetic silencing of NER protein XPD in MM cells that was found to promote clonal expansion of MM cells (Peng et al. 2005). As a consequence, it might contribute to MM pathogenesis. NER has also been studied in the context of autologous bone marrow transplantation (ASCT) in MM. It has been observed that the combination of variant alleles of NER proteins XPD K75Q and XRCC3 T241M correlates with prolonged time to treatment failure in MM patients treated with ASCT (Vangsted et al. 2007). This finding is very important, since polymorphisms in XPD and XRCC3 could potentially predict the outcome in MM patients undergoing ASCT.

Finally, there is recent evidence that DNA repair polymorphisms are related to treatment response rate (RR) in MM. In a study conducted in Spain, it was found that polymorphisms ERCC1 rs735482 and Ku80 rs1051685 were associated with higher RR and longer overall survival in patients with relapsed/refractory myeloma treated with thalidomide (Cibeira et al. 2011). Similarly, RR was better in MM patients with SNPs in ERCC5 rs17655 (Cibeira et al. 2011). Although sample size is relatively small and belongs to a specific group of patients, this study provides very useful information, which, if confirmed in other studies, could be used in clinical practice to predict favorable outcome in relapsed myeloma.

Abnormalities of enzymes involved in DNA repair mechanisms and their influence on hematological malignancies are summarized in Table 1.

4. Conclusions

Genomic instability is the hallmark of all hematological malignancies. Since DNA repair mechanisms are responsible for correcting DNA damage and preserving genomic integrity, it is obvious that abnormalities of these mechanisms are strongly related to hematological cancers. Mutations and polymorphisms of DNA repair genes are associated with alteration of an individual's susceptibility to malignancy. In addition, they are related to prognosis, drug resistance and clinical outcome and represent potential targets for therapy.

We have presented the existing published data that show association of DNA repair mechanisms with the most common myeloid and lymphoid malignancies. Many studies have focused on investigation of the role of Base Excision Repair, Nucleotide Excision Repair, Non Homologous End Joining and Homologous Recombination in hematological

MALIGNANCY	DEFECTS
AML	<p>NER: - XPDGlu751C/Asp312G → ↓ risk of RD - XPDAsp312Asn → ↓ risk of RD/ ↓ survival in AML with normal karyotype - XPD Lys751Gln A>C → ↑ AML risk - XPC Ala499Val → ↓ OS in normal karyotype - XPA 4A>GGG → chemoresistant disease, ↓ OS</p> <p>BER: - XRCC3 Thr241Met → ↑ AML risk, especially inv(16)/t(16;16) AML</p> <p>HR: -Rad51G135C → 2x ↑ or ↓ AML risk? - Rad51-135-172-C haplotype → ↓ AML risk</p> <p>NHEJ: - ↑ NHEJ activity and repair infidelity</p> <p>DSBR: - ATM variant 4138CT → poor response to chemotherapy in Chinese - hypermethylation of BRCA1</p> <p>MMR: -MMR deficiency in relapsed/refractory AML.</p>
ALL	<p>BER: - XRCC1 194Trp allele → ↓ risk ALL in Thais, ↑ risk in Indians - XRCC1 399Gln → ↑ risk of ALL in Indians, Chinese, Thais - XRCC1 haplotypes B&C → ↑ risk of ALL</p> <p>NER: - ERCC1 8092C>A CC genotype → ↑ risk of ALL in Chinese</p> <p>DSBR: - E185Q in NBS1 → ↑ risk of ALL</p> <p>MMR: - MLH1 Ile-219/Ile-219+ CYP2A1*2A/ +GSTM1/ +CYP2E1*5 → ↑ risk of ALL</p>
MDS	<p>HR: -RAD51-G135C → ↑ risk of MDS?</p> <p>NHEJ: - ↓ Ligase IV expression in MDS vs. controls, ↑ Ku70 expression → aggressive disease</p>
CML	<p>NER: -XPB binds to BCR domain → dysfunction of XPB → role in BC?</p> <p>DSBR: -BCR/ABL(+) cells → ↑ DSBs, high mutation rate in HR/NHEJ → genomic instability</p> <p>HR: - DNA-PKcs expression in hematopoietic cells transfected with BCR/ABL/ CD34+ cells from CML patients</p> <p>MMR: - hMSH3 ↓ in MMR</p>
NHL	<p>DR: - MGMT Ile143Val and MGMT Lys178Arg → ↑ risk of NHL (FL,DLBCL,MCL,T-Cell Lymphomas)</p> <p>BER: - XRCC1 Arg194Trp → ↑ risk of NHL, ↓ risk of DLBCL</p> <p>NER: - XPD K751QCC → ↓ risk of DLBCL - XPD R156R → ↑ risk of FL - XPG Asp1104His → ↑ risk of NHL - hHR23B → Burkitt lymphoma?</p> <p>DSBR: -Loss of ATM in MCL - MRE11 GCTCA → ↓ risk of DLBCL - MRE11 rs601341 → ↓ risk of FL - BLM rs441399 → ↑ risk of FL - BRCA2 372 H/H → ↓ risk of NHL (FL & DLBCL) - RAG1 820R → ↑ risk of NHL</p>

	<p>NHEJ: -Ligase 9I var allele→↓ risk of NHL (FL & DLBCL) -WRN V114I variant→↓ risk of NHL -WRN Cys1367Arg→↓ risk of NHL MMR: - hMLH1, hPMS1→↓ risk for B-NHL and MCL blastoid type -MMR genes inactivation→ role in ATL disease progression</p>
HL	<p>BER:- XRCC1 Arg399Gln→↑ risk for HL -XRCC3 Thr241Met, XRCC1 Arg194Trp→↑ risk for HL - polymorphisms XRCC1/XRCC3& XRCC1/XPC→↑ risk for HL DSB: - SNPs of ATM with defective function</p>
CLL	<p>BER: -XRCC1 399Gln→ ↑in CLL with unfavorable prognosis NER: -XPD Lys751Gln→↑ in CLL with unfavorable prognosis -XPF Arg415Gln→ ↑ in CLL with unfavorable prognosis DSB: Inactivation of ATM HR:- Rad51 G135C→↑ in CLL with favorable prognosis -.↑ Rad51 foci in CLL exposed to chlorambucil→ resistance to chlorambucil ? -↑ HR activity in CLL cells exposed to alkylating agents→ resistance to alkylating agents NHEJ:- ↓ DNA-PKcs expression→ chemosensitivity -↑ DNA-PK activity→ resistance to chlorambucil -↑ DNA-PKcs expression in CLL patients with poor prognosis→ disease progression?</p>
MM	<p>DR: -MGMT ↓/hypermethylated in MM NER: -XPD K75Q→↑ prolonged TTR in MM patients with ASCT -ERCC1 rs735482→↑ RR and OS in relapsed/refractory MM HR: - Rad50 overexpression in MM patients - ↑ HR activity in MM cell lines -XRCC3 T24↑ 1M→↑ prolonged TTR in MM patients with ASCT NHEJ: -XRCC4 rs96248 Allele A→↑ risk of MM -Overexpression of XRCC4 -Ku80 rs1051685 GG→↑ risk for MM - Corrupt NHEJ in MM cell lines - Ku80 rs1051685→↑ RR and OS in relapsed/refractory MM</p>

DR= Direct Repair, BER= Base Excision Repair, NER= Nucleotide Excision Repair, MMR= MisMatch Repair, DSB= Double Strand Break Repair, HR= Homologous Recombination, NHEJ= Non Homologous End Joining, AML= Acute Myeloid Leukemia, ALL= Acute Lymphoblastic Leukemia, MDS= Myelodysplastic Syndromes, CML= Chronic Myeloid Leukemia, NHL= Non Hodgkin Lymphoma, FL= Follicular Lymphoma, DLBCL= Diffuse Large B- Cell Lymphoma, MCL= Mantle Cell Lymphoma, ATL= Adult T- cell Leukemia, HL= Hodgkin's Lymphoma, , CLL= Chronic Lymphocytic Leukemia, MM= Multiple Myeloma, MGUS= Monoclonal Gammopathy of Undetermined Significance, TTR= Time to Treatment Failure, ASCT= Autologous Stem Cell Transplantation, RD= Resistant Disease, BC= Blast Crisis, OS=Overall Survival

Table 1. Defects of DNA repair mechanisms in hematological malignancies

diseases, whereas few data is reported regarding the impact of Direct Repair and Mismatch Repair on these malignancies. The most commonly studied diseases are acute myeloid leukemia and Non Hodgkin's Lymphoma. Among all the data reviewed, none has clinical

application yet, therapeutic or prognostic. Further analysis of DNA repair pathways alterations is essential in order to elucidate their pathogenetic role in hematological malignancies and establish alternative therapeutic options that could be used in a clinical level, in order to help improve prognosis and survival of patients with hematological cancers.

5. Abbreviations

AGT/MGMT	O ⁶ -alkylguanine transferase
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
ATL	Adult T-cell Leukemia
ASCT	Autologous Stem Cell Transplantation
BC	Blast crisis in chronic myeloid leukemia
BER	Base Excision Repair
B-NHL	Non Hodgkin's Lymphoma of B-origin
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CP	Chronic Phase in chronic myeloid leukemia
CR	Complete Remission
CSR	Class Switch Recombination
dbSNP	Single Nucleotide Polymorphism Database
DDR	DNA Damage Response
DLBCL	Diffuse Large B-Cell Lymphoma
DR	Direct Repair
DSB	Double Strand Break
FL	Follicular Lymphoma
GG-NER	Global Genome Nucleotide Excision Repair
HL	Hodgkin's Lymphoma
HR	Homologous Recombination
IgV	Immunoglobulin Variable region
LP-BER	Long Patch Base Excision Repair
MCL	Mantle Cell Lymphoma
MDS	Myelodysplastic Syndromes
MGUS	Monoclonal Gammopathy of Undetermined Significance
MM	Multiple Myeloma
MMR	Mismatch Repair
MSI	Microsatellite Instability
MZL	Marginal Zone Lymphoma
NCBI	National Center for Biotechnology Information
NER	Nucleotide Excision Repair
NHEJ	Non Homologous End Joining
NHGRI	National Human Genome Research Institute
NHL	Non Hodgkin's Lymphoma
OR	Odd Ratio

OS	Overall Survival
PCL	Plasma-Cell Leukemia
RD	Resistant Disease
ROS	Reactive Oxygen Species
RR	Response Rate
SNP	Single Nucleotide Polymorphism
SP-BER	Short-Patch Base Excision Repair
TC-NER	Transcription Coupled Nucleotide Excision Repair
TTR	Time to Treatment Failure

6. References

- Aloyz R, Grzywacz K, Xu ZY, Loignon M, Alaoui-Jamali MA, Panasci L. 2004. Imatinib sensitizes CLL lymphocytes to chlorambucil. *Leukemia* 18: 409-414.
- Altieri F, Grillo C, Maceroni M, Chichiarelli S. 2008. DNA damage and repair: from molecular mechanisms to health implications. *Antioxid Redox Signal* 10: 891-937.
- Amrein L, Loignon M, Goulet AC, Dunn M, Jean-Claude B, Aloyz R, Panasci L. 2007. Chlorambucil cytotoxicity in malignant B lymphocytes is synergistically increased by 2-(morpholin-4-yl)-benzo[h]chomen-4-one (NU7026)-mediated inhibition of DNA double-strand break repair via inhibition of DNA-dependent protein kinase. *J Pharmacol Exp Ther* 321: 848-855.
- Amrein L, Davidson D, Shawi M, Petrucelli LA, Miller WH, Jr., Aloyz R, Panasci L. 2011. Dual inhibition of the homologous recombinational repair and the nonhomologous end-joining repair pathways in chronic lymphocytic leukemia therapy. *Leuk Res*.
- Austen B, Powell JE, Alvi A, Edwards I, Hooper L, Starczynski J, Taylor AM, Fegan C, Moss P, Stankovic T. 2005. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. *Blood* 106: 3175-3182.
- Bakkenist CJ, Kastan MB. 2004. Initiating cellular stress responses. *Cell* 118: 9-17.
- Batar B, Guven M, Baris S, Celkan T, Yildiz I. 2009. DNA repair gene XPD and XRCC1 polymorphisms and the risk of childhood acute lymphoblastic leukemia. *Leuk Res* 33: 759-763.
- Baumann Kreuziger LM, Steensma DP. 2008. RAD51 and XRCC3 polymorphism frequency and risk of myelodysplastic syndromes. *Am J Hematol* 83: 822-823.
- Bello VE, Aloyz RS, Christodouloupoulos G, Panasci LC. 2002. Homologous recombinational repair vis-a-vis chlorambucil resistance in chronic lymphocytic leukemia. *Biochem Pharmacol* 63: 1585-1588.
- Ben-Yehuda D, et al. 1996. Microsatellite instability and p53 mutations in therapy-related leukemia suggest mutator phenotype. *Blood* 88: 4296-4303.
- Bergsagel PL, Kuehl WM. 2005. Molecular pathogenesis and a consequent classification of multiple myeloma. *J Clin Oncol* 23: 6333-6338.
- Bianco T, Chenevix-Trench G, Walsh DC, Cooper JE, Dobrovic A. 2000. Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. *Carcinogenesis* 21: 147-151.
- Boyer JC, Risinger JI, Farber RA. 1998. Stability of microsatellites in myeloid neoplasias. *Cancer Genet Cytogenet* 106: 54-61.

- Calasanz MJ, Cigudosa JC, Otero MD, Ferreira C, Ardanaz MT, Fraile A, Carrasco JL, Sole F, Cuesta B, Gullon A. 1997. Cytogenetic analysis of 280 patients with multiple myeloma and related disorders: primary breakpoints and clinical correlations. *Genes Chromosomes Cancer* 18: 84-93.
- Champlin RE, Golde DW. 1985. Chronic myelogenous leukemia: recent advances. *Blood* 65: 1039-1047.
- Choudhury A, Elliott F, Iles MM, Churchman M, Bristow RG, Bishop DT, Kiltie AE. 2008. Analysis of variants in DNA damage signalling genes in bladder cancer. *BMC Med Genet* 9: 69.
- Christodouloupoulos G, Malapetsa A, Schipper H, Golub E, Radding C, Panasci LC. 1999. Chlorambucil induction of HsRad51 in B-cell chronic lymphocytic leukemia. *Clin Cancer Res* 5: 2178-2184.
- Cibeira MT, Fernandez de Larrea C, Navarro A, Diaz T, Fuster D, Tovar N, Rosinol L, Monzo M, Blade J. 2011. Impact on response and survival of DNA repair single nucleotide polymorphisms in relapsed or refractory multiple myeloma patients treated with thalidomide. *Leuk Res*.
- Cline SD, Hanawalt PC. 2003. Who's on first in the cellular response to DNA damage? *Nat Rev Mol Cell Biol* 4: 361-372.
- da Silva Silveira V, Canalle R, Scrideli CA, Queiroz RG, Bettiol H, Valera ET, Tone LG. 2009. Polymorphisms of xenobiotic metabolizing enzymes and DNA repair genes and outcome in childhood acute lymphoblastic leukemia. *Leuk Res* 33: 898-901.
- de Laat WL, Jaspers NG, Hoeijmakers JH. 1999. Molecular mechanism of nucleotide excision repair. *Genes Dev* 13: 768-785.
- den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 15: 7-12.
- . 2003. Mutation nomenclature. *Curr Protoc Hum Genet* Chapter 7: Unit 7 13.
- Deutsch E, et al. 2001. BCR-ABL down-regulates the DNA repair protein DNA-PKcs. *Blood* 97: 2084-2090.
- Doll DC, List AF. 1989. Myelodysplastic syndromes. *West J Med* 151: 161-167.
- Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. 1996. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 2: 561-566.
- Economopoulou P, et al. 2009. Expression analysis of proteins involved in the non homologous end joining DNA repair mechanism, in the bone marrow of adult de novo myelodysplastic syndromes. *Ann Hematol*.
- El-Zein R, Monroy CM, Etzel CJ, Cortes AC, Xing Y, Collier AL, Strom SS. 2009. Genetic polymorphisms in DNA repair genes as modulators of Hodgkin disease risk. *Cancer* 115: 1651-1659.
- Elliott SL, et al. 2011. Mitoxantrone in combination with an inhibitor of DNA-dependent protein kinase: a potential therapy for high risk B-cell chronic lymphocytic leukaemia. *Br J Haematol* 152: 61-71.
- Enjuanes A, et al. 2008. Genetic variants in apoptosis and immunoregulation-related genes are associated with risk of chronic lymphocytic leukemia. *Cancer Res* 68: 10178-10186.
- Esteller M, Corn PG, Baylin SB, Herman JG. 2001. A gene hypermethylation profile of human cancer. *Cancer Res* 61: 3225-3229.

- Fabiani E, et al. 2009. Polymorphisms of detoxification and DNA repair enzymes in myelodysplastic syndromes. *Leuk Res* 33: 1068-1071.
- Faderl S, Kantarjian HM, Talpaz M, Estrov Z. 1998. Clinical significance of cytogenetic abnormalities in adult acute lymphoblastic leukemia. *Blood* 91: 3995-4019.
- Fang NY, et al. 2003. Oligonucleotide microarrays demonstrate the highest frequency of ATM mutations in the mantle cell subtype of lymphoma. *Proc Natl Acad Sci U S A* 100: 5372-5377.
- Fenton JA, Pratt G, Rawstron AC, Morgan GJ. 2002. Isotype class switching and the pathogenesis of multiple myeloma. *Hematol Oncol* 20: 75-85.
- Fonseca R, et al. 2004. Genetics and cytogenetics of multiple myeloma: a workshop report. *Cancer Res* 64: 1546-1558.
- Fortini P, Dogliotti E. 2007. Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. *DNA Repair (Amst)* 6: 398-409.
- Fortini P, Parlanti E, Sidorkina OM, Laval J, Dogliotti E. 1999. The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. *J Biol Chem* 274: 15230-15236.
- Friedenson B. 2007. The BRCA1/2 pathway prevents hematologic cancers in addition to breast and ovarian cancers. *BMC Cancer* 7: 152.
- Ganster C, Neesen J, Zehetmayer S, Jager U, Esterbauer H, Mannhalter C, Kluge B, Fonatsch C. 2009. DNA repair polymorphisms associated with cytogenetic subgroups in B-cell chronic lymphocytic leukemia. *Genes Chromosomes Cancer* 48: 760-767.
- Gillert E, et al. 1999. A DNA damage repair mechanism is involved in the origin of chromosomal translocations t(4;11) in primary leukemic cells. *Oncogene* 18: 4663-4671.
- Gillet LC, Scharer OD. 2006. Molecular mechanisms of mammalian global genome nucleotide excision repair. *Chem Rev* 106: 253-276.
- Gu L, Cline-Brown B, Zhang F, Qiu L, Li GM. 2002. Mismatch repair deficiency in hematological malignancies with microsatellite instability. *Oncogene* 21: 5758-5764.
- Hakem R. 2008. DNA-damage repair; the good, the bad, and the ugly. *Embo J* 27: 589-605.
- Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. 1999. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 94: 1848-1854.
- Hangaishi A, Ogawa S, Mitani K, Hosoya N, Chiba S, Yazaki Y, Hirai H. 1997. Mutations and loss of expression of a mismatch repair gene, hMLH1, in leukemia and lymphoma cell lines. *Blood* 89: 1740-1747.
- Hayden PJ, et al. 2007. Variation in DNA repair genes XRCC3, XRCC4, XRCC5 and susceptibility to myeloma. *Hum Mol Genet* 16: 3117-3127.
- Helleday T, Lo J, van Gent DC, Engelward BP. 2007. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)* 6: 923-935.
- Hill DA, et al. 2006. Risk of non-Hodgkin lymphoma (NHL) in relation to germline variation in DNA repair and related genes. *Blood* 108: 3161-3167.
- Ho PJ, Brown RD, Pelka GJ, Basten A, Gibson J, Joshua DE. 2001. Illegitimate switch recombinations are present in approximately half of primary myeloma tumors, but do not relate to known prognostic indicators or survival. *Blood* 97: 490-495.

- Hwang BJ, Ford JM, Hanawalt PC, Chu G. 1999. Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. *Proc Natl Acad Sci U S A* 96: 424-428.
- Infante-Rivard C, Mathonnet G, Sinnett D. 2000. Risk of childhood leukemia associated with diagnostic irradiation and polymorphisms in DNA repair genes. *Environ Health Perspect* 108: 495-498.
- Inokuchi K, Ikejima M, Watanabe A, Nakajima E, Orimo H, Nomura T, Shimada T. 1995. Loss of expression of the human MSH3 gene in hematological malignancies. *Biochem Biophys Res Commun* 214: 171-179.
- Janssen JW, et al. 1989. Clonal analysis of myelodysplastic syndromes: evidence of multipotent stem cell origin. *Blood* 73: 248-254.
- Jiang L, Liang J, Jiang M, Yu X, Zheng J, Liu H, Wu D, Zhou Y. 2011. Functional polymorphisms in the NBS1 gene and acute lymphoblastic leukemia susceptibility in a Chinese population. *Eur J Haematol* 86: 199-205.
- Jiricny J. 1998. Replication errors: cha(lle)nging the genome. *Embo J* 17: 6427-6436.
- . 2006. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* 7: 335-346.
- Johnson N, et al. 2007. Counting potentially functional variants in BRCA1, BRCA2 and ATM predicts breast cancer susceptibility. *Hum Mol Genet* 16: 1051-1057.
- Joseph T, Kusumakumary P, Chacko P, Abraham A, Pillai MR. 2005. DNA repair gene XRCC1 polymorphisms in childhood acute lymphoblastic leukemia. *Cancer Lett* 217: 17-24.
- Kanaar R, Wyman C, Rothstein R. 2008. Quality control of DNA break metabolism: in the 'end', it's a good thing. *Embo J* 27: 581-588.
- Kaneko H, Horiike S, Inazawa J, Nakai H, Misawa S. 1995. Microsatellite instability is an early genetic event in myelodysplastic syndrome. *Blood* 86: 1236-1237.
- Karagiannis TC, El-Osta A. 2004. Double-strand breaks: signaling pathways and repair mechanisms. *Cell Mol Life Sci* 61: 2137-2147.
- Kim JE, Minter-Dykhouse K, Chen J. 2006. Signaling networks controlled by the MRN complex and MDC1 during early DNA damage responses. *Mol Carcinog* 45: 403-408.
- Kotoula V, Hytiroglou P, Kaloutsi V, Barbanis S, Kouidou S, Papadimitriou CS. 2002. Mismatch repair gene expression in malignant lymphoproliferative disorders of B-cell origin. *Leuk Lymphoma* 43: 393-399.
- Kuptsova N, Kopecky KJ, Godwin J, Anderson J, Hoque A, Willman CL, Slovak ML, Ambrosone CB. 2007. Polymorphisms in DNA repair genes and therapeutic outcomes of AML patients from SWOG clinical trials. *Blood* 109: 3936-3944.
- Kyle RA, Rajkumar SV. 2004. Multiple myeloma. *N Engl J Med* 351: 1860-1873.
- Li L, Yang L, Zhang Y, Xu Z, Qin T, Hao Y, Xiao Z. 2010. Detoxification and DNA repair genes polymorphisms and susceptibility of primary myelodysplastic syndromes in Chinese population. *Leuk Res*.
- Liberzon E, Avigad S, Yaniv I, Stark B, Avrahami G, Goshen Y, Zaizov R. 2004. Molecular variants of the ATM gene in Hodgkin's disease in children. *Br J Cancer* 90: 522-525.
- Lieberman HB. 2008. DNA damage repair and response proteins as targets for cancer therapy. *Curr Med Chem* 15: 360-367.
- Liebisch P, Dohner H. 2006. Cytogenetics and molecular cytogenetics in multiple myeloma. *Eur J Cancer* 42: 1520-1529.

- Liu A, Takakuwa T, Fujita S, Luo WJ, Tresnasari K, Van den Berg A, Poppema S, Aozasa K. 2008. ATR alterations in Hodgkin's lymphoma. *Oncol Rep* 19: 999-1005.
- Liu J, Song B, Wang Z, Song X, Shi Y, Zheng J, Han J. 2009. DNA repair gene XRCC1 polymorphisms and non-Hodgkin lymphoma risk in a Chinese population. *Cancer Genet Cytogenet* 191: 67-72.
- Liu L, Yang L, Mi Y, Wang J, Li J, Zhang Y, Ma X, Qin T, Xu Z, Xiao Z. 2011. RAD51 and XRCC3 polymorphisms: Impact on the risk and treatment outcomes of de novo inv(16) or t(16;16)/CBFBeta-MYH11(+) acute myeloid leukemia. *Leuk Res*.
- Lockett KL, Snowwhite IV, Hu JJ. 2005. Nucleotide-excision repair and prostate cancer risk. *Cancer Lett* 220: 125-135.
- M'Kacher R, et al. 2007. Telomere shortening and associated chromosomal instability in peripheral blood lymphocytes of patients with Hodgkin's lymphoma prior to any treatment are predictive of second cancers. *Int J Radiat Oncol Biol Phys* 68: 465-471.
- Madhusudan S, Middleton MR. 2005. The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. *Cancer Treat Rev* 31: 603-617.
- Mao G, Yuan F, Absher K, Jennings CD, Howard DS, Jordan CT, Gu L. 2008. Preferential loss of mismatch repair function in refractory and relapsed acute myeloid leukemia: potential contribution to AML progression. *Cell Res* 18: 281-289.
- Martin P, Santon A, Garcia-Cosio M, Bellas C. 2006. hMLH1 and MGMT inactivation as a mechanism of tumorigenesis in monoclonal gammopathies. *Mod Pathol* 19: 914-921.
- Mathonnet G, Krajcinovic M, Labuda D, Sinnett D. 2003. Role of DNA mismatch repair genetic polymorphisms in the risk of childhood acute lymphoblastic leukaemia. *Br J Haematol* 123: 45-48.
- Meza-Espinoza JP, Peralta-Leal V, Gutierrez-Angulo M, Macias-Gomez N, Ayala-Madrigal ML, Barros-Nunez P, Duran-Gonzalez J, Leal-Ugarte E. 2009. XRCC1 polymorphisms and haplotypes in Mexican patients with acute lymphoblastic leukemia. *Genet Mol Res* 8: 1451-1458.
- Molenaar JJ, Gerard B, Chambon-Pautas C, Cave H, Duval M, Vilmer E, Grandchamp B. 1998. Microsatellite instability and frameshift mutations in BAX and transforming growth factor-beta RII genes are very uncommon in acute lymphoblastic leukemia in vivo but not in cell lines. *Blood* 92: 230-233.
- Monroy CM, Cortes AC, Lopez M, Rourke E, Etzel CJ, Younes A, Strom SS, El-Zein R. 2011. Hodgkin lymphoma risk: Role of genetic polymorphisms and gene-gene interactions in DNA repair pathways. *Mol Carcinog*.
- Monzo M, et al. 2006. Genomic polymorphisms provide prognostic information in intermediate-risk acute myeloblastic leukemia. *Blood* 107: 4871-4879.
- Morimoto H, Tsukada J, Kominato Y, Tanaka Y. 2005. Reduced expression of human mismatch repair genes in adult T-cell leukemia. *Am J Hematol* 78: 100-107.
- Noel P, Solberg LA, Jr. 1992. Myelodysplastic syndromes. Pathogenesis, diagnosis and treatment. *Crit Rev Oncol Hematol* 12: 193-215.
- Nowicki MO, Falinski R, Koptyra M, Slupianek A, Stoklosa T, Gloc E, Nieborowska-Skorska M, Blasiak J, Skorski T. 2004. BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. *Blood* 104: 3746-3753.

- Oehler VG, et al. 2007. The effects of imatinib mesylate treatment before allogeneic transplantation for chronic myeloid leukemia. *Blood* 109: 1782-1789.
- Ogino S, Gulley ML, den Dunnen JT, Wilson RB. 2007. Standard mutation nomenclature in molecular diagnostics: practical and educational challenges. *J Mol Diagn* 9: 1-6.
- Olive PL. 1998. The role of DNA single- and double-strand breaks in cell killing by ionizing radiation. *Radiat Res* 150: S42-51.
- Pakakasama S, Sirirat T, Kanchanachumpol S, Udomsubpayakul U, Mahasirimongkol S, Kitpoka P, Thithapandha A, Hongeng S. 2007. Genetic polymorphisms and haplotypes of DNA repair genes in childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer* 48: 16-20.
- Palitti F. 2004. Mechanisms of formation of chromosomal aberrations: insights from studies with DNA repair-deficient cells. *Cytogenet Genome Res* 104: 95-99.
- Park CJ, Choi BS. 2006. The protein shuffle. Sequential interactions among components of the human nucleotide excision repair pathway. *Febs J* 273: 1600-1608.
- Peng B, Hodge DR, Thomas SB, Cherry JM, Munroe DJ, Pompeia C, Xiao W, Farrar WL. 2005. Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. *J Biol Chem* 280: 4182-4187.
- Rajkumar S, Fonseca R, Lacy M, Witzig T, Lust J, Greipp P, Therneau T, Kyle R, Litzow M, Gertz M. 1999. Abnormal cytogenetics predict poor survival after high-dose therapy and autologous blood cell transplantation in multiple myeloma. *Bone Marrow Transplant* 24: 497-503.
- Roddam PL, Rollinson S, O'Driscoll M, Jeggo PA, Jack A, Morgan GJ. 2002. Genetic variants of NHEJ DNA ligase IV can affect the risk of developing multiple myeloma, a tumour characterised by aberrant class switch recombination. *J Med Genet* 39: 900-905.
- Roddam PL, Allan JM, Dring AM, Worrillow LJ, Davies FE, Morgan GJ. 2010. Non-homologous end-joining gene profiling reveals distinct expression patterns associated with lymphoma and multiple myeloma. *Br J Haematol* 149: 258-262.
- Rollinson S, Kesby H, Morgan GJ. 2006. Haplotypic variation in MRE11, RAD50 and NBS1 and risk of non-Hodgkin's lymphoma. *Leuk Lymphoma* 47: 2567-2583.
- Rollinson S, Smith AG, Allan JM, Adamson PJ, Scott K, Skibola CF, Smith MT, Morgan GJ. 2007. RAD51 homologous recombination repair gene haplotypes and risk of acute myeloid leukaemia. *Leuk Res* 31: 169-174.
- Rowley JD. 1999. The role of chromosome translocations in leukemogenesis. *Semin Hematol* 36: 59-72.
- Salles D, Mencalha AL, Ireno IC, Wiesmuller L, Abdelhay E. 2011. BCR-ABL stimulates mutagenic homologous DNA double-strand break repair via the DNA-end-processing factor CtIP. *Carcinogenesis* 32: 27-34.
- Sampath D, Plunkett W. 2007. The role of DNA repair in chronic lymphocytic leukemia pathogenesis and chemotherapy resistance. *Curr Oncol Rep* 9: 361-367.
- Sancar A. 1996. DNA excision repair. *Annu Rev Biochem* 65: 43-81.
- Scardocci A, et al. 2006. Reduced BRCA1 expression due to promoter hypermethylation in therapy-related acute myeloid leukaemia. *Br J Cancer* 95: 1108-1113.

- Schaffner C, Idler I, Stilgenbauer S, Dohner H, Lichter P. 2000. Mantle cell lymphoma is characterized by inactivation of the ATM gene. *Proc Natl Acad Sci U S A* 97: 2773-2778.
- Scharer OD. 2003. Chemistry and biology of DNA repair. *Angew Chem Int Ed Engl* 42: 2946-2974.
- Schuetz JM, MaCarthur AC, Leach S, Lai AS, Gallagher RP, Connors JM, Gascoyne RD, Spinelli JJ, Brooks-Wilson AR. 2009. Genetic variation in the NBS1, MRE11, RAD50 and BLM genes and susceptibility to non-Hodgkin lymphoma. *BMC Med Genet* 10: 117.
- Schwartz CL, Cohen HJ. 1988. Preleukemic syndromes and other syndromes predisposing to leukemia. *Pediatr Clin North Am* 35: 853-871.
- Seedhouse C, Faulkner R, Ashraf N, Das-Gupta E, Russell N. 2004. Polymorphisms in genes involved in homologous recombination repair interact to increase the risk of developing acute myeloid leukemia. *Clin Cancer Res* 10: 2675-2680.
- Sellick GS, Wade R, Richards S, Oscier DG, Catovsky D, Houlston RS. 2008. Scan of 977 nonsynonymous SNPs in CLL4 trial patients for the identification of genetic variants influencing prognosis. *Blood* 111: 1625-1633.
- Seviour EG, Lin SY. 2010. The DNA damage response: Balancing the scale between cancer and ageing. *Ageing (Albany NY)* 2: 900-907.
- Shammas MA, Shmookler Reis RJ, Koley H, Batchu RB, Li C, Munshi NC. 2009. Dysfunctional homologous recombination mediates genomic instability and progression in myeloma. *Blood* 113: 2290-2297.
- Shen M, et al. 2007. Polymorphisms in DNA repair genes and risk of non-Hodgkin's lymphoma in New South Wales, Australia. *Haematologica* 92: 1180-1185.
- . 2006. Polymorphisms in DNA repair genes and risk of non-Hodgkin lymphoma among women in Connecticut. *Hum Genet* 119: 659-668.
- . 2010. Polymorphisms in DNA repair genes and risk of non-Hodgkin lymphoma in a pooled analysis of three studies. *Br J Haematol* 151: 239-244.
- Shi JY, Ren ZH, Jiao B, Xiao R, Yun HY, Chen B, Zhao WL, Zhu Q, Chen Z, Chen SJ. 2011. Genetic variations of DNA repair genes and their prognostic significance in patients with acute myeloid leukemia. *Int J Cancer* 128: 233-238.
- Sill H, Goldman JM, Cross NC. 1996. Rarity of microsatellite alterations in acute myeloid leukaemia. *Br J Cancer* 74: 255-257.
- Sipahimalani P, et al. 2007. A systematic evaluation of the ataxia telangiectasia mutated gene does not show an association with non-Hodgkin lymphoma. *Int J Cancer* 121: 1967-1975.
- Slupianek A, Poplawski T, Jozwiakowski SK, Cramer K, Pytel D, Stoczynska E, Nowicki MO, Blasiak J, Skorski T. 2011. BCR/ABL stimulates WRN to promote survival and genomic instability. *Cancer Res* 71: 842-851.
- Smadja NV, Bastard C, Brigaudeau C, Leroux D, Fruchart C. 2001. Hypodiploidy is a major prognostic factor in multiple myeloma. *Blood* 98: 2229-2238.
- Smedby KE, et al. 2006. Variation in DNA repair genes ERCC2, XRCC1, and XRCC3 and risk of follicular lymphoma. *Cancer Epidemiol Biomarkers Prev* 15: 258-265.
- Stanczyk M, Sliwinski T, Cuchra M, Zubowska M, Bielecka-Kowalska A, Kowalski M, Szmraj J, Mlynarski W, Majsterek I. 2011. The association of polymorphisms in

- DNA base excision repair genes XRCC1, OGG1 and MUTYH with the risk of childhood acute lymphoblastic leukemia. *Mol Biol Rep* 38: 445-451.
- Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS, Cooper DN. 2009. The Human Gene Mutation Database: 2008 update. *Genome Med* 1: 13.
- Strom SS, Estey E, Ouitschoorn UM, Garcia-Manero G. 2010. Acute myeloid leukemia outcome: role of nucleotide excision repair polymorphisms in intermediate risk patients. *Leuk Lymphoma* 51: 598-605.
- Tai YT, Podar K, Kraeft SK, Wang F, Young G, Lin B, Gupta D, Chen LB, Anderson KC. 2002. Translocation of Ku86/Ku70 to the multiple myeloma cell membrane: functional implications. *Exp Hematol* 30: 212-220.
- Takagi M, et al. 2004. Identification and characterization of polymorphic variations of the ataxia telangiectasia mutated (ATM) gene in childhood Hodgkin disease. *Blood* 103: 283-290.
- Takeda N, Shibuya M, Maru Y. 1999. The BCR-ABL oncoprotein potentially interacts with the xeroderma pigmentosum group B protein. *Proc Natl Acad Sci U S A* 96: 203-207.
- Takeuchi S, et al. 1997. Microsatellite instability and other molecular abnormalities in childhood acute lymphoblastic leukaemia. *Br J Haematol* 98: 134-139.
- Tasak T, Lee S, Spira S, Takeuchi S, Hatta Y, Nagai M, Takahara J, Koeffler HP. 1996. Infrequent microsatellite instability during the evolution of myelodysplastic syndrome to acute myelocytic leukemia. *Leuk Res* 20: 113-117.
- Taylor AM, Metcalfe JA, Thick J, Mak YF. 1996. Leukemia and lymphoma in ataxia telangiectasia. *Blood* 87: 423-438.
- Timuragaoglu A, Demircin S, Dizlek S, Alanoglu G, Kiris E. 2009. Microsatellite instability is a common finding in multiple myeloma. *Clin Lymphoma Myeloma* 9: 371-374.
- Tumer TB, Yilmaz D, Tanrikut C, Sahin G, Ulusoy G, Arinc E. 2010. DNA repair XRCC1 Arg399Gln polymorphism alone, and in combination with CYP2E1 polymorphisms significantly contribute to the risk of development of childhood acute lymphoblastic leukemia. *Leuk Res* 34: 1275-1281.
- Vangsted A, Gimsing P, Klausen TW, Nexø BA, Wallin H, Andersen P, Hokland P, Lillevang ST, Vogel U. 2007. Polymorphisms in the genes ERCC2, XRCC3 and CD3EAP influence treatment outcome in multiple myeloma patients undergoing autologous bone marrow transplantation. *Int J Cancer* 120: 1036-1045.
- Velangi MR, Matheson EC, Morgan GJ, Jackson GH, Taylor PR, Hall AG, Irving JA. 2004. DNA mismatch repair pathway defects in the pathogenesis and evolution of myeloma. *Carcinogenesis* 25: 1795-1803.
- Vogel U, Olsen A, Wallin H, Overvad K, Tjønneland A, Nexø BA. 2005. Effect of polymorphisms in XPD, RAI, ASE-1 and ERCC1 on the risk of basal cell carcinoma among Caucasians after age 50. *Cancer Detect Prev* 29: 209-214.
- Vorechovsky I, Luo L, Dyer MJ, Catovsky D, Amlot PL, Yaxley JC, Foroni L, Hammarstrom L, Webster AD, Yuille MA. 1997. Clustering of missense mutations in the ataxia-telangiectasia gene in a sporadic T-cell leukaemia. *Nat Genet* 17: 96-99.
- Voso MT, Fabiani E, D'Alo F, Guidi F, Di Ruscio A, Sica S, Pagano L, Greco M, Hohaus S, Leone G. 2007. Increased risk of acute myeloid leukaemia due to polymorphisms in detoxification and DNA repair enzymes. *Ann Oncol* 18: 1523-1528.

- Wagner JE, et al. 2004. Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. *Blood* 103: 3226-3229.
- Wang SL, Zhao H, Zhou B, Chen YL, Zou Y, Zhu XF, Li QS, Han MZ, Yang RC, Han ZC. 2006. Polymorphisms in ERCC1 and susceptibility to childhood acute lymphoblastic leukemia in a Chinese population. *Leuk Res* 30: 1341-1345.
- Wheeler DL, et al. 2007. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 35: D5-12.
- Wiemels JL, Alexander FE, Cazzaniga G, Biondi A, Mayer SP, Greaves M. 2000. Microclustering of TEL-AML1 translocation breakpoints in childhood acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 29: 219-228.
- Wiemels JL, et al. 2002. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood* 99: 3801-3805.
- Willmore E, et al. 2008. DNA-dependent protein kinase is a therapeutic target and an indicator of poor prognosis in B-cell chronic lymphocytic leukemia. *Clin Cancer Res* 14: 3984-3992.
- Wood RD. 1997. Nucleotide excision repair in mammalian cells. *J Biol Chem* 272: 23465-23468.
- Worrillow L, Roman E, Adamson PJ, Kane E, Allan JM, Lightfoot TJ. 2009. Polymorphisms in the nucleotide excision repair gene ERCC2/XPD and risk of non-Hodgkin lymphoma. *Cancer Epidemiol* 33: 257-260.
- Xu ZY, Loignon M, Han FY, Panasci L, Aloyz R. 2005. Xrcc3 induces cisplatin resistance by stimulation of Rad51-related recombinational repair, S-phase checkpoint activation, and reduced apoptosis. *J Pharmacol Exp Ther* 314: 495-505.
- Yang C, Betti C, Singh S, Toor A, Vaughan A. 2009. Impaired NHEJ function in multiple myeloma. *Mutat Res* 660: 66-73.
- Yoshida H, Naoe T, Fukutani H, Kiyoi H, Kubo K, Ohno R. 1995. Analysis of the joining sequences of the t(15;17) translocation in human acute promyelocytic leukemia: sequence non-specific recombination between the PML and RARA genes within identical short stretches. *Genes Chromosomes Cancer* 12: 37-44.
- Zhu R, Lu FJ, Zhang ZB, Zhai XW, Liu J, Lu G, Wu Y, Chen C, Xia Z. 2005. [Association of genetic polymorphism of XRCC1 with susceptibility to acute childhood leukemia]. *Wei Sheng Yan Jiu* 34: 300-302.

DNA Repair in Acute Myeloid Leukemia and Myelodysplastic Syndromes

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1. Introduction

Acute myeloid leukemia (AML) comprises approximately 25% of all leukemias in adults in the western world. It is a clonal disorder caused by uncontrolled proliferation and accumulation of myeloid progenitor cells in the bone marrow with impaired differentiation, leading ultimately to hematopoietic failure.

Myelodysplastic syndrome (MDS) is characterized by persistent pancytopenia, dysplastic hematopoiesis in bone marrow, increase in blast cell number and high risk of progression to AML. MDS is a disease of elderly, which makes treatment difficult (Estey 2008). The median age of AML patients is also high and is estimated to be 66 to 70 years. Standard therapeutic strategies in MDS and AML depend on various factors, of which age, comorbidities and performance status are most important. Treatment modalities vary from the best supportive care through low dose chemotherapy to intensive dose chemotherapy and allogeneic bone marrow transplantation (Robak T, Wierzbowska A 2009). Untreated MDS and AML carry extremely poor prognosis with high mortality. The search for new drugs in AML and MDS is stimulated by the significant progress in the understanding of the biology of both diseases.

Abnormal myeloid cells usually carry chromosomal anomalies, including translocations, deletions, and allelic loss. Typical cytogenetic changes seen in AML are balanced translocations such as t(8,21), t(15,17) which result in formation of a fusion gene. MDS is characterized by deletions of fragments or whole chromosomes hence loss of genetic information. DNA methylation dysregulation is one of the postulated mechanisms of leukemia development and progression. Hypermethylation of DNA generally results in a decreased expression of tumor suppressor genes and defective cell cycle control and is a hallmark of MDS and AML. Epigenetic changes augment genetic alterations occurring in cancer cells and promote tumor progression. Several sequential events in the genome are required to create a leukemic clone. Defects of DNA repair are the key mechanism of development and progression of myeloid leukemias. Most of the AML cases originate de novo, but around 10 to 20% of patients have previous exposure to myelotoxic substances. Preceding anticancer treatment or exposure to chemical toxins may result in severe damage to DNA and in case of defective DNA repair mechanisms lead to secondary AML. DNA repair mechanisms may influence not only the risk of leukemia development, but also its refractoriness to treatment.

Several major pathways of DNA repair exist: Homologous Recombination (HR), Non Homologous End Joining (NHEJ), Base Excision Repair (BER), Nucleotide Excision Repair

(NER), Mismatch Repair (MMR), Translesion DNA synthesis (TLS) (D'Andrea 2010, Shrivastav, de Haro et al 2008). HR and NHEJ are responsible for repair of DNA double strand breaks (DSB), caused mainly by ionizing radiation, free radicals, and chemical toxins including cytostatics. DSBs comprise DNA lesions most detrimental for cell survival. The other DNA repair mechanisms deal with single strand breaks and the presence of improper base or alkyl adducts in the DNA. A complementary strand is used as the repair template. Different DNA repair processes overlap in their function and usually one problem can be repaired in 2 different ways. In normal cells all of DNA repair pathways are active and balanced. In the case of irreversible DNA damage, cells are directed to apoptosis. When the damage is moderate and repair processes inadequate the cells accumulate dangerous mutations and genomic instability occurs. This is the first step of neoplastic transformation. The defective function of one of the DNA repair pathways often results in overexpression of the other one. Increased processes of DNA repair may lead to resistance to cytostatics and radiotherapy (Pallis, Karamouzis 2010). The role of DNA damage and repair processes in pathogenesis and treatment of cancer was first noted in patients with inherited syndromes with defective DNA repair mechanisms such as Fanconi anemia. Much information comes also from neoplastic disorders induced by factors known to damage DNA, such as ionizing radiation or cytostatic therapy.

2. Secondary AML and MDS

Secondary AML and MDS are most common therapy related neoplasms. Two main types of treatment-related AML exist depending on type of cytostatics administered. Secondary leukemia due to topoisomerase II inhibitors such as podophilotoxins and anthracycline antibiotics, used in variety of solid tumors, occurs usually after short period of time (1 year) following chemotherapy. Characteristic features include chromosome 11q23 anomalies, total or partial deletion of chromosome 7 and certain balanced translocations such as t(8,21) or t(15,17). The mechanism of development of that type of treatment related AML is not clearly understood, but defects in DSB repair are thought to be a key mechanism. Anthracycline antibiotics intercalate into DNA and stabilize DNA-topoisomerase II complex and promote DSB formation. The cytostatics induce DSB at sites concerning hematopoietic transcription factors such as MLL, AML1/CBFB, RARA and additionally decrease rejoining of generated DSB. HR and NHEJ proper function is therefore crucial for restoring genome integrity. Otherwise, accumulating mutations lead to malignant transformation, thus patients with impaired DNA repair may be predisposed to chemotherapy induced leukemia (Guillem, Tormo 2008).

The other type of secondary leukemia is concerned with the previous use of alkylating agents. AML is diagnosed usually 5-7 years after chemotherapy and often follows MDS phase. Cytogenetic events common in this type of secondary leukemia are total or partial deletion of chromosome 5 and 7. Alkylating agents produce damage to the DNA forming monoadducts and diadducts. The process may result in interstrand and intrastrand cross links, producing single and double strand breaks. The damage caused by alkylating agents activates various DNA repair pathways. Monoadducts are usually repaired by NER and BER. Diadducts are managed by NER and HR. MMR by its influence on HR also takes part in repair processes induced in response to alkylator treatment. Cells with defective MMR function show an increased expression of RAD51, one of components of HR, and an increased microsatellite instability (Worilow, Allan et al. 2006).

3. HR defects in AML and MDS

HR, together with NHEJ, is responsible for repair of DSB, the most important DNA lesion. DSB are produced naturally, especially during a normal programmed genom rearrangement and after the exposure to DNA toxic agents. HR requires homologous sequence to that of the broken end to start the repair process and in human cells deals mainly with DSB located within the replication forks. HR is more accurate than NHEJ.

Impaired HR is a hallmark of Fanconi anemia (FA), a rare inherited disorder. Different 13 FA proteins work together in HR DNA repair pathway and a defect of 1 of those proteins results in similar clinical phenotype: short stature, skeletal defects, bone marrow failure and hypersensitivity to DNA damaging agents such as mitomycin C. The patients are at high risk of developing AML and solid tumors, mainly gynecological. Diagnostic test for FA is based on detection of defective DNA repair: examination of chromosome breakage after exposure to mitomycin C or diepoxybutane. Chromosomal aberrations typical for FA patients with AML are also found in de novo AML, thus the knowledge based on this genetic disorder help us to understand the biology of AML. DNA repair defects typical for Fanconi anemia put those patients at high risk of AML and solid tumor development, especially breast, ovarian and pancreatic cancer. Defects in HR activity similar to those detected in AML patients with Fanconi anemia, were seen in AML secondary to previous anticancer treatment. Their occurrence in patients with de novo AML is rare.

Single nucleotide G/C polymorphism in position 135 in gene encoding main protein active in HR pathway, RAD51, is correlated with AML predisposition (Seedhouse, Foulkner 2004). A polymorphism at codon 241 of another HR gene, XRCC3, results in Thr to Met substitution. Both polymorphisms were known previously to increase solid cancer susceptibility. The presence of both RAD51-135 C and XRCC3-241 Met protein variants increased the risk of secondary and primary AML development, 8 and 4 fold respectively.

Observations made in patients with solid tumors with defective HR suggest increased sensitivity of cancer cells to cis-platin and to DNA repair inhibitors such as PARP or ATM inhibitors. So far such strategies are still at preclinical phase.

4. NHEJ defects in AML and MDS

NHEJ, together with HR, constitutes main pathways to repair DNA double strand breaks. NHEJ rejoins broken fragments with little requirement for homology and is extremely important in avoiding radiation toxicity. Translocation and mutations at the junction of the broken ends happen, that is why NHEJ is responsible for tumorigenic processes. Proper and balanced functions of NHEJ and HR are necessary to maintain genom integrity.

Chromosomal instability in myeloid neoplasms results from deregulated NHEJ and inadequate DSB repair. The rate of NHEJ in leukemic blasts was 2-7 fold higher than in normal hematopoietic cells *in vitro* and resulted in increased misrepair, especially large deletions (Gaymes, Mufti et al. 2002).

The number of double strand breaks is increased by the excessive reactive oxygen species (ROS) production. Certain genetic changes such as FMS-like tyrosine kinase 3 (FLT3) mutation in AML and RAS mutations in MDS are responsible for induction of ROS generation and lead to genomic instability. FLT3 mutations occur in half of the patients with AML. Activating mutation is seen in 30-35% of cases while internal tandem duplication (FLT3/ITD) in 20-25% of AML cases. The presence of FLT3/ITD carries extremely bad

prognosis in AML and is correlated with increased ROS generation and increased double strand breaks in DNA (Sallmyr, Fan et al. 2008). In FLT3/ITD-containing cells NHEJ is defective, which results in accumulating aberrant DNA structures (Seedhouse, Hunter et al. 2006). Thus the cells depend on another DNA repair pathway. Upregulation of RAD51, the main component of HR pathway, was noted in patients with FLT3/ITD, defective in NHEJ, and could be partially responsible for resistance to chemotherapy (Seedhouse, Hunter et al 2006).

Increased ROS generation and accumulation of double strand breaks are seen also in patients with N- RAS mutation in MDS and AML. In addition to enhanced survival and proliferation of the cells, N-RAS mutation results in ineffective NHEJ leading to DNA instability (Rassool, Gaymes et al. 2007).

5. BER in AML and MDS

BER pathway is concerned with the removal of the base changed by alkylation, oxidation or ionizing radiation. BER also takes part in the single strand breaks repair. A defective base is detected and removed and the gap is filled by DNA polymerase, then the fragments are joined by XRCC1 /ligase III complex.

Malfunction of BER pathway may contribute to cancer development. Data concerning polymorphism of XRCC1, one of the genes belonging to the BER pathway, in solid tumors are not conclusive, some studies show predisposition to the cancer in a wild type allele, the other do not. In AML studies, polymorphism of this gene (XRCC1 Arg399Gln) protects from development of the disease, especially of a secondary type. Patients with treatment related AML are more likely to have the wild -type of XRCC1 399Arg allele (Seedhouse, Bainton 2002).

Important components of BER pathway are the poly(ADP-ribose) polymerases (PARP) family containing 18 members. Those proteins allow for access to DNA repair enzymes in the case of single strand DNA breaks. After recruitment of PARP proteins the strands are cut, repaired and rejoined. PARP1 is the best studied protein in the PARPs family. Inhibition of PARP1 results in conversion of single to double strand breaks increasing the need for HR repair. Cancer cells with defective HR processes switch to PARP mediated BER mechanisms. Hence, PARP1 inhibitors are extremely active in tumors deficient in HR pathway and thus converting DNA repair to BER. Such treatment could be alternative for selected AML patients with impaired HR (Gaymes, Shall 2009).

6. NER in AML and MDS

NER is able to eliminate a wide variety of DNA damage, for example, long adducts such as pyrimidine dimers or crosslinks caused by chemotherapy. It is responsible also for the elimination of DNA damage resulting from UV radiation and chemical substances. NER is necessary both in maintaining global genom integrity and in repair of actively transcribed genes. Long sequences of improper oligonucleotides can be excised in a multi-step process requiring helicases and nucleases followed by ligation of the repaired DNA fragments.

Genes belonging to XP (xeroderma pigmentosum) group B and D encode helicases, enzymes responsible for unwinding DNA prior to transcription or NER. XPB protein is bound and modified by p210, the product of fusion gene bcr-abl responsible for chronic myeloid leukemia. Defective DNA helicases enhance genetic instability observed in this disorder.

The role of NER in AML pathogenesis and prognosis has also been investigated. Common polymorphisms in XPD gene belonging to NER pathway are associated with the risk of AML development and the outcome of the disease. XPD Lys 751 Gln variant is an independent prognostic marker for disease free survival and overall survival in elderly patients with AML (Allan, Smith 2004). Glutamine variant has altered enzymatic function with an impaired cellular response to genotoxins and is associated with the worse outcome as compared to lysine one. Heterozygotes had an intermediate AML outcome while homozygotes in the glutamine variant had the shortest overall and disease free survival. Homozygosity for the glutamine variant was also correlated with an increased risk of developing secondary AML after chemotherapy, but not after radiotherapy. Homozygosity did not affect de novo AML incidence. Moreover, the presence of the glutamine variant of XPD gene was associated with unfavorable cytogenetic profile in AML including patients with 5q and 7 q deletions (Smith, Worrillow et al. 2007). The same single nucleotide polymorphisms of XPD gene (XPD in Lys 751 Gln) together with the polymorphism of another gene in the same family (XPC Ala499 Val) were tested recently in AML patients with normal cytogenetics (Strom, Estey et al. 2010). Each polymorphism was an independent adverse prognostic factor for overall survival. Patients with the combination of variants in both genes had a significantly shorter overall survival (median 12 months) than carriers of wild type genes (median 44 months $p=0.001$).

7. MMR in AML and MDS

Processes of MMR play an important role in maintaining genome stability by detecting and repairing small insertions, deletions or misplaced bases which may occur during replication. Defects of MMR result in an increased rate of spontaneous mutations. Multiple replication errors occur in repetitive DNA sequences leading to microsatellite instability. Inadequate MMR may increase the risk of solid tumors and myeloid neoplasms (Ben-Yehuda, Krichevsky et al. 1996; Seedhouse, Das-Gupta et al. 2003). Microsatellite instability was observed in secondary leukemia and in elderly patients with AML (Das-Gupta, Seedhouse et al 2001), but not in de-novo young AML patients. The majority of patients showed multifocal changes. However, not all studies have found the increased microsatellite instability in AML patients (Rimsza et al 2000). MMR defects correlated with the presence of abnormalities of chromosome 5 and 7 (Ben-Yehuda, Krichevsky et al. 1996, Das-Gupta, Seedhouse et al. 2001). A high incidence of p53 mutations was also observed in this group of patients (Ben-Yehuda, Krichevsky et al. 1996). Improper p53 function may additionally enhance genom instability.

8. Conclusions

DNA repair defects are key events in multistep evolution of the neoplastic clone. The inherited improper function of DNA repair mechanisms may lead to accelerated genom instability, including tumor suppressor genes, resulting in neoplastic transformation. Increasing understanding of mechanisms leading to development and progression of myeloid malignancies may have future implications in the modern treatment. Leukemia is frequently specified by loss of a certain pathway, which may be the target of personalized treatment. So far the modification of DNA repair in AML and MDS is still at preclinical phase. Several potential problems with strategies influencing DNA repair arise. Most

important is the tendency for secondary malignancies because of genome instability and a high risk of rapidly increasing refractoriness.

DNA repair mechanism	Detected defect	Correlation with AML	Authors
Homologous Repair	Polymorphism in genes: RAD51, XRCC3,	Increase risk of AML development	Seedhouse, Foulkner 2004
Non Homologous End Joining	Ineffective processes in patients with FLT3 mutations	Correlates with refractoriness of the disease	Seedhouse, Hunter et al. 2006
Base Excision Repair	Polymorphism in gene XRCC1 (Arg399Gln)	Protects from AML development as compared with the wild type of allele	Seedhouse, Bainton 2002
Nucleotide Excision Repair	Polymorphism in gene XPD (Lys751Gln)	Adverse effect on overall survival and disease free survival in glutamine variant	Allan, Smith 2004
Mismatch Repair	Microsatellite instability	Increases risk of secondary leukemia; correlates with high risk cytogenetics	Das-Gupta, Seedhouse et al. 2001

Table 1. DNA repair defects in AML

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10. References

- Allan, J.M.; Smith, A.G. et al. 2004. Genetic variation in XPD predicts treatment outcome and risk of acute myeloid leukemia following chemotherapy. *Blood* 104, 3872-3877.
- Ben-Yehuda, D.; Krichevsky, S. et al. 1996. Microsatellite instability and p53 mutations in therapy related leukemia suggest mutator phenotype. *Blood* 88, 4296-4303.
- Casorelli, I.; Offman, J. et al. 2003. Drug treatment in the development of mismatch repair defective acute leukemia and myelodysplastic syndrome. *DNA repair*, 2, 547-559.
- D'Andrea, A.D. 2010. Targeting DNA pathways in AML. *Best Pract Res Clin Haematol*. 23, 469-473.
- Das-Gupta, E.P., Seedhouse C. et al. 2001. Microsatellite instability occurs in defined subsets of patients with acute myeloblastic leukaemia. *Br J Haematol* 14, 307-312.

- Estey, E. 2007. Acute myeloid leukemia and myelodysplastic syndromes in older patients. *J. Clin. Oncol.* 25, 908-1915.
- Gaymes T.J.; Mufti, G.J. et al. 2002. Myeloid leukemias have increased activity of the nonhomologous end-joining pathway and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer. *Cancer Res* 62, 2791-2797.
- Gaymes, T.J.; Shall, S. et al. 2009. Inhibitors of poly ADP-ribose polymerase (PARP) induce apoptosis of Myeloid leukemic cells: potential for therapy of myeloid leukemia and myelodysplastic syndromes. *Hematologica* 94, 638-646.
- Guillem, V.; Tormo, M. 2008. Influence of DNA damage and repair upon the risk of treatment related leukemia. *Leuk Lymphoma* 49, 204-217.
- Pallis, A.G.; Karamouzis, M.V. 2010. DNA repair pathways and their implication in cancer treatment. *Cancer Metastasis Rev* 29, 677-685.
- Rassool, F.V.; Gaymes, T.J. et al. 2007. Reactive oxygen species drive increased DNA damage and error-prone repair in a mouse model of myeloid leukemia disease progression. *Cancer Res* 67, 8762-8771.
- Rimsza, L.M.; Kopecky, K.J. et al. 2000. Microsatellite instability is not a defining feature of acute Myeloid leukemogenesis in adults: results of a retrospective study of 132 patients and review of the literature. *Leukemia* 14, 1044-1051.
- Robak, T.; Wierzbowska, A. 2009. Current and emerging therapies for acute myeloid leukemia. *Clin. Ther.*, 31, 2349-2370.
- Sallmyr, A.; Fan J et al. 2008. Internal tandem duplication in of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage and misrepair : implications for poor prognosis in AML. *Blood* 111, 3173-3182.
- Seedhouse, C.; Faulkner, R. et al. 2004. Polymorphisms in genes involved in homologous recombination repair interact to increase the risk of developing acute myeloid leukemia. *Clin Cancer Res* 10, 2675-2680.
- Seedhouse, C.H.; Hunter, H.M. DNA repair contributes to the drug-resistant phenotype of primary acute myeloid leukemia cells with FLT3 internal tandem duplication and is reversed by the FLT3 inhibitor PKC412. *Leukemia* 20, 2130-2136.
- Seedhouse, C.; Bainton R. et al. 2002. The genotype distribution of the XRCC1 gene indicates a role for base cision repair in the development of therapy-related acute myeloblastic leukemia. *Blood* 100, 3761-3766.
- Seedhouse, C.H.; Das-Gupta, E.P.; Russell, N.H. 2003. Methylation of hMLH1 promoter and its association with microsatellite instability in acute myeloid leukemia. *Leukemia* 17, 83-88.
- Shaheen, M, Allen, C. et al. 2011. Synthetic lethality : exploiting the addiction of cancer to DNA repair. *Blood* republished 2011 march 25.
- Shrivastav, M., De Haro, L.P. et al. 2008. Regulation of DNA double-strand break repair pathway choice. *Cell Research* 18, 134-147.
- Sloand, E.M. 2008 Myelodysplastic syndromes: introduction. *Semin. Hematol.* 45, 1-2.
- Smith, A.G., Worrillow, L.J. et al. 2007. A common genetic variant in XPD associates with risk of 5q- and 7q-deleted acute myeloid leukemia. *Blood* 109,1233-1236.
- Strom, S.S., Estey, E.H. et al. 2010. AML outcome: role of nucleotide excision repair polymorphisms in intermediate risk patients. *Leuk Lymphoma* 51, 598-605.

Worrillow, L.J., Alla, J.M. Deregulation of homologous recombination DNA repair in alkylating agent- treated stem cell clones: a possible role in the aetiology of chemotherapy- induced leukemia. *Oncogene* 25, 1709-1720.

Therapeutic Modulation of DNA Damage and Repair Mechanisms in Blood Cells

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1. Introduction

Hematopoietic stem cells (HSCs) are a rare population of pluripotent cells that predominantly reside in the bone marrow. Under the appropriate microenvironmental cues, HSCs can undergo self-renewal, expansion, and differentiation into all types of progenitor and terminally differentiated blood cells required for survival of the host (Figure 1). Due to the importance of this cell population for survival, protection of its genome from endogenous and exogenous genotoxic insults is a necessity. However, the intracellular molecular signaling network in hematopoietic cells that control surveillance of the genome as well as maintain genome stability is still largely unexplored. As more is learned regarding how these cells detect a genotoxic event and seek to repair the damaged nucleotides (i.e. DNA adducts), it will become even more feasible to design strategies to protect these life-sustaining cells when the host is exposed to a genotoxic event.

Maintenance of genome stability in both the hematopoietic stem and progenitor cell (HSPC) populations is essential for the sustainment of normal hematopoiesis. For example, transient depletion of bone-marrow derived HSC induced by irradiation or chemotherapy can induce these primitive cells to expand so that the bone marrow can be fully reconstituted; blood-cell development can then continue with minimal disruption. However, once therapy-mediated DNA damage is too high, a DNA-damage threshold is reached resulting in subsequent cell death, myelosuppression, and if not treated, life-threatening bone-marrow failure (Figure 1). With the basal level of DNA repair relatively low in these cells, this does present a challenge to maintain normal hematopoiesis in individuals exposed to prolonged or high levels of genotoxic stress. The reduced ability to repair DNA damage in HSPCs that give rise to multiple mature blood-cell lineages can cause detrimental and long-lasting effects to the host resulting in abnormal cell function, cell death, cellular transformation, and eventually leukemogenesis (Figure 1). Numerous studies have shown that HSPCs are intrinsically more sensitive than other cell types and tissues mostly due to intrinsic limitations in DNA-repair capacity. Buschfort-Papewalis *et al* previously demonstrated that when human HSPCs (phenotypically defined as CD34⁺ cells) or differentiated cells (phenotypically defined as CD34⁻ cells) from the same donor were exposed to alkylating agents, an overall decrease in repair capacity of the more primitive CD34⁺ cells compared the more differentiated cells CD34⁻ cells was observed. When human CD34⁺ cells were exposed to a variety of chemotherapeutic drugs, single-strand DNA breaks as well as DNA adducts were found at higher levels and persisted for longer time periods than in CD34⁻ cells (Buschfort-Papewalis *et al.*, 2002), providing evidence that the kinetics of DNA repair are slower overall in the

CD34⁺ cells which is the most likely reason for their enhanced sensitivity to irradiation and chemotherapy.

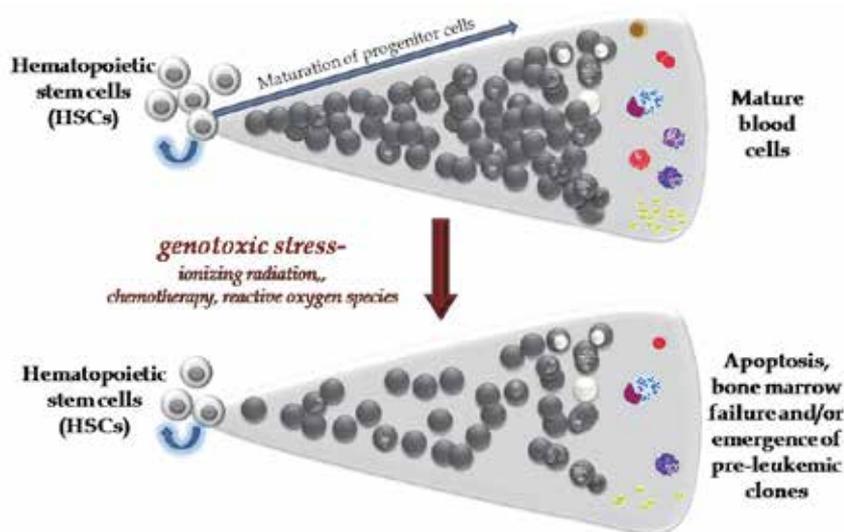


Fig. 1. Hematopoiesis and impact of genotoxic stress. Hematopoietic stem cells (HSCs) undergo self-renewal or differentiate into progenitor cells and ultimately into mature terminally differentiated blood cells. When HSC are exposed to exogenous genotoxic stress such as ionizing radiation or chemotherapy and intrinsic stress such as high levels of reactive oxygen species, the bone marrow can become myelosuppressed. If the stress is transient and not of high intensity, the HSC can repopulate the bone marrow. However, if the stress is of sufficient and prolonged intensity, a large number of cells will undergo apoptosis which will lead to bone marrow failure and potentially emergence of pre-leukemic cells (white cells) and ultimately transformation and full blown leukemia.

In this chapter, an overview and analysis of investigations that employ *in vitro* and *in vivo* model systems to study how hematopoietic cells in mouse and man respond to genotoxic stress is presented. In addition, we summarize new therapeutic strategies designed to protect and limit therapy-induced stress to the hematopoietic system and the animal models used to develop and test these therapies.

2. DNA repair pathways in hematopoietic stem and progenitor cells

Mammalian cells are equipped to varying degrees with multiple DNA repair pathways in which to defend against the accumulation of DNA damage caused by environmental insults such as reactive oxygen species, ionizing irradiation, and chemotherapy. The DNA repair pathways- O⁶-methylguanine DNA methyltransferase (MGMT) direct repair, nucleotide-excision repair, base-excision repair, mismatch repair, non-homologous DNA end-joining, and homologous recombination- all play prominent roles in maintaining genome stability. A brief overview of the major DNA repairs pathways that are operative in the vast majority of mammalian cells including hematopoietic stem and progenitor is presented below and detailed reviews of these pathways have been previously published (Bekker-Jensen & Mailand, 2010; Niedernhofer, 2008; Seita et al., 2010)

2.1 The direct repair protein O⁶-methylguanine DNA methyltransferase (MGMT)

MGMT is also referred to as O⁶-methylguanine DNA methyltransferase and in contrast to the other DNA repair pathways which rely on a series of DNA repair proteins, acts singly to repair DNA damage caused by DNA adducts (Gerson, 2004). MGMT repairs DNA damage mediated by endogenous alkylation or by chloroethylating and methylating agents used in anti-cancer therapies. In a one-step stoichiometric reaction, MGMT removes adducts from the O⁶ position of guanine (Gerson, 2002). Each MGMT molecule repairs one O⁶ lesion; MGMT is then inactivated and degraded. For chloroethylating agents such as carmustine (BCNU) and lomustine (CCNU), if lesions at the O⁶ position are not repaired by MGMT, an intramolecular rearrangement occurs over the next 18-24 hours resulting in an interstrand crosslink with the paired cytosine on the opposite DNA strand (Sorrentino, 2002). Since this covalent crosslink between guanine and cytosine is poorly repaired, DNA replication is blocked and caspase 3-mediated apoptosis ensues. In regards to methylating agents such as temozolomide (TMZ), procarbazine, streptozotocin, and dacarbazine, formation of O⁶-methyl adducts disrupts hydrogen bonding between the methylated guanine and the corresponding cytosine. As DNA replication proceeds, O⁶-methyl guanine pairs with thymidine instead of cytosine and this mismatch ultimately results in a guanine to adenine switch resulting in a point mutation. As a result, the mismatch repair pathway is activated. During this repair process, single-strand gaps occur in the DNA and once this occurs in the same region on the opposite DNA strand, double-strand breaks occur. This leads to erroneous mismatch repair cycles and ultimately leads to apoptosis. Chloroethylating agents are far more potent than methylating agents in terms of cellular cytotoxicity. For chloroethylating agents, formation of only 5-10 O⁶-chloroethyl adducts per cell results in cell death. In contrast, for methylating agents, formation of approximately 6000 O⁶-methyl adducts per cell is required for cellular toxicity.

MGMT is ubiquitously expressed in all tissues with the bone marrow express substantially lower levels (Sorrentino, 2002). The kinetics of MGMT regeneration following DNA repair also vary amongst tissues; MGMT expression in tumor cells regenerates relatively faster compared to normal tissues (Kreklau et al., 2001). The role of MGMT activity in repairing mutations that would otherwise lead to cancer is confirmed in studies using transgenic or knock-out mice. Indeed transgenic mice that overexpressed MGMT in the thymus were significantly less susceptible to thymic lymphomas caused by methylating agents compared to control littermates (Dumenco et al., 1993). MGMT knock-out mice exhibited an increased incidence of tumors compared to wild-type mice (Glassner et al., 1999). These observations set the stage for developing strategies that can selectively protect normal tissues while simultaneously disrupting DNA repair in cancer cells. MGMT removes two kinds of alkyl adducts—O⁶-alkylguanine and O⁴ on thymine—leaving behind an intact guanine or thymine in the DNA strand (Park & Gerson, 2005). The MGMT DNA repair protein is referred to as a “suicide” protein since after a single repair event, each molecule is ubiquitinated and ultimately degraded by the proteasome (Gerson, 2002, 2004). Inactivation of the *MGMT* gene via promoter methylation as well as over-expression of its gene product have been found in multiple types of cancer including glioma, melanoma, lymphoma, colorectal cancer and breast cancer and can lead to cell sensitivity or resistance to alkylators such as temozolomide, CCNU, and BCNU. Therefore establishing the expression level of MGMT expression in a particular cancer could help guide the selection of appropriate treatment modalities (Pegg, 2011).

2.2 Nucleotide excision repair (NER)

NER is a type of repair in which a small series of nucleotide bases in each direction from the damaged DNA adduct are removed. There are two types of pathways associated with NER-global genome and transcription-coupled repair-the specific pathway used depends on how the DNA damage is recognized (Park & Gerson, 2005). With global genome repair, xeroderma-pigmentosum complementation group C (XPC) and proteins recognize DNA damage. Many proteins are then recruited to remove the damaged bases as well as to repair the damage including XPA, RPA, transcription factor IIIH, XPG, and polymerases delta or epsilon (Park & Gerson, 2005). With transcription-coupled NER, DNA damage leads to a halt in gene transcription and "sensor" proteins are rapidly recruited to the site for initiation of repair including MSH2, CSA, XBA2, XPB, XPG, BRCA1, and BRCA2 (Park & Gerson, 2005). Defects in NER genes are the underlying cause of severe genetic disease states such as Xeroderma pigmentosum and Cockayne's Syndrome.

2.3 Base excision repair (BER)

BER removes a misplaced or damaged base in the DNA strand. DNA glycosylases first remove a damaged base by creating an apurinic or apyrimidinic site within which AP endonuclease (APE-1) cuts the 5' end of DNA so that the damaged base can be removed and replaced with the correct base. There are two pathways within BER which can be classified into "simple" and "complex." The DNA glycosylases are different between the two pathways. In the simple pathway, N-methylpurine DNA glycosylase (MPG) removes a damaged site leaving an apurinic or apyrimidinic site without nicking the DNA backbone(Park & Gerson, 2005). With the complex BER pathway, the DNA glycosylase such as 8-oxoguanine DNA glycosylase (OGG1) removes the damaged base and also nicks the DNA backbone(Park & Gerson, 2005). β -polymerase fills in the missing base and DNA ligase seals the nick in the DNA backbone completing BER(Limp-Foster & Kelley, 2000). Interestingly, BER protein knockout mice are embryonic lethal which in contrast to NER, could explain why no known genetic diseases have been implicated with BER protein loss.

2.4 Mismatch repair (MMR) pathway

The MMR pathway recognizes single mismatches or misaligned sequence repeats (Belcheva et al., 2010; Martin et al., 2010). Msh2 will link with either Msh6 or Msh3 when recognized sequences are detected. Following detection, the Mlh1-Pms2 complex will coordinate the DNA endonuclease removal of damage, DNA re-synthesis, and ligation to complete the repair (Schmutte et al., 2001). Many diseases have been implicated in MMR deficiencies including lymphomas and stem-cell derived leukemias(Park & Gerson, 2005).

2.5 Non-homologous DNA end-joining (NHEJ)

Double strand breaks are most often repaired by NHEJ which can be caused by free radicals or ionizing radiation. Double-strand breaks can also occur following a malfunction during V(D)J recombination in T and B lymphocytes(Park & Gerson, 2005). This pathway is quite complex and uses does not always accurately repair the DNA which could be detrimental to the cell or in some situations such as during V(D)J recombination can add sequence diversity to genes encoding the T and B lymphocyte antigen-specific receptors. The ends of the broken DNA strands are held by a protein complex comprised of Ku70, Ku80, and DNA-PKcs. The repair is completed by XRCC4-DNA ligase IV (Lieber, 2010; Nick McElhinny et

al., 2000). Seita et al have demonstrated that in human HSPCs, that cycling status can dictate selection of the repair pathway that is operative. For example, in quiescent human HSPCs, the NHEJ pathway is preferentially used (Seita et al., 2010).

2.6 Homologous recombination (HR)

Homologous recombination (HR) can also repair double-strand breaks; however, it often can lead to further DNA damage with misalignments, deletions, and rearrangements. The PI-3 kinase, ATM binds to the DNA following a double strand break acting to phosphorylate target proteins involved in repair including Mre11/Rad50/Nbs, BRCA1, BRCA2, RAD 51, BLM, and WRN(Park & Gerson, 2005). Abnormalities in homologous recombination are linked to many genetic diseases including Ataxia Telangiectasia, Werner's syndrome and Bloom's syndrome. In contrast to NHEJ, HR repair is generally considered superior in regards to ensuring that all DNA adducts are accurately repaired. As discussed below, in the study by Seita *et al*, they find that cycling HSPCs in contrast to quiescent HSPCs, utilized the HR pathway to repair DNA strand breaks (Seita et al., 2010). Abnormalities in nucleotide excision repair, telomere maintenance, or non-homologous DNA end-joining in mice have shown increased deficiencies in hematopoietic stem cells (HSCs) as they age implicating DNA damage as a driving force for stem-cell aging(Naka & Hirao, 2011). The continued research in employing gene therapy targeting increased DNA repair proteins in HSCs will continue to be an area of research as the limiting factor in anti-cancer treatments is normal tissue toxicity typically myelosuppression(Niedernhofer, 2008) and will be discussed in detail below.

3. DNA damage responses in hematopoiesis

Understanding the DNA-damage response and DNA-repair pathways that control the sensitivities of HSPCs to DNA-damaging agents will be key as the field continues to develop new pharmacological and cellular-based therapies to protect and maintain genome stability in these life-sustaining cells. The maintenance of HSC in a quiescent state *in vivo* is essential for long-term survival. Quiescence is widely considered to be an essential protective mechanism for stem cells that minimizes endogenous stress caused by cellular respiration and DNA replication. What molecular cues are essential for maintenance of "stem-ness" *in vivo* is a rapidly growing area of investigation and a complex network of proteins control the DNA-damage response and repair pathways. The tumor suppressor protein, p53, clearly plays a pivotal role in the regulation and promotion of senescence, apoptosis, and cell cycle arrest in a variety of cell types(Vousden & Lane, 2007). Several laboratories have utilized p53-deficient mice to investigate the role of this protein in hematopoiesis. Due to the dual functions of p53 in promoting survival versus cell death, results have been somewhat difficult to interpret. In transplantation studies, reconstitution of the bone marrow with p53-null murine HSC has resulted in increased, equivalent or decreased levels of engraftment in the mouse. Most likely, small nuances in experimental design are the most likely reason for these discrepancies and underscore the complexity of the p53-signaling network in hematopoiesis (Chen et al., 2008; Liu et al., 2009; Marusyk et al., 2010). In competitive repopulation assays, Dumble *et al* demonstrated that with increased p53 expression, HSCs exhibited decreased self-renewal capabilities (Dumble et al., 2007). In murine studies by Liu *et al*(Liu & Gerson, 2006; Liu et al., 2009), p53 was highly expressed in primitive Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) wild-type cells compared to the more differentiated hematopoietic myeloid cells. The maintenance of LSK frequency appeared to

be dependent on p53 expression since LSK frequency of bone marrow cells derived from p53 $-/-$ mice was significantly decreased. *In vivo* bromodeoxyuridine assays suggested that while p53 promoted HSC quiescence, p53 absence promoted entry of HSCs into cycle (Liu et al., 2009).

The mechanisms by which HSPCs respond to DNA double-strand breaks (DSBs) induced by genotoxic stress, such as ionizing radiation (IR), is an emerging area of investigation. Two provocative studies conducted by Mohrin et al and Milyavsky et al studied IR-induced DNA damage responses of HSPC compartments derived from mice (Mohrin et al., 2010) and humans (Milyavsky et al., 2010). The molecular mechanisms by which murine and human HSPCs respond to IR appear to differ substantially (Seita et al., 2010). In the mouse model, Mohrin et al. found that murine HSPCs exposed to 2 Gy of IR were significantly more resistant than differentiated myeloid progenitors. Furthermore, significant differences in the radiosensitivity of quiescent versus proliferating murine HSPCs were observed. The role of the *ataxia telangiectasia mutated* (ATM) protein in resistance to IR was also studied. ATM is a serine-threonine protein kinase and a key sensor of DNA damage which is rapidly recruited to DNA double-strand breaks; upon activation at the strand break, ATM plays a major role in activation of the DNA-damage checkpoint. In HSCs derived from ATM-null mice, quiescent and proliferating HSCs exhibited similar radiation sensitivities, indicating the importance of this protein in monitoring DNA damage in these populations. Further studies indicated that different modes of DNA repair are used to repair IR-induced DNA damage in quiescent versus cycling HSPCs. Quiescent HSPCs preferentially utilized the NHEJ pathway, which as mentioned previously is a fairly inefficient DNA repair pathway and is much more error prone than the HR pathway. High basal levels of NHEJ activity were detected in the quiescent HSPCs and this activity increased by ~2-fold following exposure to IR. In contrast to the quiescent HSPCs, the cycling HSPCs preferentially switched to the HR pathway to repair IR-induced double-strand breaks. The HR pathway is able to repair IR-induced DNA damage more accurately than the NHEJ pathway. These data are consistent with the concept that cell-cycle status may influence the integrity of the DNA-repair processes and hence, influence long-term genome stability. For example, irradiated quiescent HSPCs gave rise to progeny that have genomic abnormalities at a higher frequency than cycling HSPCs. In particular when compared to non-irradiated resting HSPCs, greater than 30% of the cells derived from IR-treated resting HSPCs exhibited genomic rearrangements such as reciprocal translocations, interstitial deletions, and complex rearrangements. When mice were transplanted with non-irradiated or irradiated quiescent HSPCs (CD45.1+ cells) into CD45.2 lethally irradiated recipient mice, there was decreased engraftment in mice transplanted with the IR-treated versus non-treated HSPCs. While no transplanted mice developed leukemia or showed signs of abnormal hematopoiesis, further analyses demonstrated the presence of increased genome alterations in mice transplanted with IR-treated quiescent HSPC donor cells. These data suggest a DNA-repair threshold exists in which repair of DNA damage in the mouse may not be fully accomplished in the quiescent HSPCs. Collectively, these findings also suggest that mouse HSC quiescence and reliance on NHEJ to repair IR may be an important mechanism contributing to mutagenesis at the stem-cell level. (Mohrin et al., 2010) This may serve as the starting point for the emergence of certain blood-cell cancers. In addition, the accumulation of mutations in quiescent HSPCs over time may account for many of the stem-cell based hematological abnormalities observed during aging. While the upside is that the quiescent state of murine HSPCs preserves these cells for use in the future, the downside is

that at least in mice, HSPCs in the quiescent state may be intrinsically more vulnerable to mutagenesis following DNA damage, particularly if the DNA damage pathways, such as NHEJ are operative and are not as reliable in repairing the DNA damage.

Milyavsky et al. focused on the response of different hematopoietic cell populations isolated from human cord blood to IR-mediated DNA damage (Milyavsky et al., 2010). In contrast to the murine study, human HSPCs were found to have increased sensitivity to IR than the more differentiated progenitor cells. One likely reason for the increased sensitivity to IR was that the IR-mediated DNA damage led to slower repair of DNA damage in the human HSPC following IR exposure. In this study, Milyavsky et al determined the relative sensitivity of 3 human hematopoietic subpopulations isolated from cord blood and phenotypically defined as : (1) HSC- Lineage-negative (Lin⁻), CD34⁺CD38⁻ or Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻ fraction; (2) myeloid progenitor population (MPP)-Lin⁻CD34⁺CD38⁻CD90⁻CD45RA⁻ population which contains fewer HSCs and many MPPs; (3) progenitor population-more mature progenitor/precursor cells, including CFCs, which are Lin⁻CD34⁺CD38⁺.

To monitor the DNA damage and repair kinetics of the different hematopoietic cell populations, neutral comet assays and subnuclear foci of phosphorylated H2AX (γ H2AX) were used as measures of IR-induced DNA double-strand breaks. Different cell population were treated with 15 Gy IR and damage monitored at 30-60 minutes post-IR exposure. In the progenitor cells, 19.4% of the breaks were repaired by 30 minutes and 36.4% of the breaks had been repaired by 60 minutes. In contrast, in the more primitive population-HSC/MPP-no repair of DNA-strand breaks was observed at 30 minutes post-IR; and at 60 minutes, 29.9% of the breaks had been repaired. The delay in repair of the double-strand DNA breaks indicated that the more quiescent HSC/MPP cells differed in their repair capacity from the differentiated progenitor population. Similar differences in DNA repair between the primitive and more differentiated cell populations were noted with the levels of γ H2AX foci. At one hour post-exposure to IR, similar levels of γ H2AX foci were evident in both the HSC/MPP and the progenitor populations. By 12 hours post-IR exposure, more γ H2AX foci still remained in the HSC/MPP compared to the progenitor population (7.1 versus 2.7 foci/nucleus respectively) further confirming the differential DNA repair capabilities of the two populations. Additionally these data correlated with decreased survival in the HSC versus the more differentiated progenitor cells in clonogenic assays.

To assess the viability of irradiated HSPCs in the bone-marrow microenvironment, the SCID-repopulating assay was used (Milyavsky et al., 2010). The nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were transplanted with cord blood CD34⁺ cells and 5-10 weeks post-transplant cohorts of mice received 3 Gy IR. The bone marrow was harvested from the mice 1.5 hours after IR delivery and the viability of the more primitive human HSC (Lin⁻CD34⁺CD38⁻) versus the more differentiated (Lin⁻CD34⁺CD38⁺) was determined by flow cytometry. The *in vivo* data correlated with the *in vitro* DNA damage and repair studies, for there was a 2-fold increase in IR-induced apoptosis in the more primitive Lin⁻CD34⁺CD38⁻ versus the Lin⁻CD34⁺CD38⁺ cells. (Milyavsky et al., 2010) The authors went on to show that the primitive versus more differentiated hematopoietic cells utilize the p53/Bcl-2 pathway but to different degrees. Both *in vitro* and *in vivo* studies indicated that decreased p53 expression or over-expression of Bcl2 in cord blood progenitors blocked IR-induced apoptosis indicating the involvement of the p53-BCL2 pathway in regulating apoptosis and conferring some degree of radioprotection. However, in the secondary transplant setting, lack of p53 expression in the

HSC resulted in higher levels of γ H2AX foci in the engrafted cells and this correlated with decreased levels of engraftment. In contrast, in mice transplanted with the Bcl-2-overexpressing HSCs, engraftment was within a normal range. To begin to define in more detail, the intracellular proteins involved in regulating the DNA damage response to IR, global microarray expression profiling indicated that the apoptosis-stimulating protein of p53 (ASPP1) was preferentially expressed and found to play a major role in mediating stem-cell radiosensitivity. ASPP1 is involved in the activation of p53-dependent apoptosis. Expression levels of ASPP1 were higher in HSC versus the more differentiated populations. The critical role of ASPP1 in response of primitive hematopoietic cells to IR was further confirmed in knockdown experiments; knockdown of ASPP1 in a CD34⁺CD90⁺ primitive cell population versus knockdown in a more differentiated CD34⁺CD90⁻ cell population revealed that only in the primitive CD34⁺CD90⁺ cells with decreased ASPP1 was there an increased resistance to IR exposure (Milyavsky et al., 2010). These studies clearly highlight the differences between these two species and underscore the importance of evaluating DNA damage responses in different models and cell types. It is becomingly increasing clear that DNA damage thresholds that dictate survival versus cell death do vary among different cell types and the mechanisms that regulate these responses will be different and need to be understood in order to develop relevant, effective, and safe strategies to protect bone marrow cells from genotoxic stresses.

4. Increasing cell cycle arrest by pharmacological intervention to improve DNA-repair activity in hematopoietic cells exposed to genotoxic stress

The use of growth-factor support such as granulocyte-colony stimulating factor, granulocyte/macrophage-colony stimulating factor, or erythropoietin can help increase the resistance to DNA-damaging agents and help facilitate recovery of the bone marrow following genotoxic therapy. However, these treatments are very expensive and can have adverse side effects. With the explosion of small-molecule development and drug discovery and as more is learned regarding how different cell types repair DNA damage, it may be possible to modulate DNA damage and repair pathways to increase protection and stability of the hematopoietic genome. These types of strategies could be used to prevent life-threatening myelosuppression in the case of radiation accidents or disasters as well as an adjuvant to aggressive cancer therapies that typically induce severe myelosuppression or complete non-irreversible myeloablation. In terms of IR exposure, the DNA double-strand DNA breaks caused by IR are cell-cycle dependent. The early G1 and late S phases are fairly resistant to IR effects, but the G1/S transition and the G2/M phases are the most sensitive to IR. It has been shown previously that extension of the G1 period following exposure to a genotoxic stress can enhance resistance; it is possible that this could allow for a longer period of time to repair the DNA before entering the cell cycle (Johnson et al., 2010). The cyclin-dependent kinases (CDK2, CDK4, and CDK6) are involved in promoting the G1 to S cell-cycle transition. Johnson et al demonstrated significant increases in the radioprotection of human cell lines and mice exposed to CDK4/6 small molecule inhibitors such as PD0332991 (Pfizer, Inc.) that block entry into cell cycle. If wild-type mice were treated with the selective inhibitors, a reversible cell-cycle arrest ensued in the most primitive HSPCs but not in cells already cycling the bone marrow or in other tissues (Johnson et al., 2010). The inhibitor-mediated inhibition of CDK4/6 function abrogated the total-body irradiation whether the inhibitor was given before or even up to several hours after delivery of total

body irradiation (TBI). The authors also determined if inhibition of CDK4/6 could be used in the context of an anti-cancer therapy such as IR as to protect the mice from life-threatening hematotoxicity but also to see if inhibition of CDK4/6 would have any impact on tumor-cell kill (Johnson et al., 2010). In a genetically engineered melanoma mouse model, male *TyrRas Ink4a/Arf*^{-/-} develop autochthonous melanomas due to melanocyte-specific promoter expression of mutant H-Ras. In this model, the growth of the tumors was not inhibited by CDK4/6 inhibitor treatment. When tumor-bearing mice were treated with 7.5 Gy TBI, tumor growth slowed substantially for ~20 days. When the tumor-bearing mice were treated with 1 dose of the CDK4/6 inhibitor 4 hours before TBI, the tumors still responded to the irradiation and growth slowed as in control mice but also there was a substantial decrease in irradiation-induced myelosuppression and mortality. The relative contribution of a prolonged G1 arrest versus a block in the G1 to S transition in enhancing radioprotection of murine HSPCs is not completely clear. Likewise, how these intriguing results will translate into the radioprotection of human bone-marrow cells and whether cancer-initiating mutations could permanently reside in the genome will require further investigation.

5. Human gene-therapy clinical trials that target HSPCs-safety, efficacy, and vectors

HSPCs have been studied for the past three decades as a relevant target for gene therapy due to feasibility with which they can be harvested, *ex vivo* manipulated, and transplanted back into the patient. While the field of hematopoietic-stem cell gene therapy has had set backs and successes, many hurdles still remain in moving this from an experimental to a well accepted treatment modality. Investigations originally focused on utilization of retrovirus vectors for transduction of murine HSC (Williams et al., 1984). As studies progressed and investigators moved to humanized mouse models and large animal models, the promise shown in the murine transplant models did not always hold up. The first attempts of using a gene-therapy approach in human hematopoietic cells were also not very promising. Low-gene transfer and engraftment levels of genetically modified cells in humans were noted (Brenner et al., 1993b; Dunbar et al., 1995). Recent technical advances, however, including identification of suitable cytokine cocktails that minimize stem-cell commitment and differentiation during *ex vivo* culture, use of Retronectin-enhanced gene transfer, as well as enrichment of stem and progenitor cells prior to transduction have improved the efficiency of gene transfer into human cells and resulted in substantial successes in human gene-therapy trials particularly in X-SCID (Abonour et al., 2000; Cavazzana-Calvo et al., 2000) but also some unexpected abnormal events. In terms of the success of the trial, the selective advantage of the corrected hematopoietic cells *in vivo* clearly was a determining factor in generating sufficient numbers of corrected progenitors and lymphocytes in these patients. In spite of these improvements, however, the efficiency of gene transfer into primitive human hematopoietic cells and the engraftment of sufficient numbers of these transduced cells into patients still remains a major impediment. The very early gene-therapy trials using T-lymphocyte populations or hematopoietic stem/progenitor cells into subjects with adenosine deaminase deficiency associated with SCID revealed no signs of genotoxicity (Aiuti et al., 2009). However, in the first trial of X-SCID gene therapy, in which vector and transduction protocol were optimized, the reality

that oncoretroviral-mediated insertional mutagenesis could serve as an initiator of leukemia in some patients transplanted with genetically engineered autologous hematopoietic cells is concerning. These early success stories coupled with the adverse events reported in the X-SCID (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b) and chronic granulomatous disease (Metais & Dunbar, 2008; Ott et al., 2006) trials demonstrate that gene therapy based approaches have a place in the clinic, but at the same time, more research into all aspects of these approaches and the disease state to be treated require more investigation. When the first cases of insertional mutagenesis were reported in the X-SCID trials, the community responded in a very positive and aggressive way to unravel the underlying cause of the T-cell leukemia in several of X-SCID patients. Retroviral insertion into the genome near the LMO-2 and other growth-promoting loci in the transplanted hematopoietic cells was defined very early after the adverse events were reported. In addition, as preclinical studies in nonhuman primates, dogs, and mice have progressed over the past decade, development of leukemias most likely linked or initiated by vector-mediated insertional mutagenesis have been reported in these animal models (Li et al., 2005; Li et al., 2002; Modlich et al., 2005; Nienhuis et al., 2006; Seggewiss et al., 2006; Zhang et al., 2008). Since these reports, a large effort has focused on designing vectors with better safety profiles, well-defined transduction protocols, as well as improved small and large animal models for long-term evaluation of efficacy and safety testing. As the field of hematopoietic gene therapy continues to progress, it is highly likely that vector design coupled with optimal transduction protocols and better understanding of the underlying hematological disease will result in cures (Trobridge et al., 2005).

5.1 Retroviral vectors

Many improvements in vector design resulting in improved transgene expression and safe guards against generation of replication-competent viruses have been summarized in detail elsewhere (Trobridge, 2011; Yi et al., 2011). A brief summary of retroviral vector types and transduction considerations are discussed below.

5.1.1 Gamma-retroviral vectors

Gamma-retroviral vectors were the first to be used for the purpose of marking murine hematopoietic stem and progenitor cells (Williams et al., 1984). Due to their long history in development, they have been the most commonly used so far as vectors for clinical studies. It has been widely appreciated for some time now, that cell division of the retrovirally transduced cells is essential for stable integration of gamma-retroviral vectors into the host genome (Lewis & Emerman, 1994; Miller et al., 1990). Due to the highly quiescent nature of the hematopoietic cells, cytokines that promote cell division in primitive hematopoietic cells have been utilized over the years and in combination with Retronectin-coated plates-to enhance proximity of retroviral particles and cells-have resulted in fairly high levels of transfer into hematopoietic stem and progenitor cells derived from mice, canines and primates (Horn et al., 2002b; Kiem et al., 1999; Kurre et al., 2002; Rosenzweig et al., 1999). There are generally 3 disadvantages in using a gamma-retroviral vector to transduce hematopoietic cells. Silencing of the transgene expressed by the gamma-retroviral vectors can occur over time and this has been well documented in murine transplant studies (Halene et al., 1999; Klug et al., 2000; Robbins et al., 1998). Additionally, while gene transfer into primitive hematopoietic cells is fairly efficient, the cytokine-mediated entry into cell

cycle *ex vivo* can lead to a loss or primitive hematopoietic cells residing in the graft, which could compromise engraftment kinetics (Tisdale et al., 1998). The final downside of gamma-retroviral vectors is their preferential integration near transcript start sites (Wu et al., 2003), which in the proper molecular context can increase the chances of insertional mutagenesis and development of leukemia as discussed above. Alternative vector systems such as lentiviral or foamy viral vectors, are being investigated in a variety of contexts and may have better integration properties such that only minimal cytokine exposure and *ex vivo* culture time are required to achieve acceptable gene transfer into repopulating hematopoietic stem and progenitor cells (Horn et al., 2004a; Josephson et al., 2002; Miyoshi et al., 1999; Trobridge et al., 2005).

5.1.2 Lentiviral vectors

Lentiviral vectors do not necessarily require mitosis in order to enter the nucleus and integrate into the genome. This vector system has been shown to successfully transduce a variety of nondividing target cells (Case et al., 1999; Naldini et al., 1996; Reiser et al., 1996). However, lentiviral-mediated transduction was relatively higher still, when the target cells were induced to undergo cell division during the transduction period (Naldini et al., 1996; Russell & Miller, 1996; Trobridge & Russell, 2004). Lentiviral vectors can also efficiently transduce SCID-repopulating cells in the NOD/SCID mouse model (Miyoshi et al., 1999), as well as long-term repopulating cells in canines (Horn et al., 2004b) and monkeys (An et al., 2001; An et al., 2000; Horn et al., 2002a).

As more long-term repopulation studies are completed, it is becoming clear that lentiviral vectors in contrast to gamma-retroviral vectors allow for efficient transduction, optimal reconstitution, and quicker recovery following transplantation (Goerner et al., 1999; Goerner et al., 2001; Horn et al., 2002a). It is possible that lentiviral vectors can transduce larger numbers of immature hematopoietic repopulating cell compared to gamma-retroviral vectors. One obstacle to extending these observations to large animal models is that human immunodeficiency virus type 1 (HIV1)-based lentiviral vectors in nonhuman primates have a low transduction rate in Old World monkey cells (Song et al., 2005; Stremlau et al., 2004; Stremlau et al., 2005). Peter-Kiem and colleagues have found, however, that human and pigtail macaque (*Macaca nemestrina*) but not baboon (*Papio cynocephalus anubis*) CD34⁺ cells could be efficiently transduced with lentiviral vectors (Beard et al., 2007; Trobridge et al., 2005).

5.1.3 Foamy virus vectors

Foamy (*spuma*) retroviral vectors are the newest vector system to undergo extensive development and testing for use as a gene-transfer vector for HSC gene therapy. The vector backbones clearly possess several characteristics that make this vector attractive for gene transfer into primitive hematopoietic cell lineages. The vector has the ability to allow for subcloning of larger therapeutic transgenes than the other vector systems. Importantly for use in the clinic, the foamy virus does not cause any known pathogenesis in humans which further strengthens the safety of this novel viral vector (Trobridge et al., 2002). Foamy viral vectors require mitosis for integration into the host genome. In contrast to gamma-retroviral and lentiviral vectors, foamy vectors can form a stable intermediate in quiescent cells (Trobridge & Russell, 2004) which can exist for days. Therefore, this allows for increased opportunities for the foamy viral intermediate to integrate into the genome of the

transduced cell once it divides at a later time point, presumably even after it is transplanted. This is possibly one reason why foamy viral vectors have shown promise in the transduction of SCID-repopulating cells derived from quiescent mobilized peripheral blood (Josephson et al., 2004). How well lentiviral versus foamy viral vectors can transduce canine hematopoietic stem and progenitor cells has been investigated. Equivalent levels of gene transfer were evident in both myeloid and lymphocyte lineages (Beard & Kiem, 2009). Interestingly, when the retroviral insertion sites of lentiviruses and foamy viruses were compared, different insertion profiles was found between the two viral vectors. (De Palma et al., 2005; Dunbar, 2005; Hematti et al., 2004; Laufs et al., 2004; Nowrouzi et al., 2006; Trobridge et al., 2006). Lentiviral vectors integrated more frequently within the coding region of genes compared to gamma-retroviruses which exhibited a strong preference for the 5' end of genes most often near the enhancer/promoter regions. Another attractive feature of the foamy vectors is that they do not preferentially integrate near transcript start sites, which would predict there is less likely chance of activating near-by gene expression (Trobridge et al., 2005). Investigations continue to interrogate if the genome-insertion profile of lentiviral vectors will result in any difference in the potential mutagenic properties of these vector systems (Modlich et al., 2006; Montini et al., 2006).

5.2 Viral envelopes for pseudotyping vectors

Selection of an appropriate viral envelope for pseudotyping a particular vector is critical and can influence the transduction efficiency and engraftment capability of the target population. For example, if the levels of the host receptor that is specific for the viral envelope is high, then optimal levels of gene transfer can be expected (Kurre et al., 1999; Kurre et al., 2001b; Orlic et al., 1996; Sabatino et al., 1997). Gamma retroviral vectors were originally pseudotyped with the amphotropic envelope in human ((Brenner et al., 1993a; Dunbar et al., 1995; Kohn et al., 1995) and nonhuman primate (Bodine et al., 1993; van Beusechem et al., 1992). As studies progressed, it was discovered that hematopoietic cells in general have low levels of the amphotropic receptor on the most primitive hematopoietic cells; thus, providing a reason for the low levels of gene transfer into the primitive hematopoietic cells (Orlic et al., 1996). Other viral envelope glycoproteins have been discovered and tested (Akkina et al., 1996; Cone & Mulligan, 1984); these include the gibbon ape leukemia virus (GALV) envelope (Wilson et al., 1989), the feline endogenous retrovirus (RD114) envelope (Porter et al., 1996), and the vesicular stomatitis virus G glycoprotein (VSVG) envelope (Emi et al., 1991). When GALV- versus amphotropic-pseudotyped gamma-retroviral vectors were compared, GALV-pseudotyped vectors had higher marking than vectors with amphotropic envelopes in both human progenitors (von Kalle et al., 1994) and baboon repopulating cells (Kiem et al., 1999). The VSVG envelope has undergone extensive testing and has been attractive to the community since it can be substantially concentrated via ultracentrifugation to increase viral titers (Burns et al., 1993). Establishing stable packaging lines that also provide high titer has been problematic since the VSVG can be toxic to the packaging cells (Yang et al., 1995; Yee et al., 1994). The RD114 envelope has some advantages in that it is not toxic, and due to its stability, viral titers can be increased by ultracentrifugation. The RD114 envelope is also resistant to inactivation by human and macaque sera (Kelly et al., 2001; Sandrin et al., 2002). The disadvantage of this envelope, is that transduction efficiencies into human hematopoietic cells is variable and is due to differing levels of RD114 receptor expression on the human cells (Green et al., 2004).

5.3 Optimal conditions for retroviral-mediated transduction of HSPCs

The development of an optimal cytokine cocktail for retroviral transduction of primitive hematopoietic cells has been challenging. To optimize gene transfer into quiescent HSCs, the cytokine combination needs to promote entry into cell cycle but at the same time provide a cytokine milieu that minimizes differentiation, maintains the ability of the cells to engraft efficiently in the host and still undergo self renewal. A number of cytokine cocktails have promoted reasonable levels of gene transfer. (Dunbar et al., 1996; Hematti et al., 2004; Horn et al., 2002b; Kiem et al., 1998; Kiem et al., 1997a; Kiem et al., 2002; Kurre et al., 2001a; Tisdale et al., 1998; Wu et al., 2000). For example, 72 hr to 96 hr transduction periods for gamma-retroviral transductions and 18 hr to 24hr for lentiviral and foamy virus transductions has yielded reasonable levels of gene marking *in vitro* and *in vivo*. The immobilization of the fibronectin fragment, Retronectin (originally referred to as CH-296) on plates prior to transduction improves transduction efficiencies of numerous cell types (Donahue et al., 2001; Hanenberg et al., 1996; Kiem et al., 1998; Vassilopoulos et al., 2001; Wu et al., 2000) by co-localizing VLA-4⁺ and/or VLA-5⁺ target cells and viral particles on the plates, and in addition, via integrin-mediated signaling, promotes survival and improves stem-cell engraftment (Dao et al., 1998; Donahue et al., 2001).

6. Expression of drug-resistance genes in HSPCs

A strategy for protecting HSPCs from genotoxic stress caused by alkylator therapy is to overexpress DNA-repair proteins in these cells. As long as expression levels of the DNA repair protein are adequate during the treatment phase, this is an attractive approach since the DNA damage caused by the therapy could be adequately repaired in the hematopoietic cells. Depending on the doses of therapy used, one could use alkylators to preferentially expand transduced versus non-transduced cells, or if the treatment is an actual cancer therapy which would need to be administered at higher levels and more frequently, this approach could be used to kill cancer cells and protect the highly susceptible bone-marrow cells from the therapy.

In terms of utilizing a gene-therapy approach to serve as a chemoprotectant of the HSPCs, there are three central questions regarding the introduction of genes into hematopoietic cells that render them resistant to chemotherapy. First, is it possible for genetically modified cells to be amplified *in vivo* and still maintain self-renewal capacity? Second, is sufficient and sustained expression of the chemotherapy-resistant gene obtained so that low dose-induced selection of transduced cells or bone-marrow toxicity typically associated with intensive cancer chemotherapy can be significantly reduced? Third, will hematopoiesis proceed in a normal manner in the post-chemotherapy recovery period? There is a theoretical concern that HSC depletion or exhaustion may ensue. In this case, small numbers of transduced stem cells would be pushed to preferentially expand and differentiate on a continuous basis. Although self-renewal would be presumably be an integral part of this process, overtime preferential differentiation beyond normal hematopoietic limits may lead to extinction of the transduced stem-cell pool (Pollok, 2003). The expression of chemoprotective gene products such as the multidrug resistance protein (MDR1) (Abonour et al., 2000; Hanania et al., 1995; Hesdorffer et al., 1998; Hildinger et al., 1998; Schiedlmeier et al., 2000), dihydrofolate reductase (DHFR) (Allay et al., 1998; Brenner et al., 1993b; Corey et al., 1990; Persons et al., 2004; Warlick et al., 2002; Williams et al., 1987), cytidine deaminase gene (Beausejour et al., 2001; Eliopoulos et al., 2002; Momparler et al., 1996; Rattmann et al., 2006) and MGMT (Cai

et al., 2008; Cai et al., 2006; Cai et al., 2011; Cai et al., 2005; Chinnasamy et al., 1998b; Davis et al., 2000; Davis et al., 1999; Hickson et al., 1998; Jansen et al., 2001; Jansen et al., 2002; Moritz et al., 1995; Neff et al., 2003; Ragg et al., 2000; Sawai et al., 2001) have been thoroughly investigated as potential candidates to protect hematopoietic cells residing in the bone marrow. A number of retroviral vectors derived from gamma-retroviruses, lentiviruses, and foamy viruses as described above continue to be tested for delivery of drug-resistance genes to primitive hematopoietic cells.

7. *In vivo* selection and protection of chemoresistant HSPCs

Studies using small and large animal models continue to investigate the consequences and potential of using genotoxic stress to select *in vivo* for retrovirally transduced HSPCs. A powerful tool for *in-vivo* selection of HSPCs is overexpression of a mutant form of the MGMT DNA repair protein, MGMT^{P140K}, which is resistant to the MGMT inhibitor, O⁶-benzylguanine (O⁶-BG) (Figure 2). Overexpression of MGMT^{P140K} in HSC derived from mice, canines, nonhuman primates, and humanized xenograft models, shows promise as an approach for selection and protection of the MGMT^{P140K}-transduced HSPCs following administration of O⁶-BG in combination with alkylators such as BCNU, CCNU, or TMZ (Gerson, 2002).

If MGMT^{P140K} expression is adequate in the HSC, it should also protect the HSPC from high-dose alkylator therapy required in some cancer treatments and thereby prevent therapy-induced myelotoxicity (Figure 3). Generation of HSPC that efficiently repair DNA damage due to chemotherapy may protect patients from life-threatening cytopenias commonly observed following dose-intensified therapy. A case in point, in recent phase II clinical trials, patients with nitrosourea-resistant gliomas were simultaneously treated with O⁶-BG to deplete MGMT in the cancer cells, followed by treatment with the DNA-damaging agents, BCNU or temozolomide (Quinn et al., 2009; Quinn et al., 2002). Although lack of tumor progression was transiently observed in some patients, effective dose-escalation therapy could not be achieved due to severe hematopoietic toxicity. These studies provide clinical proof that strategies protecting HSC during dose-intensified therapy are indeed clearly needed in relapsed patients requiring high-dose alkylator therapy. There are two ways in which expression of a chemoresistant gene such as an MGMT could be used for hematopoietic gene therapy (Figure 4). First, cells transduced with an oncoretrovirus vector that co-expresses the MGMT mutant gene as well as another therapeutic gene that does not otherwise have a selective proliferative advantage could be selected or enriched for *in vivo*. In this situation, selection of transduced cells using a low-dose chemotherapeutic regimen would be desirable so as not to cause unnecessary alkylator-mediated DNA damage to other tissues. However, the requirement for alkylators which are highly genotoxic and potentially mutagenic, has dampened enthusiasm for using overexpression of MGMT mutant proteins as an *in vivo*-selection tool. Second, as mentioned above, expression of the MGMT mutant would be utilized as a means of protecting hematopoietic cells from high-dose alkylator therapy designed to kill tumor cells. In the cancer patient setting, the use of MGMT to actually protect the hematopoietic cells from alkylator-based regimens used to treat cancers, such as glioblastoma and melanoma is a reasonable and clinically appropriate use of this chemoprotective mechanism. The use of bicistronic oncoretrovirus vectors that co-express MGMT mutant proteins and the enhanced green fluorescent protein (EGFP) have aided in the evaluation of *in vivo* selection of transduced hematopoietic cells (Figure 4).

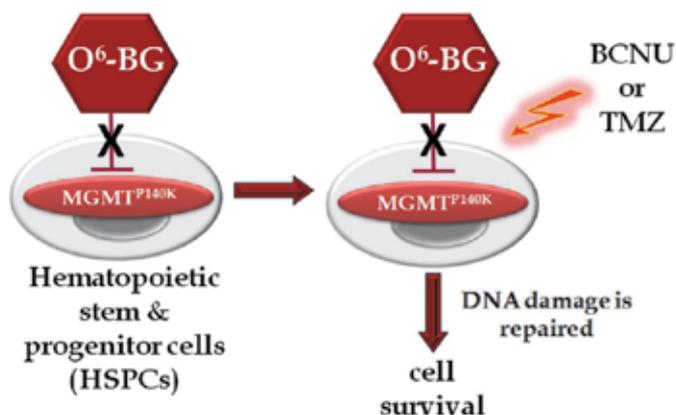


Fig. 2. Creation of chemoresistant HSPCs by expression of a mutant MGMT protein. HSPCs are transduced with a retroviral vector that encodes for a mutant MGMT protein such as MGMT^{P140K}. If expression levels of the MGMT^{P140K} transgene are sufficient and sustained, then the transduced cells will be able to repair the DNA damage caused by alkylators such as BCNU and TMZ. Nontransduced HSPCs will only express wild-type MGMT and therefore will not survive since wild-type activity will be inhibited by the MGMT inhibitor, O⁶-benzylguanine (O⁶-BG). While many mutant MGMT molecules have been tested, the MGMT^{P140K} has shown the most consistency in terms of *in vivo* selection in a variety of animal models.

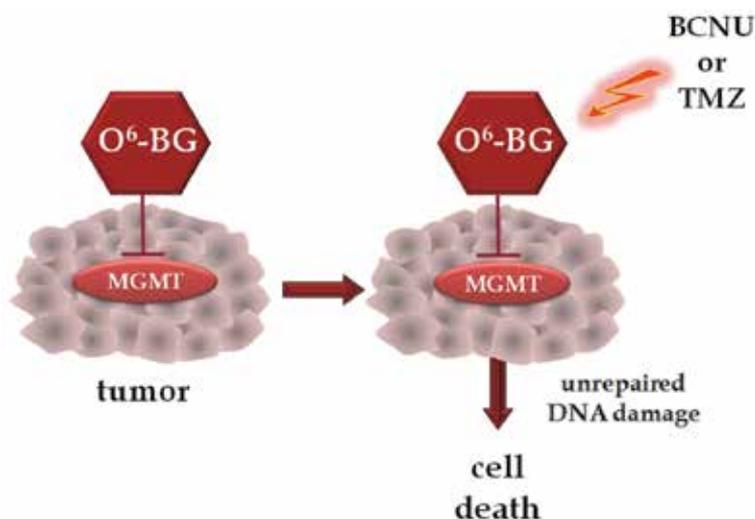


Fig. 3. Increased tumor-cell killing by sensitizing tumor cells to standard-of-care alkylator therapy. When tumor cells are exposed to MGMT inhibitors such as O⁶-BG, wild-type MGMT is irreversibly inhibited. However, *de novo* synthesis of MGMT is not blocked and will continue. Therefore, a series of bolus injections or continuous infusion of the MGMT inhibitor may be necessary to sufficiently inhibit MGMT-mediated DNA repair protein. If MGMT inhibition is complete, subsequent exposure to alkylators such as BCNU or TMZ will lead to sustained levels of DNA damage and cell death.

Protection of hematopoietic stem and progenitor cells by expression of MGMT^{P140K}

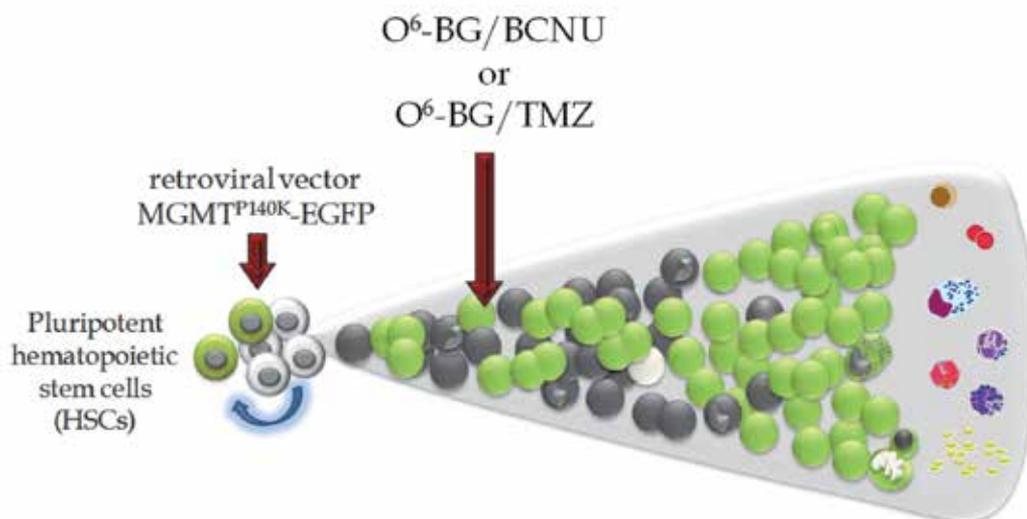


Fig. 4. *In vivo* selection of MGMT^{P140K}-EGFP-transduced hematopoietic cells. The enhanced green fluorescent protein (EGFP) has been used in a number of *in vivo* studies to follow the selection and emergence of MGMT^{P140K}-positive cells. In a successful *in vivo*-selection protocol, EGFP-positive cells (green cells) increase over time in multiple hematopoietic lineages following exposure to O⁶-BG/BCNU or O⁶-BG/TMZ.

Several groups have evaluated the efficacy of chemoprotection using different mutant forms of MGMT that are highly resistant to O⁶-BG (Davis et al., 2000; Pollok, 2003; Ragg et al., 2000; Sawai et al., 2001). Ragg *et al* compared efficacy of *in vivo* selection using MGMT^{P140A}, P140K, and wild-type MGMT. This study showed that MGMT^{P140K} expression resulted in improved protection of the bone-marrow HSCs from an O⁶-BG/BCNU chemotherapy regimen compared to the MGMT^{P140A} mutant or wild-type MGMT (Ragg et al., 2000). When compared to wild-type MGMT, the MGMT^{P140A} mutant was 40-fold more resistant to O⁶-BG and the MGMT^{P140K} mutant was 1000-fold more resistant. These data support the hypothesis that the combination therapy of O⁶-BG/BCNU effectively selects for higher numbers of murine HSC cells compared to BCNU treatment alone and that partial selection with BCNU was presumably due to survival of transduced differentiated progenitors, but inefficient selection of MGMT^{P140K}-transduced stem cells. This hypothesis was confirmed in secondary transplants studies.

TMZ continues to be intensely studied as an alternative to BCNU and may have several advantages over BCNU in terms of DNA repair and multi-organ toxicity. Myelosuppression is the primary toxicity associated with TMZ therapy and pulmonary and renal toxicity is less often observed. Therefore, it has been proposed that dose-intensified therapy to effectively kill tumors with high levels of MGMT may be more feasible with a combination

of O⁶-BG and TMZ in contrast to O⁶-BG and BCNU. Reese *et al* demonstrated *in vitro* that murine and human clonogenic cells were indeed protected from O⁶-BG and TMZ by expression of MGMT^{G156A} and were also protected to a significantly higher degree than a SW40 colon cancer line that expresses high levels of MGMT (Reese *et al.*, 1999). Sawai *et al* demonstrated effective protection and sequential selection of murine stem cells *in vivo* by expression of MGMT^{P140K} and repeated cycles of O⁶-BG and TMZ (Sawai *et al.*, 2001).

Koc *et al* treated mice with nonmyeloablative doses of O⁶-BG and BCNU and transplanted cohorts of mice with MGMT^{G156A}-transduced bone marrow cells (Koc *et al.*, 1999). Subsequently, mice were inoculated with SW480 colon cancer cells that express high levels of wild-type MGMT. In contrast to control mice, there was a 9-fold increase in resistance of clonogenic cells to BCNU and tumor growth was significantly delayed in tumor-bearing mice following multiple cycles of O⁶-BG/BCNU. These studies indicated that marrow protection from high-dose chemotherapy and simultaneous decline in tumor growth was feasible even using a tumor that is resistant to standard alkylator therapy.

7.1 Disadvantages of murine transplantation models for assessing utility of transgene over-expression in HSPCs

The use of murine stem-cell transplantation modeling clearly set a solid foundation on which to initially determine the promise of genetically modifying long-term repopulating stem cells and helped move the field of hematopoietic gene therapy forward (Dick *et al.*, 1985; Keller *et al.*, 1985; Williams *et al.*, 1984). Yet, inter-species differences between mice and humans do need to be taken into consideration as the field evaluates feasibility gene-therapy approaches for the clinic. The life span of mice is shorter compared to humans and the high gene-transfer levels obtained in murine HSCs has not been obtainable in human clinical trials. This could be due to relative levels of receptors on mouse versus human hematopoietic cells. For example, transduction of murine hematopoietic progenitor cells with murine ecotropic envelope pseudotyped gamma-retroviral vectors was highly efficient (Dick *et al.*, 1985). In contrast, transduction of canine and human progenitors with a gamma-retroviral vector pseudotyped with the amphotropic envelope used in clinical trials was much less efficient than transduction of mouse progenitors with the ecotropic envelope (Brenner *et al.*, 1993a; Stead *et al.*, 1988). This was due at least in part to low levels of the amphotropic receptor on human hematopoietic stem and progenitor cells (Orlic *et al.*, 1996). A variety of inter-species differences can dictate the transduction efficiency of primitive hematopoietic cells in mice and man including differences in cell-cycle status, telomerase activity, and repopulation capacity of the hematopoietic stem cells (Abkowitz *et al.*, 1996; Abkowitz *et al.*, 1995; Cheshier *et al.*, 1999; Guttorp *et al.*, 1990; Miller *et al.*, 1996; Naldini *et al.*, 1996; Shepherd *et al.*, 2007; Trobridge & Russell, 2004; Trobridge & Kiem, 2010). For example, in mice, a single HSC is capable of engrafting and reconstituting the entire murine hematopoietic system (Dick *et al.*, 1985; Osawa *et al.*, 1996). Additionally, sources of the hematopoietic cells could yield conflicting results; for mouse studies, Lin⁻, Sca-1⁺, c-kit⁺ (LSK) or bone-marrow derived from 5-fluorouracil-treated mice are used in contrast to human CD34⁺ cells that are the target cell population for the SCID-repopulating assay and for human gene therapy trials (Drew *et al.*, 2002; Okuno *et al.*, 2002). In contrast, except for studies using pre-leukemic or leukemic cells, the reconstitution capacity of HSCs in non-human primates and dogs appears to always be derived from polyclonal populations of HSCs. Importantly, in studies evaluating the *in vivo* safety profiles of gene-transfer

vectors, mouse cells tend to be more easily transformed than human cells, which could complicate decisions regarding the utility of new gene-therapy strategies (Hahn et al., 1999; Hahn & Weinberg, 2002; Land et al., 1983). The ease of transforming murine cells could also over-estimate the likelihood of an adverse mutagenic event and hence, not provide a realistic simulation of what to expect in human gene-therapy trials.

The long-term outcome of MGMT^{P140K} expression in conjunction with extended alkylator dosing is a critical variable in assessing the safety of this approach and the potential for mutagenic events due to incomplete repair of DNA adducts. Recently, the long-term effect of MGMT^{P140K}-mediated DNA repair and the possibility for emergence of leukemic cells was followed in a murine serial bone-marrow transplant model; the clonality of hematopoiesis in animals transplanted with *in vivo* selected MGMT^{P140K}-expressing cells was followed all the way through tertiary transplant recipients (Giordano et al., 2011). In this study, a gamma-retroviral MGMT^{P140K}-IRES-EGFP vector was utilized and the target cells were from 5-fluorouracil-treated C57Bl/6 mice. At approximately one month post-transplantation, weekly cycles of alkylator therapy were administered for 5 weeks and consisted of 25 mg/kg O⁶-BG followed 1 hour later by injection of either BCNU, 10 mg/kg, ACNU, 6 mg/kg, or TMZ, 70 mg/kg and followed over time. In secondary and tertiary recipients, mice received four weekly treatment doses. The bone-marrow cells were subsequently analyzed for selection of MGMT^{P140K+} cells and for proviral insertion sites at 3–5 weeks following the last cycle of chemotherapy. Due to the repetitive doses of therapy, all cohorts had levels of MGMT^{P140K}-transduced cells that approached 100%. While polyclonal reconstitution could be found in three primary, one secondary, and three out of four tertiary transplant recipients analyzed, monoclonal or biclonal integration patterns were observed in most 2° or 3° recipients with insertions found in intron 1 of the *Usp10* gene or downstream of the *Tubb3* gene, respectively. The administration of high- versus low-dose alkylator therapy could also dictate the clonality of the *in vivo*-selected transduced cells. In earlier studies, the selection of MGMT^{P140K}-transduced hematopoietic cells using low doses of chemotherapy allowed for the preservation of polyclonal hematopoiesis (Ball et al., 2007; Beard et al., 2009; Gerull et al., 2007). However, the outcome of myeloablative dose of therapy in large animal models still needs to be investigated.

7.2 *In vivo* selection of HSPCs in humanized mouse models

With the development of immunodeficient mouse strains, such as the nonobese diabetic/severe combined immunodeficiency mouse (NOD/SCID), human hematopoiesis can be established and gene-therapy strategies targeting primitive human CD34⁺ cells can be evaluated. (Ishikawa et al., 2005; Ohbo et al., 1996). Human hematopoietic cells that home and engraft in the bone marrow of the NOD/SCID mouse are called SCID-repopulating cells (SRC). These cells proliferate and undergo multi-lineage differentiation (Kamel-Reid & Dick, 1988; Larochelle et al., 1996; Lee et al., 2001; Wang et al., 1997; Wang et al., 1998). The NOD/SCID xenograft model has been used to study selection and protection of human cells. Although efficient transfer of genes into SRC derived from umbilical cord blood CD34⁺ cells has been demonstrated (Conneally et al., 1997; Conneally et al., 1998; Demaison et al., 2000; Hennemann et al., 1999; Kelley et al., 2000; Marandin et al., 1998; van Beusechem et al., 1992), we reported that retrovirus-mediated gene transfer into SRC derived from G-CSF-mobilized peripheral blood was markedly less efficient (Pollok et al., 2001). With this in mind, we previously tested to what extent small numbers of transduced G-CSF-mobilized

peripheral blood CD34⁺ cells could be *in vivo* selected by expression of the P140K mutant and administration of O⁶-BG/BCNU. Our laboratory demonstrated effective *in vivo* selection of primitive human hematopoietic cells in NOD/SCID mice following injection of O⁶-BG/BCNU. Up to 100% of human cells derived from umbilical cord blood or G-CSF-mobilized peripheral blood were resistant to O⁶-BG/BCNU treatment. Nonlethal doses of chemotherapy, consisting of 20 mg/kg O⁶-BG and 5 mg/kg BCNU, were used and resulted in only mild cytopenia in the NOD/SCID mice. MGMT^{P140K}-transduced cells underwent multi-lineage differentiation *in vivo* and hematopoietic cells were present after *in vivo* treatment that possessed clonogenic activity and expressed high levels of the MGMT^{P140K} activity (Cai et al., 2006).

In regards to assessing whether HSPCs can be protected from a chemotherapy regimen used to kill cancer cells, a combination therapy consisting of an O⁶-BG double bolus and BCNU has been studied by us and others. Kreklau et al showed significant regeneration of wild-type MGMT activity in tumors 24 hours post-O⁶-BG injection (Kreklau et al., 2001). For optimal numbers of covalent crosslinks between guanine and cytosine to form, it is highly desirable that MGMT activity be kept to a minimum during the first 24 hours following alkylator therapy. Therefore a high-dose chemotherapy regimen consisting of an O⁶-BG double-bolus injection (30 mg/kg followed by 15 mg/kg 8 hours later) and BCNU (10 mg/kg, administered one hour after first O⁶-BG dose) that efficiently inhibits wild-type MGMT in tumor cells for 24 hours was utilized. Using NOD/SCID mice transplanted with MGMT^{P140K}-selected murine bone marrow cells and engrafted with a human glioma, SF767, that expresses high levels of wild-type MGMT, we demonstrated significant regression of a human glioma (Kreklau et al., 2003).

Utilization of the NOD/SCID xenograft as a model to study *in vivo* selection and protection of human HSPCs during chemotherapy provides a useful model for testing new vector designs for expression of chemoresistant genes and evaluating the effect of *ex vivo* manipulation of stem and progenitor cells on hematopoiesis *in vivo*. Development of strategies to protect larger numbers of primitive clonogenic cells is needed. HSCs derived from adult sources can be nonresponsive to cytokine stimulation that is needed for integration of oncoretrovirus vectors into the host genome (Veena et al., 1998). In addition, adult HSCs lose their pluripotency due to cytokine-mediated differentiation (Gothot et al., 1998; Guenechea et al., 1999). One promising alternative to protect larger numbers of stem cells is to use other virus vectors such as lentivirus or foamy virus that are reported to more efficiently transduce nondividing stem cells. Zielske and Gerson demonstrated significant protection of human clonogenic cells *in vitro* using a lentivirus vector that expresses the MGMT^{G156A} mutant (Zielske & Gerson, 2002).

Repetitive low-dose treatment for *in vivo* selection of MGMT^{P140K}-transduced cells has been successful in mice and large animal models. (Beard et al., 2010; Neff et al., 2005) Numerous transplant studies have convincingly proven that long-term repopulating murine HSCs could be selected *in vivo* with O⁶-BG/BCNU, O⁶-BG /TMZ, or O⁶-BG /CCNU. (Cai et al., 2008; Davis et al., 2004; Jansen et al., 2002; Kreklau et al., 2003; Milsom et al., 2004; Persons et al., 2003; Persons et al., 2004; Ragg et al., 2000; Sawai et al., 2003; Sawai et al., 2001) In regards to modeling of this approach with human HSPCs, we and others previously demonstrated that MGMT^{P140K}-transduced SCID-repopulating cells and their progeny could be selected *in vivo* in NOD/SCID mice. (Pollok et al., 2003; Zielske et al., 2003) Human HSPCs derived from umbilical cord blood (UCB) or granulocyte colony-stimulating factor - mobilized peripheral blood (MPB) that expressed MGMT^{P140K} could be selected *in vivo* by

nonmyeloablative doses of O⁶-BG and BCNU. Gerson and colleagues also reported similar results using MGMT^{P140K}-transduced UCB in the NOD/SCID xenograft model. (Zielske et al., 2003) Additionally, our laboratory went on to investigate the extent to which MGMT^{P140K}-transduced human SCID-repopulating cells and progeny could be protected *in vivo* by MGMT^{P140K} expression during delivery of high-dose alkylator therapy administered that kills cancer cells. In this study, we compared the outcome of administering a low-dose O⁶-BG /BCNU regimen versus a high-dose regimen in NOD/SCID mice transplanted with MGMT^{P140K}-transduced mobilized peripheral blood CD34⁺ cells. We found that, at least in the NOD/SCID xenograft model, when human MPB were transduced with an oncoretroviral vector that expresses MGMT^{P140K}, only low numbers of human MPB cells were protected following delivery of the myeloablative regimen and that these cells were limited to mature lymphoid and myeloid cells. (Cai et al., 2006) In all these studies, NOD/SCID mice were used and analysis of long-term reconstitution in secondary recipient mice was not determined. We recently determined to what degree long-term human SCID-repopulating cells could be selected *in vivo* by alkylator therapy and to compare the levels of selection in primary and secondary NOD/SCID and NSG mice. Our data demonstrate that human hematopoietic cells of multiple lineages were capable of expressing MGMT^{P140K} for at least 4 months in primary recipients and *in vivo*-selected populations while not as robust as non-selected populations, were able to home and engraft in the bone marrow of secondary recipient NSG months for at least an additional 2 months. In contrast to the NOD/SCID xenograft model, the NSG bone-marrow microenvironment appears to allow for optimal reconstitution and feasibility of long-term follow up of human hematopoiesis (Cai, 2011). The SCID-repopulating assay, while not perfect, does provide an opportunity for testing and refining clinically promising gene-transfer vectors and *ex vivo* transduction conditions. However, the downside is that while SCID-repopulating cells can be transduced with retroviral vectors, lack of correlation with the levels of gene marking in large animal studies and human gene therapy trials is the reality (Horn et al., 2003; Mezquita et al., 2008).

7.3 *In vivo* selection of chemoresistant HSPCs in large animal models

Achieving high transduction levels in HSPCs using large animal models has been challenging and hence, these may be the models that most closely simulate the low transduction rates observed in human gene-therapy trials to date (Bodine et al., 1993; Kiem et al., 1996b; Kiem et al., 1997b). While this model can be expensive, meaningful numbers of dogs can generally be studied and data obtained more closely represents what may be expected in the human situation than studies conducted in mice or humanized mouse models. (Suter et al., 2004). Additionally, since the dog leukocyte antigen type I and II loci are fully characterized, this provides the opportunity to evaluate gene-therapy based approaches in an allogeneic transplantation setting (Ladiges et al., 1990; Maris & Storb, 2002; Nyberg et al., 2004; Suter et al., 2004; Venkataraman et al., 2007; Wagner et al., 1999). Due to the clinical applicability of the results obtained in the dog model, a large amount of effort has been devoted to optimizing the conditions in the dog model which include optimizing the procedures to mobilize HSCs, culture these cells *ex vivo*, as well as transduce them with retroviral vectors and achieve efficient engraftment *in vivo* (Goerner et al., 1999; Goerner et al., 2001; Horn et al., 2004a; Kiem et al., 2007; Kiem et al., 1996a; Kiem et al., 1999). In terms long-term primary and secondary transplantation studies in canines, more than 80% of the granulocytes can now be marked by *in vivo* selection of cells expressing the MGMT^{P140K}

(Beard et al., 2009; Neff et al., 2005). However, it is critical that some studies be performed in nonhuman primates since the hematopoietic cells derived from nonhuman primates can interact and respond to many of the human cytokines used for mobilization of HSCs as well as for transduction protocols. The availability of the macaque SHIV (simian-human immunodeficiency virus) model, that can be used to test gene-therapy strategies for acquired immunodeficiency syndrome (AIDS) is a clear advantage.(Joag, 2000; Trobridge & Kiem, 2010). Additionally in terms of long-term monitoring of animals transplanted with transduced cells, the relative similarity of the human genome to the genome of the nonhuman primates is most likely a highly relevant model for investigating retroviral-insertions sites and monitoring for retroviral-mediated insertional mutagenesis (Calmels et al., 2005).

The use of nonhuman primates to simulate potential outcomes in human gene therapy trials continues to develop. It is likely that the number of transduced cells will need to be expanded in diseases in which there is not an intrinsic growth advantage in diseases such as thalassemia and other hemoglobinopathies as well as expansion of cells that express anti-HIV transgenes. A recent report of selection in rhesus macaques resulted in only transient selection *in vivo*(Larochelle et al., 2009), but efficient and stable long-term selection of >60% in the pigtailed macaque using lentiviral vectors and 80% in the baboon model using gamma-retroviral vectors have been obtained(Trobridge & Kiem, 2010) Beard and colleagues show convincing data that MGMT^{P140K}-transduced HSPCs of multiple lineages could be selected *in vivo* in both macaques and baboon nonhuman primates. Animals with different levels of base-line engraftment were all efficiently selected with a combination of O⁶-BG and BCNU and selection of transduced cells was sustained over time. With over 2 years of follow up in some animals, there have been no signs of clonal emergence or hematopoietic malignancy. Detailed analysis of retroviral integration sites indicated the presence of multiple clones(Beard et al., 2010). One critical difference in these two studies showing transient versus sustained *in vivo* selection in the nonhuman primate models was that animals with the stable increases in selected cells had higher marking levels before initiating treatment with O⁶-BG and the alkylating agent.

8. Genotoxicity concerns for *in-vivo* selection, retroviral-vector integration and potential mutagenicity of alkylator regimens

All retroviral vectors will stably integrate into the genome of the host. Depending on where the vector integrates, its proximal integration near promoters of genes could cause increased activation of proto-oncogenes, inactivation of tumor suppressor genes, or modulate expression of genes that do not lead to noticeable changes in cellular metabolism and growth. The risk of biologically relevant proviral-mediated insertional oncogenesis has been previously documented (Baum et al., 2003). As mentioned previously, the most publicized adverse event due to insertional mutagenesis was documented in the X-SCID trial and was predominantly caused by dysregulated expression of the LMO2 proto-oncogene (Baum et al., 2003; Baum et al., 2006; Ferguson et al., 2005; Hacein-Bey-Abina et al., 2003b; Williams & Baum, 2003). Following the reports that retroviral-insertional mutagenesis was directly linked to the clonal expansion that caused leukemia in the French X-linked SCID gene therapy trial(Hacein-Bey-Abina et al., 2003b), a comprehensive study of large animals that had been previously transplanted with gamma-retroviral-transduced HSCs was conducted

(Kiem et al., 2004). In rhesus macaques, baboons, and dogs that had high levels of marked hematopoietic cells, oligoclonal or monoclonal expansion was not evident. However, at ~5 years post-transplantation, in one rhesus macaque transplanted with gamma-retroviral vector-transduced CD34⁺ cells acute myeloid leukemia did develop (Seggewiss et al., 2006). Analysis of the leukemic cells tumor showed two clonal retroviral-vector insertions one of which was located near the anti-apoptotic gene, BCL2-A1. It is important to note that the X-SCID disease itself could also be an underlying factor that contributed to the leukemogenesis observed in some patients in the X-SCID trial.

O⁶-methylating and chloroethylating agents such as TMZ and BCNU (Debiak et al., 2004; Hong et al., 1999; Kaina et al., 2001; Roos et al., 2007; Sanada et al., 2004) place an alkyl lesion at the O⁶ position of guanine. The O⁶-alkylguanine adducts can be highly mutagenic and cause cell death. The mechanisms by which O⁶-methylating agents, such as TMZ, is via futile cycles of mismatch repair pathway which ultimately lead to double-strand DNA breaks. For the chloroethylating agents, a variety of intracellular mechanisms can lead to cell death; the major toxicity is through the creation of interstrand DNA cross-links that block replication (Gerson, 2004; Kaina, 2004; Margison et al., 2002; Rasouli-Nia et al., 1994). The formation of chloroethyl and methyl lesions at the O⁶ position of guanine by alkylating agents are highly toxic to cells. In addition, other DNA alkyl adducts formed by exposure to these drugs, actually represent the vast majority of the adducts-N³ position of adenine; N⁷ position of guanine; O² position of thymine and O⁴ position of thymine(Gerson, 2004). The long-term impact of these lesions if left unrepaired is not known but clearly has the potential to lead to genome instability and subsequent emergence of transformed cells. A malignant phenotype has been described for some of these adducts in cell lines and rodent tissues(Maher et al., 1990; Sukumar & Barbacid, 1990; Vogel et al., 1996). While this is still an area of active investigation, there is no evidence to date that overexpression of mutant forms of MGMT and exposure to alkylating agents can lead to leukemia. To this end, several groups have demonstrated that hematopoietic cells transduced with retroviral vectors that express MGMTmutant proteins do not show an increased in the frequency of mutations or chromosomal aberrations upon challenge with O⁶ alkylating agents(Allay et al., 1997; Chinnasamy et al., 1998a; Chinnasamy et al., 1998b; Dumenco et al., 1993; Fairbairn et al., 2000; Liu et al., 1994; Liu et al., 1999; Reese et al., 2001). Additionally, there have been no signs of hematopoietic clonal expansion in a canine large animal model in which animals transplanted animals MGMT^{P140K}-transduced cells received alkylator therapy over a prolonged time period that also included some escalation in alkylator dose. There was a significant selection of the transduced cells over time, but whether dosing levels were indeed high enough to push the envelope on creating a mutagenic event in the genome and whether the regimen reached the dose range required to kill cancer cells is not clear at this time. (Neff et al., 2005). At least in the canines analyzed so far in which up to a 3-year follow up has been completed, there were no signs of mono- or oligo-clonal hematopoiesis or full blown leukemia in animals that underwent the MGMT^{P140K}-dependent *in vivo* chemoselection. (Neff et al., 2006; Neff et al., 2005). In a rhesus macaque that was transplanted with DHFR-transduced cells and underwent an anti-folate *in-vivo* selection regimen, an acute leukemia emerged at 5 years post-transplant and 3 years post-selection. (Seggewiss et al., 2006). Two proviral insertions were found in the leukemic clone; one of the insertion sites was located near the anti-apoptotic gene BCL2-A1. Investigation and

testing in large animal models over a prolonged time period will be able to uncover whether potential toxicities linked to a particular transduction and/or chemoselection approach will yield clinically relevant information.

9. Conclusions

The potential for *in vivo* selection-induced stem-cell exhaustion does remain a concern. The use of small molecule MGMT inhibitors in combination with alkylating agents represent the most potent *in vivo* selection regimen characterized in all animal models studied to date. (Hobin & Fairbairn, 2002; Sawai et al., 2003). Due to the alkylator-mediated death and loss of hematopoietic cells that do not express the MGMT transgene, as well as the immense pressure that is placed on the MGMT^{P140K}-expressing cells to repopulate the marrow, these populations are forced to undergo extreme degrees of expansion. At what point the gene-modified HSC can no longer self-renew and undergo exhaustion and depletion is not clear. While we and others have demonstrated that O⁶-BG-resistant MGMT-transduced human CD34⁺ cells can be selected *in vivo*, the dose level and number of cycles could be critical factors that will tip the balance between adequate self-renewal and re-population versus stem-cell exhaustion and subsequent bone-marrow failure. While human CD34⁺ cells could be selected *in vivo* using chemoselection, a high-dose O⁶-BG/BCNU regimen appeared to result in a loss of transduced primitive human cells in the NOD-SCID xenograft model (Cai et al., 2006; Cai et al., 2011; Pollok et al., 2003). Our work clearly highlights the need to further define whether *in vivo* selection regimens place a detrimental proliferative stress on transduced HSPCs. (Neff et al., 2006; Neff et al., 2005). The result of using myeloablative alkylator regimens which would be required in a cancer therapy setting will require further investigations in large animal models to determine the long-term efficacy and safety of this approach.

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11. References

- Abkowitz, J. L., et al. (1996). Evidence That Hematopoiesis May Be a Stochastic Process *in vivo*. *Nat Med*, Vol. 2, No. 2, pp. 190-197, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- Abkowitz, J. L., et al. (1995). Behavior of Hematopoietic Stem Cells in a Large Animal. *Proc Natl Acad Sci U S A*, Vol. 92, No. 6, pp. 2031-2035, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Abonour, R., et al. (2000). Efficient Retrovirus-Mediated Transfer of the Multidrug Resistance 1 Gene into Autologous Human Long-Term Repopulating Hematopoietic Stem Cells. *Nat Med*, Vol. 6, No. 6, pp. 652-658, ISSN 1078-8956 (Print) 1078-8956 (Linking)

- Aiuti, A., et al. (2009). Gene Therapy for Immunodeficiency Due to Adenosine Deaminase Deficiency. *N Engl J Med*, Vol. 360, No. 5, pp. 447-458, ISSN 1533-4406 (Electronic) 0028-4793 (Linking)
- Akkina, R. K., et al. (1996). High-Efficiency Gene Transfer into Cd34+ Cells with a Human Immunodeficiency Virus Type 1-Based Retroviral Vector Pseudotyped with Vesicular Stomatitis Virus Envelope Glycoprotein G. *J Virol*, Vol. 70, No. 4, pp. 2581-2585, ISSN 0022-538X (Print) 0022-538X (Linking)
- Allay, E., et al. (1997). Potentiation of Lymphomagenesis by Methylnitrosourea in Mice Transgenic for Lmo1 Is Blocked by O6-Alkylguanine DNA-Alkyltransferase. *Oncogene*, Vol. 15, No. 17, pp. 2127-2132, ISSN 0950-9232 (Print) 0950-9232 (Linking)
- Allay, J. A., et al. (1998). *In vivo* Selection of Retrovirally Transduced Hematopoietic Stem Cells. *Nat Med*, Vol. 4, No. 10, pp. 1136-1143, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- An, D. S., et al. (2001). Lentivirus Vector-Mediated Hematopoietic Stem Cell Gene Transfer of Common Gamma-Chain Cytokine Receptor in Rhesus Macaques. *J Virol*, Vol. 75, No. 8, pp. 3547-3555, ISSN 0022-538X (Print) 0022-538X (Linking)
- An, D. S., et al. (2000). Marking and Gene Expression by a Lentivirus Vector in Transplanted Human and Nonhuman Primate Cd34(+) Cells. *J Virol*, Vol. 74, No. 3, pp. 1286-1295, ISSN 0022-538X (Print) 0022-538X (Linking)
- Andrews, R. G., et al. (1992). Cd34+ Marrow Cells, Devoid of T and B Lymphocytes, Reconstitute Stable Lymphopoiesis and Myelopoiesis in Lethally Irradiated Allogeneic Baboons. *Blood*, Vol. 80, No. 7, pp. 1693-1701, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Ball, C. R., et al. (2007). Stable Differentiation and Clonality of Murine Long-Term Hematopoiesis after Extended Reduced-Intensity Selection for Mgmt P140k Transgene Expression. *Blood*, Vol. 110, No. 6, pp. 1779-1787, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Baum, C., et al. (2003). Side Effects of Retroviral Gene Transfer into Hematopoietic Stem Cells. *Blood*, Vol. 101, No. 6, pp. 2099-2114, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Baum, C., et al. (2006). Mutagenesis and Oncogenesis by Chromosomal Insertion of Gene Transfer Vectors. *Hum Gene Ther*, Vol. 17, No. 3, pp. 253-263, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Beard, B. C., et al. (2007). Comparison of Hiv-Derived Lentiviral and Mlv-Based Gammaretroviral Vector Integration Sites in Primate Repopulating Cells. *Mol Ther*, Vol. 15, No. 7, pp. 1356-1365, ISSN 1525-0024 (Electronic) 1525-0016 (Linking)
- Beard, B. C. & Kiem H. P. (2009). Canine Models of Gene-Modified Hematopoiesis. *Methods Mol Biol*, Vol. 506, No. pp. 341-361, ISSN 1064-3745 (Print) 1064-3745 (Linking)
- Beard, B. C., et al. (2009). Long-Term Polyclonal and Multilineage Engraftment of Methylguanine Methyltransferase P140k Gene-Modified Dog Hematopoietic Cells in Primary and Secondary Recipients. *Blood*, Vol. 113, No. 21, pp. 5094-5103, ISSN 1528-0020 (Electronic) 0006-4971 (Linking)
- Beard, B. C., et al. (2010). Efficient and Stable Mgmt-Mediated Selection of Long-Term Repopulating Stem Cells in Nonhuman Primates. *J Clin Invest*, Vol. 120, No. 7, pp. 2345-2354, ISSN 1558-8238 (Electronic) 0021-9738 (Linking)
- Beausejour, C. M., et al. (2001). Selection of Drug-Resistant Transduced Cells with Cytosine Nucleoside Analogs Using the Human Cytidine Deaminase Gene. *Cancer Gene Ther*, Vol. 8, No. 9, pp. 669-676, ISSN 0929-1903 (Print) 0929-1903 (Linking)

- Bekker-Jensen, S. & Mailand N. (2010). Assembly and Function of DNA Double-Strand Break Repair Foci in Mammalian Cells. *DNA Repair (Amst)*, Vol. 9, No. 12, pp. 1219-1228, ISSN 1568-7856 (Electronic) 1568-7856 (Linking)
- Belcheva, A., et al. (2010). Missing Mismatch Repair: A Key to T Cell Immortality. *Leuk Lymphoma*, Vol. 51, No. 10, pp. 1777-1778, ISSN 1029-2403 (Electronic) 1026-8022 (Linking)
- Berenson, R. J., et al. (1988). Antigen Cd34+ Marrow Cells Engraft Lethally Irradiated Baboons. *J Clin Invest*, Vol. 81, No. 3, pp. 951-955, ISSN 0021-9738 (Print) 0021-9738 (Linking)
- Bodine, D. M., et al. (1993). Long-Term *in vivo* Expression of a Murine Adenosine Deaminase Gene in Rhesus Monkey Hematopoietic Cells of Multiple Lineages after Retroviral Mediated Gene Transfer into Cd34+ Bone Marrow Cells. *Blood*, Vol. 82, No. 7, pp. 1975-1980, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Brenner, M. K., et al. (1993a). Gene Marking to Determine Whether Autologous Marrow Infusion Restores Long-Term Haemopoiesis in Cancer Patients. *Lancet*, Vol. 342, No. 8880, pp. 1134-1137, ISSN 0140-6736 (Print) 0140-6736 (Linking)
- Brenner, M. K., et al. (1993b). Gene-Marking to Trace Origin of Relapse after Autologous Bone-Marrow Transplantation. *Lancet*, Vol. 341, No. 8837, pp. 85-86, ISSN 0140-6736 (Print) 0140-6736 (Linking)
- Burns, J. C., et al. (1993). Vesicular Stomatitis Virus G Glycoprotein Pseudotyped Retroviral Vectors: Concentration to Very High Titer and Efficient Gene Transfer into Mammalian and Nonmammalian Cells. *Proc Natl Acad Sci U S A*, Vol. 90, No. 17, pp. 8033-8037, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Buschfort-Papewalis, C., et al. (2002). Down-Regulation of DNA Repair in Human Cd34(+) Progenitor Cells Corresponds to Increased Drug Sensitivity and Apoptotic Response. *Blood*, Vol. 100, No. 3, pp. 845-853, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Cai, S., et al. (2008). *In vivo* Selection of Hematopoietic Stem Cells Transduced at a Low Multiplicity-of-Infection with a Foamy Viral Mgmt(P140k) Vector. *Exp Hematol*, Vol. 36, No. 3, pp. 283-292, ISSN 0301-472X (Print) 0301-472X (Linking)
- Cai, S., et al. (2006). *In vivo* Effects of Myeloablative Alkylator Therapy on Survival and Differentiation of MgmtP140k-Transduced Human G-Csf-Mobilized Peripheral Blood Cells. *Mol Ther*, Vol. 13, No. 5, pp. 1016-1026, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Cai, S., et al. (2011). Humanized Bone Marrow Mouse Model as a Preclinical Tool to Assess Therapy-Mediated Hematotoxicity. *Clin Cancer Res*, Vol. No., ISSN 1078-0432 (Electronic) 1078-0432 (Linking)
- Cai, S., et al. (2005). Mitochondrial Targeting of Human O6-Methylguanine DNA Methyltransferase Protects against Cell Killing by Chemotherapeutic Alkylating Agents. *Cancer Res*, Vol. 65, No. 8, pp. 3319-3327, ISSN 0008-5472 (Print) 0008-5472 (Linking)
- Cai, S. W., H;Bailey,B;Pollok,K (2011). Differential Secondary Reconstitution of *in vivo*-Selected Human Scid-Repopulating Cells in Nod/Scid Versus Nod/Scid/γ Chainnull Mice. *Bone Marrow Research*, Vol. 2011, No. p. 11 pages, ISSN
- Calmels, B., et al. (2005). Recurrent Retroviral Vector Integration at the Mds1/Evi1 Locus in Nonhuman Primate Hematopoietic Cells. *Blood*, Vol. 106, No. 7, pp. 2530-2533, ISSN 0006-4971 (Print) 0006-4971 (Linking)

- Case, S. S., et al. (1999). Stable Transduction of Quiescent Cd34(+)Cd38(-) Human Hematopoietic Cells by Hiv-1-Based Lentiviral Vectors. *Proc Natl Acad Sci U S A*, Vol. 96, No. 6, pp. 2988-2993, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Cavazzana-Calvo, M., et al. (2000). Gene Therapy of Human Severe Combined Immunodeficiency (Scid)-X1 Disease. *Science*, Vol. 288, No. 5466, pp. 669-672, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Chen, J., et al. (2008). Enrichment of Hematopoietic Stem Cells with Slam and Lsk Markers for the Detection of Hematopoietic Stem Cell Function in Normal and Trp53 Null Mice. *Exp Hematol*, Vol. 36, No. 10, pp. 1236-1243, ISSN 0301-472X (Print) 0301-472X (Linking)
- Cheshier, S. H., et al. (1999). *In vivo* Proliferation and Cell Cycle Kinetics of Long-Term Self-Renewing Hematopoietic Stem Cells. *Proc Natl Acad Sci U S A*, Vol. 96, No. 6, pp. 3120-3125, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Chinnasamy, N., et al. (1998a). Modulation of O6-Alkylating Agent Induced Clastogenicity by Enhanced DNA Repair Capacity of Bone Marrow Cells. *Mutat Res*, Vol. 416, No. 1-2, pp. 1-10, ISSN 0027-5107 (Print) 0027-5107 (Linking)
- Chinnasamy, N., et al. (1998b). Chemoprotective Gene Transfer Ii: Multilineage *in vivo* Protection of Haemopoiesis against the Effects of an Antitumour Agent by Expression of a Mutant Human O6-Alkylguanine-DNA Alkyltransferase. *Gene Ther*, Vol. 5, No. 6, pp. 842-847, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Cone, R. D. & Mulligan R. C. (1984). High-Efficiency Gene Transfer into Mammalian Cells: Generation of Helper-Free Recombinant Retrovirus with Broad Mammalian Host Range. *Proc Natl Acad Sci U S A*, Vol. 81, No. 20, pp. 6349-6353, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Conneally, E., et al. (1997). Expansion *in vitro* of Transplantable Human Cord Blood Stem Cells Demonstrated Using a Quantitative Assay of Their Lympho-Myeloid Repopulating Activity in Nonobese Diabetic-Scid/Scid Mice. *Proc Natl Acad Sci U S A*, Vol. 94, No. 18, pp. 9836-9841, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Conneally, E., et al. (1998). Efficient Retroviral-Mediated Gene Transfer to Human Cord Blood Stem Cells with *in vivo* Repopulating Potential. *Blood*, Vol. 91, No. 9, pp. 3487-3493, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Corey, C. A., et al. (1990). Serial Transplantation of Methotrexate-Resistant Bone Marrow: Protection of Murine Recipients from Drug Toxicity by Progeny of Transduced Stem Cells. *Blood*, Vol. 75, No. 2, pp. 337-343, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Dao, M. A., et al. (1998). Adhesion to Fibronectin Maintains Regenerative Capacity During Ex Vivo Culture and Transduction of Human Hematopoietic Stem and Progenitor Cells. *Blood*, Vol. 92, No. 12, pp. 4612-4621, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Davis, B. M., et al. (2004). *In vivo* Selection for Human and Murine Hematopoietic Cells Transduced with a Therapeutic Mgmt Lentiviral Vector That Inhibits Hiv Replication. *Mol Ther*, Vol. 9, No. 2, pp. 160-172, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Davis, B. M., et al. (2000). Limiting Numbers of G156a O(6)-Methylguanine-DNA Methyltransferase-Transduced Marrow Progenitors Repopulate Nonmyeloablated Mice after Drug Selection. *Blood*, Vol. 95, No. 10, pp. 3078-3084, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Davis, B. M., et al. (1999). Characterization of the P140k, Pvp(138-140)Mlk, and G156a O6-Methylguanine-DNA Methyltransferase Mutants: Implications for Drug Resistance

- Gene Therapy. *Hum Gene Ther*, Vol. 10, No. 17, pp. 2769-2778, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- De Palma, M., et al. (2005). Promoter Trapping Reveals Significant Differences in Integration Site Selection between Mlv and Hiv Vectors in Primary Hematopoietic Cells. *Blood*, Vol. 105, No. 6, pp. 2307-2315, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Debiak, M., et al. (2004). Loss of Atm Sensitizes against O6-Methylguanine Triggered Apoptosis, Sces and Chromosomal Aberrations. *DNA Repair (Amst)*, Vol. 3, No. 4, pp. 359-368, ISSN 1568-7864 (Print) 1568-7856 (Linking)
- Demaison, C., et al. (2000). A Defined Window for Efficient Gene Marking of Severe Combined Immunodeficient-Repopulating Cells Using a Gibbon Ape Leukemia Virus-Pseudotyped Retroviral Vector. *Hum Gene Ther*, Vol. 11, No. 1, pp. 91-100, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Dick, J. E., et al. (1985). Introduction of a Selectable Gene into Primitive Stem Cells Capable of Long-Term Reconstitution of the Hemopoietic System of W/W^v Mice. *Cell*, Vol. 42, No. 1, pp. 71-79, ISSN 0092-8674 (Print) 0092-8674 (Linking)
- Donahue, R. E., et al. (2001). Fibronectin Fragment Ch-296 Inhibits Apoptosis and Enhances Ex Vivo Gene Transfer by Murine Retrovirus and Human Lentivirus Vectors Independent of Viral Tropism in Nonhuman Primate Cd34+ Cells. *Mol Ther*, Vol. 3, No. 3, pp. 359-367, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Drew, E., et al. (2002). Cd34 Is a Specific Marker of Mature Murine Mast Cells. *Exp Hematol*, Vol. 30, No. 10, pp. 1211-1218, ISSN 0301-472X (Print) 0301-472X (Linking)
- Dumble, M., et al. (2007). The Impact of Altered P53 Dosage on Hematopoietic Stem Cell Dynamics During Aging. *Blood*, Vol. 109, No. 4, pp. 1736-1742, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Dumenco, L. L., et al. (1993). The Prevention of Thymic Lymphomas in Transgenic Mice by Human O6-Alkylguanine-DNA Alkyltransferase. *Science*, Vol. 259, No. 5092, pp. 219-222, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Dunbar, C. E. (2005). Stem Cell Gene Transfer: Insights into Integration and Hematopoiesis from Primate Genetic Marking Studies. *Ann N Y Acad Sci*, Vol. 1044, No. pp. 178-182, ISSN 0077-8923 (Print) 0077-8923 (Linking)
- Dunbar, C. E., et al. (1995). Retrovirally Marked Cd34-Enriched Peripheral Blood and Bone Marrow Cells Contribute to Long-Term Engraftment after Autologous Transplantation. *Blood*, Vol. 85, No. 11, pp. 3048-3057, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Dunbar, C. E., et al. (1996). Improved Retroviral Gene Transfer into Murine and Rhesus Peripheral Blood or Bone Marrow Repopulating Cells Primed *in vivo* with Stem Cell Factor and Granulocyte Colony-Stimulating Factor. *Proc Natl Acad Sci U S A*, Vol. 93, No. 21, pp. 11871-11876, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Eliopoulos, N., et al. (2002). Human Cytidine Deaminase as an Ex Vivo Drug Selectable Marker in Gene-Modified Primary Bone Marrow Stromal Cells. *Gene Ther*, Vol. 9, No. 7, pp. 452-462, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Emi, N., et al. (1991). Pseudotype Formation of Murine Leukemia Virus with the G Protein of Vesicular Stomatitis Virus. *J Virol*, Vol. 65, No. 3, pp. 1202-1207, ISSN 0022-538X (Print) 0022-538X (Linking)

- Fairbairn, L. J., et al. (2000). Enhancing Hemopoietic Drug Resistance: A Rationale for Reconsidering the Clinical Use of Mitozolomide. *Cancer Gene Ther*, Vol. 7, No. 2, pp. 233-239, ISSN 0929-1903 (Print) 0929-1903 (Linking)
- Ferguson, C., et al. (2005). Hematopoietic Stem Cell Gene Therapy: Dead or Alive? *Trends Biotechnol*, Vol. 23, No. 12, pp. 589-597, ISSN 0167-7799 (Print) 0167-7799 (Linking)
- Gerson, S. L. (2002). Clinical Relevance of Mgmt in the Treatment of Cancer. *J Clin Oncol*, Vol. 20, No. 9, pp. 2388-2399, ISSN 0732-183X (Print) 0732-183X (Linking)
- Gerson, S. L. (2004). Mgmt: Its Role in Cancer Aetiology and Cancer Therapeutics. *Nat Rev Cancer*, Vol. 4, No. 4, pp. 296-307, ISSN 1474-175X (Print) 1474-175X (Linking)
- Gerull, S., et al. (2007). *In vivo* Selection and Chemoprotection after Drug Resistance Gene Therapy in a Nonmyeloablative Allogeneic Transplantation Setting in Dogs. *Hum Gene Ther*, Vol. 18, No. 5, pp. 451-456, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Giordano, F. A., et al. (2011). Clonal Inventory Screens Uncover Monoclonality Following Serial Transplantation of Mgmt(P140k)-Transduced Stem Cells and Dose-Intense Chemotherapy. *Hum Gene Ther*, Vol. No., ISSN 1557-7422 (Electronic) 1043-0342 (Linking)
- Glassner, B. J., et al. (1999). DNA Repair Methyltransferase (Mgmt) Knockout Mice Are Sensitive to the Lethal Effects of Chemotherapeutic Alkylating Agents. *Mutagenesis*, Vol. 14, No. 3, pp. 339-347, ISSN 0267-8357 (Print) 0267-8357 (Linking)
- Goerner, M., et al. (1999). The Use of Granulocyte Colony-Stimulating Factor During Retroviral Transduction on Fibronectin Fragment Ch-296 Enhances Gene Transfer into Hematopoietic Repopulating Cells in Dogs. *Blood*, Vol. 94, No. 7, pp. 2287-2292, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Goerner, M., et al. (2001). Sustained Multilineage Gene Persistence and Expression in Dogs Transplanted with Cd34(+) Marrow Cells Transduced by Rd114-Pseudotype Oncoretrovirus Vectors. *Blood*, Vol. 98, No. 7, pp. 2065-2070, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Gothot, A., et al. (1998). Cell Cycle-Related Changes in Repopulating Capacity of Human Mobilized Peripheral Blood Cd34(+) Cells in Non-Obese Diabetic/Severe Combined Immune-Deficient Mice. *Blood*, Vol. 92, No. 8, pp. 2641-2649, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Green, B. J., et al. (2004). Biodistribution of the Rd114/Mammalian Type D Retrovirus Receptor, Rdr. *J Gene Med*, Vol. 6, No. 3, pp. 249-259, ISSN 1099-498X (Print) 1099-498X (Linking)
- Guenechea, G., et al. (1999). Delayed Engraftment of Nonobese Diabetic/Severe Combined Immunodeficient Mice Transplanted with Ex Vivo-Expanded Human Cd34(+) Cord Blood Cells. *Blood*, Vol. 93, No. 3, pp. 1097-1105, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Guttorp, P., et al. (1990). A Stochastic Model for Haematopoiesis in Cats. *IMA J Math Appl Med Biol*, Vol. 7, No. 2, pp. 125-143, ISSN 0265-0746 (Print) 0265-0746 (Linking)
- Hacein-Bey-Abina, S., et al. (2003a). A Serious Adverse Event after Successful Gene Therapy for X-Linked Severe Combined Immunodeficiency. *N Engl J Med*, Vol. 348, No. 3, pp. 255-256, ISSN 1533-4406 (Electronic) 0028-4793 (Linking)
- Hacein-Bey-Abina, S., et al. (2003b). Lmo2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for Scid-X1. *Science*, Vol. 302, No. 5644, pp. 415-419, ISSN 1095-9203 (Electronic) 0036-8075 (Linking)

- Hahn, W. C., et al. (1999). Creation of Human Tumour Cells with Defined Genetic Elements. *Nature*, Vol. 400, No. 6743, pp. 464-468, ISSN 0028-0836 (Print) 0028-0836 (Linking)
- Hahn, W. C. & Weinberg R. A. (2002). Rules for Making Human Tumor Cells. *N Engl J Med*, Vol. 347, No. 20, pp. 1593-1603, ISSN 1533-4406 (Electronic) 0028-4793 (Linking)
- Halene, S., et al. (1999). Improved Expression in Hematopoietic and Lymphoid Cells in Mice after Transplantation of Bone Marrow Transduced with a Modified Retroviral Vector. *Blood*, Vol. 94, No. 10, pp. 3349-3357, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Hanania, E. G., et al. (1995). Chemotherapy Resistance to Taxol in Clonogenic Progenitor Cells Following Transduction of Cd34 Selected Marrow and Peripheral Blood Cells with a Retrovirus That Contains the Mdr-1 Chemotherapy Resistance Gene. *Gene Ther*, Vol. 2, No. 4, pp. 285-294, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Hanenberg, H., et al. (1996). Colocalization of Retrovirus and Target Cells on Specific Fibronectin Fragments Increases Genetic Transduction of Mammalian Cells. *Nat Med*, Vol. 2, No. 8, pp. 876-882, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- Hematti, P., et al. (2004). Distinct Genomic Integration of Mlv and Siv Vectors in Primate Hematopoietic Stem and Progenitor Cells. *PLoS Biol*, Vol. 2, No. 12, p. e423, ISSN 1545-7885 (Electronic) 1544-9173 (Linking)
- Hennemann, B., et al. (1999). Optimization of Retroviral-Mediated Gene Transfer to Human Nod/Scid Mouse Repopulating Cord Blood Cells through a Systematic Analysis of Protocol Variables. *Exp Hematol*, Vol. 27, No. 5, pp. 817-825, ISSN 0301-472X (Print) 0301-472X (Linking)
- Hesdorffer, C., et al. (1998). Phase I Trial of Retroviral-Mediated Transfer of the Human Mdr1 Gene as Marrow Chemoprotection in Patients Undergoing High-Dose Chemotherapy and Autologous Stem-Cell Transplantation. *J Clin Oncol*, Vol. 16, No. 1, pp. 165-172, ISSN 0732-183X (Print) 0732-183X (Linking)
- Hickson, I., et al. (1998). Chemoprotective Gene Transfer I: Transduction of Human Haemopoietic Progenitors with O6-Benzylguanine-Resistant O6-Alkylguanine-DNA Alkyltransferase Attenuates the Toxic Effects of O6-Alkylating Agents *in vitro*. *Gene Ther*, Vol. 5, No. 6, pp. 835-841, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Hildinger, M., et al. (1998). Fmev Vectors: Both Retroviral Long Terminal Repeat and Leader Are Important for High Expression in Transduced Hematopoietic Cells. *Gene Ther*, Vol. 5, No. 11, pp. 1575-1579, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Hobin, D. A. & Fairbairn L. J. (2002). Genetic Chemoprotection with Mutant O6-Alkylguanine-DNA-Alkyltransferases. *Curr Gene Ther*, Vol. 2, No. 1, pp. 1-8, ISSN 1566-5232 (Print) 1566-5232 (Linking)
- Hong, M. Y., et al. (1999). Relationship between DNA Adduct Levels, Repair Enzyme, and Apoptosis as a Function of DNA Methylation by Azoxymethane. *Cell Growth Differ*, Vol. 10, No. 11, pp. 749-758, ISSN 1044-9523 (Print) 1044-9523 (Linking)
- Horn, P. A., et al. (2004a). Efficient Lentiviral Gene Transfer to Canine Repopulating Cells Using an Overnight Transduction Protocol. *Blood*, Vol. 103, No. 10, pp. 3710-3716, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Horn, P. A., et al. (2002a). Lentivirus-Mediated Gene Transfer into Hematopoietic Repopulating Cells in Baboons. *Gene Ther*, Vol. 9, No. 21, pp. 1464-1471, ISSN 0969-7128 (Print) 0969-7128 (Linking)

- Horn, P. A., et al. (2004b). Stem Cell Gene Transfer--Efficacy and Safety in Large Animal Studies. *Mol Ther*, Vol. 10, No. 3, pp. 417-431, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Horn, P. A., et al. (2003). Distinct Hematopoietic Stem/Progenitor Cell Populations Are Responsible for Repopulating Nod/Scid Mice Compared with Nonhuman Primates. *Blood*, Vol. 102, No. 13, pp. 4329-4335, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Horn, P. A., et al. (2002b). Highly Efficient Gene Transfer into Baboon Marrow Repopulating Cells Using Galv-Pseudotype Oncoretroviral Vectors Produced by Human Packaging Cells. *Blood*, Vol. 100, No. 12, pp. 3960-3967, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Ishikawa, F., et al. (2005). Development of Functional Human Blood and Immune Systems in Nod/Scid/Il2 Receptor {Gamma} Chain(Null) Mice. *Blood*, Vol. 106, No. 5, pp. 1565-1573, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Jansen, M., et al. (2001). Protection of Hematopoietic Cells from O(6)-Alkylation Damage by O(6)-Methylguanine DNA Methyltransferase Gene Transfer: Studies with Different O(6)-Alkylating Agents and Retroviral Backbones. *Eur J Haematol*, Vol. 67, No. 1, pp. 2-13, ISSN 0902-4441 (Print) 0902-4441 (Linking)
- Jansen, M., et al. (2002). Hematoprotection and Enrichment of Transduced Cells *in vivo* after Gene Transfer of Mgmt(P140k) into Hematopoietic Stem Cells. *Cancer Gene Ther*, Vol. 9, No. 9, pp. 737-746, ISSN 0929-1903 (Print) 0929-1903 (Linking)
- Joag, S. V. (2000). Primate Models of Aids. *Microbes Infect*, Vol. 2, No. 2, pp. 223-229, ISSN 1286-4579 (Print) 1286-4579 (Linking)
- Johnson, S. M., et al. (2010). Mitigation of Hematologic Radiation Toxicity in Mice through Pharmacological Quiescence Induced by Cdk4/6 Inhibition. *J Clin Invest*, Vol. 120, No. 7, pp. 2528-2536, ISSN 1558-8238 (Electronic) 0021-9738 (Linking)
- Josephson, N. C., et al. (2004). Transduction of Long-Term and Mobilized Peripheral Blood-Derived Nod/Scid Repopulating Cells by Foamy Virus Vectors. *Hum Gene Ther*, Vol. 15, No. 1, pp. 87-92, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Josephson, N. C., et al. (2002). Transduction of Human Nod/Scid-Repopulating Cells with Both Lymphoid and Myeloid Potential by Foamy Virus Vectors. *Proc Natl Acad Sci U S A*, Vol. 99, No. 12, pp. 8295-8300, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Kaina, B. (2004). Mechanisms and Consequences of Methylating Agent-Induced Sces and Chromosomal Aberrations: A Long Road Traveled and Still a Far Way to Go. *Cytogenet Genome Res*, Vol. 104, No. 1-4, pp. 77-86, ISSN 1424-859X (Electronic) 1424-8581 (Linking)
- Kaina, B., et al. (2001). Ber, Mgmt, and Mmr in Defense against Alkylation-Induced Genotoxicity and Apoptosis. *Prog Nucleic Acid Res Mol Biol*, Vol. 68, No. pp. 41-54, ISSN 0079-6603 (Print) 0079-6603 (Linking)
- Kamel-Reid, S. & Dick J. E. (1988). Engraftment of Immune-Deficient Mice with Human Hematopoietic Stem Cells. *Science*, Vol. 242, No. 4886, pp. 1706-1709, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Keller, G., et al. (1985). Expression of a Foreign Gene in Myeloid and Lymphoid Cells Derived from Multipotent Haematopoietic Precursors. *Nature*, Vol. 318, No. 6042, pp. 149-154, ISSN 0028-0836 (Print) 0028-0836 (Linking)

- Kelley, M. R., et al. (2000). Genomic Structure and Characterization of the *Drosophila* S3 Ribosomal/DNA Repair Gene and Mutant Alleles. *DNA Cell Biol*, Vol. 19, No. 3, pp. 149-156, ISSN 1044-5498 (Print) 1044-5498 (Linking)
- Kelly, P. F., et al. (2001). Rd114-Pseudotyped Oncoretroviral Vectors. Biological and Physical Properties. *Ann N Y Acad Sci*, Vol. 938, No. pp. 262-276; discussion 276-267, ISSN 0077-8923 (Print) 0077-8923 (Linking)
- Kiem, H. P., et al. (2007). Foamy-Virus-Mediated Gene Transfer to Canine Repopulating Cells. *Blood*, Vol. 109, No. 1, pp. 65-70, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Kiem, H. P., et al. (1998). Improved Gene Transfer into Baboon Marrow Repopulating Cells Using Recombinant Human Fibronectin Fragment Ch-296 in Combination with Interleukin-6, Stem Cell Factor, Flt-3 Ligand, and Megakaryocyte Growth and Development Factor. *Blood*, Vol. 92, No. 6, pp. 1878-1886, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Kiem, H. P., et al. (1996a). Long-Term Persistence of Canine Hematopoietic Cells Genetically Marked by Retrovirus Vectors. *Hum Gene Ther*, Vol. 7, No. 1, pp. 89-96, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Kiem, H. P., et al. (1997a). Gene Transfer into Marrow Repopulating Cells: Comparison between Amphotropic and Gibbon Ape Leukemia Virus Pseudotyped Retroviral Vectors in a Competitive Repopulation Assay in Baboons. *Blood*, Vol. 90, No. 11, pp. 4638-4645, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Kiem, H. P., et al. (1996b). Marrow Transplantation for Hepatitis-Associated Aplastic Anemia: A Follow-up of Long-Term Survivors. *Biol Blood Marrow Transplant*, Vol. 2, No. 2, pp. 93-99, ISSN 1083-8791 (Print) 1083-8791 (Linking)
- Kiem, H. P., et al. (1999). Improved Gene Transfer into Canine Hematopoietic Repopulating Cells Using Cd34-Enriched Marrow Cells in Combination with a Gibbon Ape Leukemia Virus-Pseudotype Retroviral Vector. *Gene Ther*, Vol. 6, No. 6, pp. 966-972, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Kiem, H. P., et al. (1997b). Prevalence of Hepatitis G Virus in Patients with Aplastic Anemia. *Blood*, Vol. 90, No. 3, pp. 1335-1336, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Kiem, H. P., et al. (2002). Ex Vivo Selection for Oncoretrovirally Transduced Green Fluorescent Protein-Expressing Cd34-Enriched Cells Increases Short-Term Engraftment of Transduced Cells in Baboons. *Hum Gene Ther*, Vol. 13, No. 8, pp. 891-899, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Kiem, H. P., et al. (2004). Long-Term Clinical and Molecular Follow-up of Large Animals Receiving Retrovirally Transduced Stem and Progenitor Cells: No Progression to Clonal Hematopoiesis or Leukemia. *Mol Ther*, Vol. 9, No. 3, pp. 389-395, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Klug, C. A., et al. (2000). Inactivation of a Gfp Retrovirus Occurs at Multiple Levels in Long-Term Repopulating Stem Cells and Their Differentiated Progeny. *Blood*, Vol. 96, No. 3, pp. 894-901, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Koc, O. N., et al. (1999). Deltamgmt-Transduced Bone Marrow Infusion Increases Tolerance to O6-Benzylguanine and 1,3-Bis(2-Chloroethyl)-1-Nitrosourea and Allows Intensive Therapy of 1,3-Bis(2-Chloroethyl)-1-Nitrosourea-Resistant Human Colon Cancer Xenografts. *Hum Gene Ther*, Vol. 10, No. 6, pp. 1021-1030, ISSN 1043-0342 (Print) 1043-0342 (Linking)

- Kohn, D. B., et al. (1995). Engraftment of Gene-Modified Umbilical Cord Blood Cells in Neonates with Adenosine Deaminase Deficiency. *Nat Med*, Vol. 1, No. 10, pp. 1017-1023, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- Kreklau, E. L., et al. (2001). Comparison of Single- Versus Double-Bolus Treatments of O(6)-Benzylguanine for Depletion of O(6)-Methylguanine DNA Methyltransferase (Mgmt) Activity *in vivo*: Development of a Novel Fluorometric Oligonucleotide Assay for Measurement of Mgmt Activity. *J Pharmacol Exp Ther*, Vol. 297, No. 2, pp. 524-530, ISSN 0022-3565 (Print) 0022-3565 (Linking)
- Kreklau, E. L., et al. (2003). Hematopoietic Expression of O(6)-Methylguanine DNA Methyltransferase-P140k Allows Intensive Treatment of Human Glioma Xenografts with Combination O(6)-Benzylguanine and 1,3-Bis-(2-Chloroethyl)-1-Nitrosourea. *Mol Cancer Ther*, Vol. 2, No. 12, pp. 1321-1329, ISSN 1535-7163 (Print) 1535-7163 (Linking)
- Kurre, P., et al. (2002). *In vivo* Administration of Interferon Gamma Does Not Cause Marrow Aplasia in Mice with a Targeted Disruption of Fanc. *Exp Hematol*, Vol. 30, No. 11, pp. 1257-1262, ISSN 0301-472X (Print) 0301-472X (Linking)
- Kurre, P., et al. (1999). Efficient Transduction by an Amphotropic Retrovirus Vector Is Dependent on High-Level Expression of the Cell Surface Virus Receptor. *J Virol*, Vol. 73, No. 1, pp. 495-500, ISSN 0022-538X (Print) 0022-538X (Linking)
- Kurre, P., et al. (2001a). Gene Transfer into Baboon Repopulating Cells: A Comparison of Flt-3 Ligand and Megakaryocyte Growth and Development Factor Versus Il-3 During Ex Vivo Transduction. *Mol Ther*, Vol. 3, No. 6, pp. 920-927, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Kurre, P., et al. (2001b). Envelope Fusion Protein Binding Studies in an Inducible Model of Retrovirus Receptor Expression and in Cd34(+) Cells Emphasize Limited Transduction at Low Receptor Levels. *Gene Ther*, Vol. 8, No. 8, pp. 593-599, ISSN 0969-7128 (Print) 0969-7128 (Linking)
- Ladiges, W. C., et al. (1990). Canine Models of Bone Marrow Transplantation. *Lab Anim Sci*, Vol. 40, No. 1, pp. 11-15, ISSN 0023-6764 (Print) 0023-6764 (Linking)
- Land, H., et al. (1983). Tumorigenic Conversion of Primary Embryo Fibroblasts Requires at Least Two Cooperating Oncogenes. *Nature*, Vol. 304, No. 5927, pp. 596-602, ISSN 0028-0836 (Print) 0028-0836 (Linking)
- Larochelle, A., et al. (2009). *In vivo* Selection of Hematopoietic Progenitor Cells and Temozolomide Dose Intensification in Rhesus Macaques through Lentiviral Transduction with a Drug Resistance Gene. *J Clin Invest*, Vol. 119, No. 7, pp. 1952-1963, ISSN 1558-8238 (Electronic) 0021-9738 (Linking)
- Larochelle, A., et al. (1996). Identification of Primitive Human Hematopoietic Cells Capable of Repopulating Nod/Scid Mouse Bone Marrow: Implications for Gene Therapy. *Nat Med*, Vol. 2, No. 12, pp. 1329-1337, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- Laufs, S., et al. (2004). Insertion of Retroviral Vectors in Nod/Scid Repopulating Human Peripheral Blood Progenitor Cells Occurs Preferentially in the Vicinity of Transcription Start Regions and in Introns. *Mol Ther*, Vol. 10, No. 5, pp. 874-881, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Lee, K., et al. (2001). G156a Mgmt-Transduced Human Mesenchymal Stem Cells Can Be Selectively Enriched by O6-Benzylguanine and Bcnu. *J Hematother Stem Cell Res*, Vol. 10, No. 5, pp. 691-701, ISSN 1525-8165 (Print) 1525-8165 (Linking)

- Lewis, P. F. & Emerman M. (1994). Passage through Mitosis Is Required for Oncoretroviruses but Not for the Human Immunodeficiency Virus. *J Virol*, Vol. 68, No. 1, pp. 510-516, ISSN 0022-538X (Print) 0022-538X (Linking)
- Li, X., et al. (2005). Ex Vivo Culture of Fanccl-/- Stem/Progenitor Cells Predisposes Cells to Undergo Apoptosis, and Surviving Stem/Progenitor Cells Display Cytogenetic Abnormalities and an Increased Risk of Malignancy. *Blood*, Vol. 105, No. 9, pp. 3465-3471, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Li, Z., et al. (2002). Murine Leukemia Induced by Retroviral Gene Marking. *Science*, Vol. 296, No. 5567, p. 497, ISSN 1095-9203 (Electronic) 0036-8075 (Linking)
- Lieber, M. R. (2010). The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annu Rev Biochem*, Vol. 79, No. pp. 181-211, ISSN 1545-4509 (Electronic) 0066-4154 (Linking)
- Limp-Foster, M. & Kelley M. R. (2000). DNA Repair and Gene Therapy: Implications for Translational Uses. *Environ Mol Mutagen*, Vol. 35, No. 2, pp. 71-81, ISSN 0893-6692 (Print) 0893-6692 (Linking)
- Liu, L., et al. (1994). Rapid Repair of O6-Methylguanine-DNA Adducts Protects Transgenic Mice from N-Methylnitrosourea-Induced Thymic Lymphomas. *Cancer Res*, Vol. 54, No. 17, pp. 4648-4652, ISSN 0008-5472 (Print) 0008-5472 (Linking)
- Liu, L. & Gerson S. L. (2006). Targeted Modulation of Mgmt: Clinical Implications. *Clin Cancer Res*, Vol. 12, No. 2, pp. 328-331, ISSN 1078-0432 (Print) 1078-0432 (Linking)
- Liu, L., et al. (1999). Reduced Lung Tumorigenesis in Human Methylguanine DNA--Methyltransferase Transgenic Mice Achieved by Expression of Transgene within the Target Cell. *Carcinogenesis*, Vol. 20, No. 2, pp. 279-284, ISSN 0143-3334 (Print) 0143-3334 (Linking)
- Liu, Y., et al. (2009). P53 Regulates Hematopoietic Stem Cell Quiescence. *Cell Stem Cell*, Vol. 4, No. 1, pp. 37-48, ISSN 1875-9777 (Electronic)
- Maher, V. M., et al. (1990). Alkylation Damage, DNA Repair and Mutagenesis in Human Cells. *Mutat Res*, Vol. 233, No. 1-2, pp. 235-245, ISSN 0027-5107 (Print) 0027-5107 (Linking)
- Marandin, A., et al. (1998). Retrovirus-Mediated Gene Transfer into Human Cd34+38low Primitive Cells Capable of Reconstituting Long-Term Cultures *in vitro* and Nonobese Diabetic-Severe Combined Immunodeficiency Mice *in vivo*. *Hum Gene Ther*, Vol. 9, No. 10, pp. 1497-1511, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Margison, G. P., et al. (2002). Mechanisms of Carcinogenicity/Chemotherapy by O6-Methylguanine. *Mutagenesis*, Vol. 17, No. 6, pp. 483-487, ISSN 0267-8357 (Print) 0267-8357 (Linking)
- Maris, M. & Storb R. (2002). Outpatient Allografting in Hematologic Malignancies and Nonmalignant Disorders--Applying Lessons Learned in the Canine Model to Humans. *Cancer Treat Res*, Vol. 110, No. pp. 149-175, ISSN 0927-3042 (Print) 0927-3042 (Linking)
- Martin, S. A., et al. (2010). Therapeutic Targeting of the DNA Mismatch Repair Pathway. *Clin Cancer Res*, Vol. 16, No. 21, pp. 5107-5113, ISSN 1078-0432 (Print) 1078-0432 (Linking)
- Marusyk, A., et al. (2010). Irradiation Selects for P53-Deficient Hematopoietic Progenitors. *PLoS Biol*, Vol. 8, No. 3, p. e1000324, ISSN 1545-7885 (Electronic) 1544-9173 (Linking)
- Metais, J. Y. & Dunbar C. E. (2008). The Mds1-Evi1 Gene Complex as a Retrovirus Integration Site: Impact on Behavior of Hematopoietic Cells and Implications for Gene Therapy. *Mol Ther*, Vol. 16, No. 3, pp. 439-449, ISSN 1525-0024 (Electronic) 1525-0016 (Linking)

- Mezquita, P., et al. (2008). Nod/Scid Repopulating Cells Contribute Only to Short-Term Repopulation in the Baboon. *Gene Ther*, Vol. 15, No. 21, pp. 1460-1462, ISSN 1476-5462 (Electronic) 0969-7128 (Linking)
- Miller, A. D., et al. (1996). A Novel Murine Retrovirus Identified During Testing for Helper Virus in Human Gene Transfer Trials. *J Virol*, Vol. 70, No. 3, pp. 1804-1809, ISSN 0022-538X (Print) 0022-538X (Linking)
- Miller, D. G., et al. (1990). Gene Transfer by Retrovirus Vectors Occurs Only in Cells That Are Actively Replicating at the Time of Infection. *Mol Cell Biol*, Vol. 10, No. 8, pp. 4239-4242, ISSN 0270-7306 (Print) 0270-7306 (Linking)
- Milsom, M. D., et al. (2004). Enhanced *in vivo* Selection of Bone Marrow Cells by Retroviral-Mediated Coexpression of Mutant O6-Methylguanine-DNA-Methyltransferase and Hoxb4. *Mol Ther*, Vol. 10, No. 5, pp. 862-873, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Milyavsky, M., et al. (2010). A Distinctive DNA Damage Response in Human Hematopoietic Stem Cells Reveals an Apoptosis-Independent Role for P53 in Self-Renewal. *Cell Stem Cell*, Vol. 7, No. 2, pp. 186-197, ISSN 1875-9777 (Electronic)
- Miyoshi, H., et al. (1999). Transduction of Human Cd34+ Cells That Mediate Long-Term Engraftment of Nod/Scid Mice by Hiv Vectors. *Science*, Vol. 283, No. 5402, pp. 682-686, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Modlich, U., et al. (2006). Cell-Culture Assays Reveal the Importance of Retroviral Vector Design for Insertional Genotoxicity. *Blood*, Vol. 108, No. 8, pp. 2545-2553, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Modlich, U., et al. (2005). Leukemias Following Retroviral Transfer of Multidrug Resistance 1 (Mdr1) Are Driven by Combinatorial Insertional Mutagenesis. *Blood*, Vol. 105, No. 11, pp. 4235-4246, ISSN 0006-4971 (Print)
- Momparler, R. L., et al. (1996). Resistance to Cytosine Arabinoside by Retrovirally Mediated Gene Transfer of Human Cytidine Deaminase into Murine Fibroblast and Hematopoietic Cells. *Cancer Gene Ther*, Vol. 3, No. 5, pp. 331-338, ISSN 0929-1903 (Print) 0929-1903 (Linking)
- Montini, E., et al. (2006). Hematopoietic Stem Cell Gene Transfer in a Tumor-Prone Mouse Model Uncovers Low Genotoxicity of Lentiviral Vector Integration. *Nat Biotechnol*, Vol. 24, No. 6, pp. 687-696, ISSN 1087-0156 (Print) 1087-0156 (Linking)
- Moritz, T., et al. (1995). Retrovirus-Mediated Expression of a DNA Repair Protein in Bone Marrow Protects Hematopoietic Cells from Nitrosourea-Induced Toxicity *in vitro* and *in vivo*. *Cancer Res*, Vol. 55, No. 12, pp. 2608-2614, ISSN 0008-5472 (Print) 0008-5472 (Linking)
- Naka, K. & Hirao A. (2011). Maintenance of Genomic Integrity in Hematopoietic Stem Cells. *Int J Hematol*, Vol. No., ISSN 1865-3774 (Electronic) 0925-5710 (Linking)
- Naldini, L., et al. (1996). *In vivo* Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. *Science*, Vol. 272, No. 5259, pp. 263-267, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Neff, T., et al. (2006). Survival of the Fittest: *In vivo* Selection and Stem Cell Gene Therapy. *Blood*, Vol. 107, No. 5, pp. 1751-1760, ISSN 0006-4971 (Print) 0006-4971 (Linking)

- Neff, T., et al. (2005). Polyclonal Chemoprotection against Temozolomide in a Large-Animal Model of Drug Resistance Gene Therapy. *Blood*, Vol. 105, No. 3, pp. 997-1002, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Neff, T., et al. (2003). Methylguanine Methyltransferase-Mediated *in vivo* Selection and Chemoprotection of Allogeneic Stem Cells in a Large-Animal Model. *J Clin Invest*, Vol. 112, No. 10, pp. 1581-1588, ISSN 0021-9738 (Print) 0021-9738 (Linking)
- Nick McElhinny, S. A., et al. (2000). Ku Recruits the Xrcc4-Ligase Iv Complex to DNA Ends. *Mol Cell Biol*, Vol. 20, No. 9, pp. 2996-3003, ISSN 0270-7306 (Print) 0270-7306 (Linking)
- Niedernhofer, L. J. (2008). DNA Repair Is Crucial for Maintaining Hematopoietic Stem Cell Function. *DNA Repair (Amst)*, Vol. 7, No. 3, pp. 523-529, ISSN 1568-7864 (Print) 1568-7856 (Linking)
- Nienhuis, A. W., et al. (2006). Genotoxicity of Retroviral Integration in Hematopoietic Cells. *Mol Ther*, Vol. 13, No. 6, pp. 1031-1049, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Nowrouzi, A., et al. (2006). Genome-Wide Mapping of Foamy Virus Vector Integrations into a Human Cell Line. *J Gen Virol*, Vol. 87, No. Pt 5, pp. 1339-1347, ISSN 0022-1317 (Print) 0022-1317 (Linking)
- Nyberg, K., et al. (2004). Workshop on Long-Term Follow-up of Participants in Human Gene Transfer Research. *Mol Ther*, Vol. 10, No. 6, pp. 976-980, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Ohbo, K., et al. (1996). Modulation of Hematopoiesis in Mice with a Truncated Mutant of the Interleukin-2 Receptor Gamma Chain. *Blood*, Vol. 87, No. 3, pp. 956-967, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Okuno, Y., et al. (2002). Differential Regulation of the Human and Murine Cd34 Genes in Hematopoietic Stem Cells. *Proc Natl Acad Sci U S A*, Vol. 99, No. 9, pp. 6246-6251, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Orlic, D., et al. (1996). The Level of Mrna Encoding the Amphotropic Retrovirus Receptor in Mouse and Human Hematopoietic Stem Cells Is Low and Correlates with the Efficiency of Retrovirus Transduction. *Proc Natl Acad Sci U S A*, Vol. 93, No. 20, pp. 11097-11102, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Osawa, M., et al. (1996). Long-Term Lymphohematopoietic Reconstitution by a Single Cd34-Low/Negative Hematopoietic Stem Cell. *Science*, Vol. 273, No. 5272, pp. 242-245, ISSN 0036-8075 (Print) 0036-8075 (Linking)
- Ott, M. G., et al. (2006). Correction of X-Linked Chronic Granulomatous Disease by Gene Therapy, Augmented by Insertional Activation of Mds1-Evi1, Prdm16 or Setbp1. *Nat Med*, Vol. 12, No. 4, pp. 401-409, ISSN 1078-8956 (Print) 1078-8956 (Linking)
- Park, Y. & Gerson S. L. (2005). DNA Repair Defects in Stem Cell Function and Aging. *Annu Rev Med*, Vol. 56, No. pp. 495-508, ISSN 0066-4219 (Print) 0066-4219 (Linking)
- Pegg, A. E. (2011). Multifaceted Roles of Alkyltransferase and Related Proteins in DNA Repair, DNA Damage, Resistance to Chemotherapy and Research Tools. *Chem Res Toxicol*, Vol. No., ISSN 1520-5010 (Electronic) 0893-228X (Linking)
- Persons, D. A., et al. (2003). Successful Treatment of Murine Beta-Thalassemia Using *in vivo* Selection of Genetically Modified, Drug-Resistant Hematopoietic Stem Cells. *Blood*, Vol. 102, No. 2, pp. 506-513, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Persons, D. A., et al. (2004). Transient *in vivo* Selection of Transduced Peripheral Blood Cells Using Antifolate Drug Selection in Rhesus Macaques That Received Transplants with

- Hematopoietic Stem Cells Expressing Dihydrofolate Reductase Vectors. *Blood*, Vol. 103, No. 3, pp. 796-803, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Pollok, K. E. (2003). *In vivo* Protection of Hematopoietic Cells from Alkylator-Mediated DNA Damage. *Curr Hematol Rep*, Vol. 2, No. 4, pp. 341-347, ISSN 1540-3408 (Print) 1540-3408 (Linking)
- Pollok, K. E., et al. (2003). *In vivo* Selection of Human Hematopoietic Cells in a Xenograft Model Using Combined Pharmacologic and Genetic Manipulations. *Hum Gene Ther*, Vol. 14, No. 18, pp. 1703-1714, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Pollok, K. E., et al. (2001). Differential Transduction Efficiency of Scid-Repopulating Cells Derived from Umbilical Cord Blood and Granulocyte Colony-Stimulating Factor-Mobilized Peripheral Blood. *Hum Gene Ther*, Vol. 12, No. 17, pp. 2095-2108, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Porter, C. D., et al. (1996). Comparison of Efficiency of Infection of Human Gene Therapy Target Cells Via Four Different Retroviral Receptors. *Hum Gene Ther*, Vol. 7, No. 8, pp. 913-919, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Quinn, J. A., et al. (2009). Phase II Trial of Temozolomide (Tmz) Plus Irinotecan (Cpt-11) in Adults with Newly Diagnosed Glioblastoma Multiforme before Radiotherapy. *J Neurooncol*, Vol. 95, No. 3, pp. 393-400, ISSN 1573-7373 (Electronic) 0167-594X (Linking)
- Quinn, J. A., et al. (2002). Phase II Trial of Carmustine Plus O(6)-Benzylguanine for Patients with Nitrosourea-Resistant Recurrent or Progressive Malignant Glioma. *J Clin Oncol*, Vol. 20, No. 9, pp. 2277-2283, ISSN 0732-183X (Print) 0732-183X (Linking)
- Ragg, S., et al. (2000). Direct Reversal of DNA Damage by Mutant Methyltransferase Protein Protects Mice against Dose-Intensified Chemotherapy and Leads to *in vivo* Selection of Hematopoietic Stem Cells. *Cancer Res*, Vol. 60, No. 18, pp. 5187-5195, ISSN 0008-5472 (Print) 0008-5472 (Linking)
- Rasouli-Nia, A., et al. (1994). On the Quantitative Relationship between O6-Methylguanine Residues in Genomic DNA and Production of Sister-Chromatid Exchanges, Mutations and Lethal Events in a Mer- Human Tumor Cell Line. *Mutat Res*, Vol. 314, No. 2, pp. 99-113, ISSN 0027-5107 (Print) 0027-5107 (Linking)
- Rattmann, I., et al. (2006). Gene Transfer of Cytidine Deaminase Protects Myelopoiesis from Cytidine Analogs in an *in vivo* Murine Transplant Model. *Blood*, Vol. 108, No. 9, pp. 2965-2971, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Reese, J. S., et al. (2001). Overexpression of Human O6-Alkylguanine DNA Alkyltransferase (Agt) Prevents Mnu Induced Lymphomas in Heterozygous P53 Deficient Mice. *Oncogene*, Vol. 20, No. 38, pp. 5258-5263, ISSN 0950-9232 (Print) 0950-9232 (Linking)
- Reese, J. S., et al. (1999). Simultaneous Protection of G156a Methylguanine DNA Methyltransferase Gene-Transduced Hematopoietic Progenitors and Sensitization of Tumor Cells Using O6-Benzylguanine and Temozolomide. *Clin Cancer Res*, Vol. 5, No. 1, pp. 163-169, ISSN 1078-0432 (Print) 1078-0432 (Linking)
- Reiser, J., et al. (1996). Transduction of Nondividing Cells Using Pseudotyped Defective High-Titer Hiv Type 1 Particles. *Proc Natl Acad Sci U S A*, Vol. 93, No. 26, pp. 15266-15271, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Robbins, P. B., et al. (1998). Consistent, Persistent Expression from Modified Retroviral Vectors in Murine Hematopoietic Stem Cells. *Proc Natl Acad Sci U S A*, Vol. 95, No. 17, pp. 10182-10187, ISSN 0027-8424 (Print) 0027-8424 (Linking)

- Roos, W. P., et al. (2007). Apoptosis in Malignant Glioma Cells Triggered by the Temozolomide-Induced DNA Lesion O6-Methylguanine. *Oncogene*, Vol. 26, No. 2, pp. 186-197, ISSN 0950-9232 (Print) 0950-9232 (Linking)
- Rosenzweig, M., et al. (1999). Efficient and Durable Gene Marking of Hematopoietic Progenitor Cells in Nonhuman Primates after Nonablative Conditioning. *Blood*, Vol. 94, No. 7, pp. 2271-2286, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Russell, D. W. & Miller A. D. (1996). Foamy Virus Vectors. *J Virol*, Vol. 70, No. 1, pp. 217-222, ISSN 0022-538X (Print) 0022-538X (Linking)
- Sabatino, D. E., et al. (1997). Amphotropic or Gibbon Ape Leukemia Virus Retrovirus Binding and Transduction Correlates with the Level of Receptor Mrna in Human Hematopoietic Cell Lines. *Blood Cells Mol Dis*, Vol. 23, No. 3, pp. 422-433, ISSN 1079-9796 (Print) 1079-9796 (Linking)
- Sanada, M., et al. (2004). Killing and Mutagenic Actions of Dacarbazine, a Chemotherapeutic Alkylating Agent, on Human and Mouse Cells: Effects of Mgmt and Mlh1 Mutations. *DNA Repair (Amst)*, Vol. 3, No. 4, pp. 413-420, ISSN 1568-7864 (Print) 1568-7856 (Linking)
- Sandrin, V., et al. (2002). Lentiviral Vectors Pseudotyped with a Modified Rd114 Envelope Glycoprotein Show Increased Stability in Sera and Augmented Transduction of Primary Lymphocytes and Cd34+ Cells Derived from Human and Nonhuman Primates. *Blood*, Vol. 100, No. 3, pp. 823-832, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Sawai, N., et al. (2003). Reduction in Hematopoietic Stem Cell Numbers with *in vivo* Drug Selection Can Be Partially Abrogated by Hoxb4 Gene Expression. *Mol Ther*, Vol. 8, No. 3, pp. 376-384, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Sawai, N., et al. (2001). Protection and *in vivo* Selection of Hematopoietic Stem Cells Using Temozolomide, O6-Benzylguanine, and an Alkyltransferase-Expressing Retroviral Vector. *Mol Ther*, Vol. 3, No. 1, pp. 78-87, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Schiedlmeier, B., et al. (2000). Quantitative Assessment of Retroviral Transfer of the Human Multidrug Resistance 1 Gene to Human Mobilized Peripheral Blood Progenitor Cells Engrafted in Nonobese Diabetic/Severe Combined Immunodeficient Mice. *Blood*, Vol. 95, No. 4, pp. 1237-1248, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Schmutte, C., et al. (2001). The Interaction of DNA Mismatch Repair Proteins with Human Exonuclease I. *J Biol Chem*, Vol. 276, No. 35, pp. 33011-33018, ISSN 0021-9258 (Print) 0021-9258 (Linking)
- Seggewiss, R., et al. (2006). Acute Myeloid Leukemia Is Associated with Retroviral Gene Transfer to Hematopoietic Progenitor Cells in a Rhesus Macaque. *Blood*, Vol. 107, No. 10, pp. 3865-3867, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Seita, J., et al. (2010). Differential DNA Damage Response in Stem and Progenitor Cells. *Cell Stem Cell*, Vol. 7, No. 2, pp. 145-147, ISSN 1875-9777 (Electronic)
- Shepherd, B. E., et al. (2007). Hematopoietic Stem-Cell Behavior in Nonhuman Primates. *Blood*, Vol. 110, No. 6, pp. 1806-1813, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Song, B., et al. (2005). Retrovirus Restriction by Trim5alpha Variants from Old World and New World Primates. *J Virol*, Vol. 79, No. 7, pp. 3930-3937, ISSN 0022-538X (Print) 0022-538X (Linking)

- Sorrentino, B. P. (2002). Gene Therapy to Protect Haematopoietic Cells from Cytotoxic Cancer Drugs. *Nat Rev Cancer*, Vol. 2, No. 6, pp. 431-441, ISSN 1474-175X (Print) 1474-175X (Linking)
- Stead, R. B., et al. (1988). Canine Model for Gene Therapy: Inefficient Gene Expression in Dogs Reconstituted with Autologous Marrow Infected with Retroviral Vectors. *Blood*, Vol. 71, No. 3, pp. 742-747, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Stremlau, M., et al. (2004). The Cytoplasmic Body Component Trim5alpha Restricts Hiv-1 Infection in Old World Monkeys. *Nature*, Vol. 427, No. 6977, pp. 848-853, ISSN 1476-4687 (Electronic) 0028-0836 (Linking)
- Stremlau, M., et al. (2005). Species-Specific Variation in the B30.2(Spry) Domain of Trim5alpha Determines the Potency of Human Immunodeficiency Virus Restriction. *J Virol*, Vol. 79, No. 5, pp. 3139-3145, ISSN 0022-538X (Print) 0022-538X (Linking)
- Sukumar, S. & Barbacid M. (1990). Specific Patterns of Oncogene Activation in Transplacentally Induced Tumors. *Proc Natl Acad Sci U S A*, Vol. 87, No. 2, pp. 718-722, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Suter, S. E., et al. (2004). Isolation and Characterization of Pediatric Canine Bone Marrow Cd34+ Cells. *Vet Immunol Immunopathol*, Vol. 101, No. 1-2, pp. 31-47, ISSN 0165-2427 (Print) 0165-2427 (Linking)
- Tisdale, J. F., et al. (1998). Ex Vivo Expansion of Genetically Marked Rhesus Peripheral Blood Progenitor Cells Results in Diminished Long-Term Repopulating Ability. *Blood*, Vol. 92, No. 4, pp. 1131-1141, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Trobridge, G., et al. (2005). Hematopoietic Stem Cell Transduction and Amplification in Large Animal Models. *Hum Gene Ther*, Vol. 16, No. 12, pp. 1355-1366, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Trobridge, G. & Russell D. W. (2004). Cell Cycle Requirements for Transduction by Foamy Virus Vectors Compared to Those of Oncovirus and Lentivirus Vectors. *J Virol*, Vol. 78, No. 5, pp. 2327-2335, ISSN 0022-538X (Print) 0022-538X (Linking)
- Trobridge, G., et al. (2002). Gene Transfer with Foamy Virus Vectors. *Methods Enzymol*, Vol. 346, No. pp. 628-648, ISSN 0076-6879 (Print) 0076-6879 (Linking)
- Trobridge, G. D. (2011). Genotoxicity of Retroviral Hematopoietic Stem Cell Gene Therapy. *Expert Opin Biol Ther*, Vol. 11, No. 5, pp. 581-593, ISSN 1744-7682 (Electronic) 1471-2598 (Linking)
- Trobridge, G. D. & Kiem H. P. (2010). Large Animal Models of Hematopoietic Stem Cell Gene Therapy. *Gene Ther*, Vol. 17, No. 8, pp. 939-948, ISSN 1476-5462 (Electronic) 0969-7128 (Linking)
- Trobridge, G. D., et al. (2006). Foamy Virus Vector Integration Sites in Normal Human Cells. *Proc Natl Acad Sci U S A*, Vol. 103, No. 5, pp. 1498-1503, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- van Bekkum, D. W. (1978). The Rhesus Monkey as a Preclinical Model for Bone Marrow Transplantation. *Transplant Proc*, Vol. 10, No. 1, pp. 105-111, ISSN 0041-1345 (Print) 0041-1345 (Linking)
- van Beusechem, V. W., et al. (1992). Long-Term Expression of Human Adenosine Deaminase in Rhesus Monkeys Transplanted with Retrovirus-Infected Bone-Marrow Cells. *Proc Natl Acad Sci U S A*, Vol. 89, No. 16, pp. 7640-7644, ISSN 0027-8424 (Print) 0027-8424 (Linking)

- Van Beusechem, V. W. & Valerio D. (1996). Gene Transfer into Hematopoietic Stem Cells of Nonhuman Primates. *Hum Gene Ther*, Vol. 7, No. 14, pp. 1649-1668, ISSN 1043-0342 (Print)1043-0342 (Linking)
- Vassilopoulos, G., et al. (2001). Gene Transfer into Murine Hematopoietic Stem Cells with Helper-Free Foamy Virus Vectors. *Blood*, Vol. 98, No. 3, pp. 604-609, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Veena, P., et al. (1998). Delayed Targeting of Cytokine-Nonresponsive Human Bone Marrow Cd34(+) Cells with Retrovirus-Mediated Gene Transfer Enhances Transduction Efficiency and Long-Term Expression of Transduced Genes. *Blood*, Vol. 91, No. 10, pp. 3693-3701, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Venkataraman, G. M., et al. (2007). An Improved Method for Dog Leukocyte Antigen 88 Typing and Two New Major Histocompatibility Complex Class I Alleles, Dla-88*01101 and Dla-88*01201. *Tissue Antigens*, Vol. 70, No. 1, pp. 53-57, ISSN 0001-2815 (Print) 0001-2815 (Linking)
- Vogel, E. W., et al. (1996). DNA Damage and Repair in Mutagenesis and Carcinogenesis: Implications of Structure-Activity Relationships for Cross-Species Extrapolation. *Mutat Res*, Vol. 353, No. 1-2, pp. 177-218, ISSN 0027-5107 (Print) 0027-5107 (Linking)
- von Kalle, C., et al. (1994). Increased Gene Transfer into Human Hematopoietic Progenitor Cells by Extended *in vitro* Exposure to a Pseudotyped Retroviral Vector. *Blood*, Vol. 84, No. 9, pp. 2890-2897, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Vousden, K. H. & Lane D. P. (2007). P53 in Health and Disease. *Nat Rev Mol Cell Biol*, Vol. 8, No. 4, pp. 275-283, ISSN 1471-0072 (Print) 1471-0072 (Linking)
- Wagner, J. L., et al. (1999). Organization of the Canine Major Histocompatibility Complex: Current Perspectives. *J Hered*, Vol. 90, No. 1, pp. 35-38, ISSN 0022-1503 (Print) 0022-1503 (Linking)
- Wang, J. C., et al. (1997). Primitive Human Hematopoietic Cells Are Enriched in Cord Blood Compared with Adult Bone Marrow or Mobilized Peripheral Blood as Measured by the Quantitative *in vivo* Scid-Repopulating Cell Assay. *Blood*, Vol. 89, No. 11, pp. 3919-3924, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Wang, J. C., et al. (1998). High Level Engraftment of Nod/Scid Mice by Primitive Normal and Leukemic Hematopoietic Cells from Patients with Chronic Myeloid Leukemia in Chronic Phase. *Blood*, Vol. 91, No. 7, pp. 2406-2414, ISSN 0006-4971 (Print) 0006-4971 (Linking)
- Warlick, C. A., et al. (2002). *In vivo* Selection of Antifolate-Resistant Transgenic Hematopoietic Stem Cells in a Murine Bone Marrow Transplant Model. *J Pharmacol Exp Ther*, Vol. 300, No. 1, pp. 50-56, ISSN 0022-3565 (Print) 0022-3565 (Linking)
- Williams, D. A. & Baum C. (2003). Medicine. Gene Therapy--New Challenges Ahead. *Science*, Vol. 302, No. 5644, pp. 400-401, ISSN 1095-9203 (Electronic) 0036-8075 (Linking)
- Williams, D. A., et al. (1987). Protection of Bone Marrow Transplant Recipients from Lethal Doses of Methotrexate by the Generation of Methotrexate-Resistant Bone Marrow. *J Exp Med*, Vol. 166, No. 1, pp. 210-218, ISSN 0022-1007 (Print) 0022-1007 (Linking)
- Williams, D. A., et al. (1984). Introduction of New Genetic Material into Pluripotent Haematopoietic Stem Cells of the Mouse. *Nature*, Vol. 310, No. 5977, pp. 476-480, ISSN 0028-0836 (Print) 0028-0836 (Linking)
- Wilson, C., et al. (1989). Formation of Infectious Hybrid Virions with Gibbon Ape Leukemia Virus and Human T-Cell Leukemia Virus Retroviral Envelope Glycoproteins and the

- Gag and Pol Proteins of Moloney Murine Leukemia Virus. *J Virol*, Vol. 63, No. 5, pp. 2374-2378, ISSN 0022-538X (Print) 0022-538X (Linking)
- Wu, T., et al. (2000). Prolonged High-Level Detection of Retrovirally Marked Hematopoietic Cells in Nonhuman Primates after Transduction of Cd34+ Progenitors Using Clinically Feasible Methods. *Mol Ther*, Vol. 1, No. 3, pp. 285-293, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Wu, X., et al. (2003). Transcription Start Regions in the Human Genome Are Favored Targets for Mlv Integration. *Science*, Vol. 300, No. 5626, pp. 1749-1751, ISSN 1095-9203 (Electronic) 0036-8075 (Linking)
- Yang, Y., et al. (1995). Inducible, High-Level Production of Infectious Murine Leukemia Retroviral Vector Particles Pseudotyped with Vesicular Stomatitis Virus G Envelope Protein. *Hum Gene Ther*, Vol. 6, No. 9, pp. 1203-1213, ISSN 1043-0342 (Print) 1043-0342 (Linking)
- Yee, J. K., et al. (1994). A General Method for the Generation of High-Titer, Pantropic Retroviral Vectors: Highly Efficient Infection of Primary Hepatocytes. *Proc Natl Acad Sci U S A*, Vol. 91, No. 20, pp. 9564-9568, ISSN 0027-8424 (Print) 0027-8424 (Linking)
- Yi, Y., et al. (2011). Current Advances in Retroviral Gene Therapy. *Curr Gene Ther*, Vol. No., ISSN 1875-5631 (Electronic) 1566-5232 (Linking)
- Zhang, X. B., et al. (2008). High Incidence of Leukemia in Large Animals after Stem Cell Gene Therapy with a Hoxb4-Expressing Retroviral Vector. *J Clin Invest*, Vol. 118, No. 4, pp. 1502-1510, ISSN 0021-9738 (Print) 0021-9738 (Linking)
- Zielske, S. P. & Gerson S. L. (2002). Lentiviral Transduction of P140k Mgmt into Human Cd34(+) Hematopoietic Progenitors at Low Multiplicity of Infection Confers Significant Resistance to Bg/Bcnu and Allows Selection *in vitro*. *Mol Ther*, Vol. 5, No. 4, pp. 381-387, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Zielske, S. P. & Gerson S. L. (2003). Cytokines, Including Stem Cell Factor Alone, Enhance Lentiviral Transduction in Nondividing Human Ltcic and Nod/Scid Repopulating Cells. *Mol Ther*, Vol. 7, No. 3, pp. 325-333, ISSN 1525-0016 (Print) 1525-0016 (Linking)
- Zielske, S. P., et al. (2003). *In vivo* Selection of Mgmt(P140k) Lentivirus-Transduced Human Nod/Scid Repopulating Cells without Pretransplant Irradiation Conditioning. *J Clin Invest*, Vol. 112, No. 10, pp. 1561-1570, ISSN 0021-9738 (Print) 0021-9738 (Linking)

DNA Repair Perspectives in Thyroid and Breast Cancer: The Role of DNA Repair Polymorphisms

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1. Introduction

One of the great challenges of modern molecular biology is the integration of new genetic information into procedures that can be implemented in rapid, cost effective and reliable methods to genotype, phenotype, identify gene function, and development treatment for the disease. One of the major impacts of such methods and procedures is the increase of our knowledge and understanding of human biology leading to the recognition of the importance of molecular factors in disease aetiology. The immediate consequence of such knowledge is an increased ability for pathology diagnostic and for the identification of pre-symptomatic individuals or those susceptible to specific diseases, improving our ability for disease prognosis and to develop more efficient therapeutic strategies.

Every organism is exposed to hazardous agents in its environment on a continual basis. As a result, organisms have evolved sophisticated pathways that are considered an environmental response machinery, to minimize the biological consequences of hazardous environmental agents. A large number of human genes, including the ones involved in the environmental response machinery, are subject to genetic variability, which can be associated with the altered efficiency of a biological pathway (Perera & Weinstein, 2000). So, an individual's risk for developing a disease stemming from an environmental exposure might be dependent on the efficiency of his/her own unique set of environmental response genes. These genes are usually involved in the metabolism of environmental carcinogens, in the repair of DNA lesions induced by exogenous and endogenous carcinogens, and in the control of the cell cycle. Individual polymorphic forms in those genes have been associated with individual susceptibility to different types of cancer namely in breast and thyroid cancer (Conde et al., 2009; Gaspar et al., 2004; Pabalan et al., 2010; Peng et al., 2011; Silva et al., 2005; Silva et al., 2006b; Silva et al., 2006a; Silva et al., 2009; Silva et al., 2010; Silva et al., 2007).

Several enzymes have evolved for the detoxification of xenobiotic compounds, and their gene expression is induced in response to the presence of numerous compounds (*e.g.*, polycyclic aromatic hydrocarbons found in tobacco smoke). An inefficient detoxification of reactive endogenous or exogenous compounds ultimately leads to lesions in DNA, which should be repaired by DNA repair mechanisms, ought to reduce cancer risk. As a

consequence, a genetic change that alters the expression of the gene or the activity of the detoxifying protein produced may increase the amount of reactive carcinogen present, thus, increasing the risk of cancer development. As an example, CYP1A1 and CYP2D6 phase I enzymes, are induced by, and act on, carcinogens found in tobacco smoke. Additionally, CYP2E1, an enzyme that metabolizes ethanol, is also a candidate because epidemiological studies suggest that breast cancer risk is increased with alcohol consumption (Hasler, 1999). Environmental carcinogens interact with DNA as a result of complex metabolisms, involving phase I and phase II enzymes giving rise to several biomarkers of lesion. There are several specific biomarkers for evaluation of specific exposures. For instance, several environmental carcinogenic compounds (e.g. Aflatoxin B1, Benzo(a)Pyrene) react with DNA and/or proteins giving rise to specific adducts (Taioli et al., 2007; Wu et al., 2002). However, apart from the specific biomarkers, exposure to these compounds leads to appearance of unspecific biomarkers as well (El-Zein et al., 2011).

In spite of being usually unspecific biomarkers of lesion, there is evidence of correlation between increased frequency of chromosomal aberration (CA), micronuclei and cancer incidence in humans, which supports the use of cytogenetic human monitoring for cancer risk assessment (Bonassi et al., 2007; Bonassi et al., 2011; Dhillon et al., 2011). The levels of the different biomarkers studied are not directly correlated with the levels of exposure, suggesting that intra-individual susceptibility plays an important role. Several studies have shown that the level of biomarkers arising from environmental and/or occupational exposure may be modulated by polymorphic genes (Bonassi et al., 2011; Dhillon et al., 2011; Pavanello & Clonfero, 2000). As an example, experiments conducted in our laboratory concerning the evaluation of occupational exposure to Polycyclic Aromatic Hydrocarbons (PAHs), showed that the levels of aromatic DNA adducts are higher in smokers than in non smokers and the individual levels of DNA adducts in smokers is dependent on the CYP1A1 *MspI* polymorphism, located in the 3' non-coding region of the gene, and that the presence of at least one allele with the restriction site leads to a significant higher level of DNA adducts when compared with the ones homozygous for the absence of this restriction site (Teixeira et al., 2002). Interestingly the same CYP1A1 polymorphism has been associated with lung cancer risk among the Japanese and Caucasian populations (Kawajiri et al., 1990; Xu et al., 1996). The dependence of biomarkers' level with individual genotypes is also observed in human tumours. For instance, in lung cancer patients the level of DNA adducts has been correlated with CYP1A1 activity (Mollerup et al., 1999), and also with the GSTP polymorphisms (Rydberg et al., 1996). Thus, the parallel study of polymorphic genes associated with cancer susceptibility, and their correlation with biomarkers of exposure to carcinogens will improve our knowledge on the correlation between environmental exposure and individual variability. Additionally, these results prove the relevance of DNA repair pathways in cancer susceptibility, working as one important tool when other mechanisms fail.

The data concerning the individual levels of biomarkers of exposure and the association between individual genetic polymorphisms and cancer risk suggest the involvement of environmental factors in some human cancers (e.g. carcinogen exposure) with individual risk factors (e.g. genetic polymorphisms). Thus, the parallel study of polymorphic genes associated with cancer susceptibility, and their correlation with biomarkers of exposure will improve our knowledge on the correlation between environmental exposure and individual variability (Rueff et al., 2002) to different types of cancer namely in breast and thyroid cancers.

1.1 Breast cancer

Breast cancer is the most common form of cancer among women, being responsible for the highest mortality rate from cancer among the female sex. However, the main causes related to this pathology remain unclear. The risk of neoplastic disease has been connected with genetic and environmental factors. In fact, genes and the environment share the stage for most, if not all, common non-familial cancers, and are related to individual susceptibility.

Several studies have identified two major susceptibility genes in breast cancer: *BRCA1* and *BRCA2* (Hedenfalk et al., 2003; Narod & Foulkes, 2004). These genes have an important role in genome maintenance, in cell cycle control and in DNA repair, controlling homologous recombination repair (Scully & Puget, 2002; Venkitaraman, 2002). Analysis in families with high risk of breast cancer showed that individuals with point mutations in these genes have a 40-80% of probability to develop breast cancer. However, mutations in these two tumour-suppressor genes account for only 5-10% of all cases of breast cancer (Fackenthal & Olopade, 2007).

Factors related to reproductive history and/or hormonal status have been found to confer increased risk (e.g., nulliparity, late age at first pregnancy, early menarche, late menopause), but the magnitude of the increased risk is generally not huge (less than 3) and the majority of breast cancer cases occur in women not demonstrably at high risk for this tumour. Recent evidence shows that there are other background genetic factors that contribute to the development of breast cancer, such as polymorphisms in DNA repair pathways that might increase cancer risk (Hunter et al., 2005; Silva et al., 2007).

1.2 Thyroid cancer

Thyroid cancer is the most frequent endocrine neoplasia, accounting for 1-5% of all cancers in women and 2% in men in most countries, being responsible for 0.32% of deaths related to malignant tumours. The incidence of this type of tumour has been responsible for 6.3% of total deaths promoted by endocrine tumours, which reflects its indolent nature. Nevertheless, 5-10% of all thyroid cancers are fatal (Inskip, 2001).

The high frequency of cancer among family members of thyroid cancer patients supports the hypothesis that hereditary factors are important in the aetiology of this tumour. Among the sporadic cases of thyroid cancer the most common histological varieties are non-familial papillary and follicular thyroid carcinomas which shows a long-term (~10-year) survival rate of more than 90%. These diseases are unusual in children and adolescents, and their incidence increases with age in adults, with the majority of cases occurring between 25 and 65 years of age. The papillary and follicular carcinomas are two to four times more frequent in women than in men, particularly during reproductive years, leading to the hypothesis that female hormones may be involved in the aetiology or pathogenesis of the disease (Grubbs et al., 2008). Exposure to ionizing radiation is the only verified cause of thyroid carcinogenesis in humans, especially when exposure occurs at a young age. However, individuals without previous exposure to ionizing radiation can also develop thyroid cancers, suggesting that other risk factors may also be involved in the aetiology of sporadic tumours. For example, dietary iodine deficiency has also been linked to this pathology. In fact, papillary tumours associated with radiation exposure usually have different forms of the activated *RET* proto-oncogene and at a higher frequency than that observed in spontaneous non-radiation induced tumours (Grubbs et

al., 2008; Sarasin et al., 1999), while follicular tumours are associated with somatic *RAS* gene mutations (Grubbs et al., 2008). Accordingly, the evidences suggested the existence of other risk factors for papillary and follicular tumours. Thus, the identification of susceptibility factors, both genetic and environmental, associated with individual predisposition to thyroid cancer could possibly give further insight into the aetiology of this malignancy.

2. DNA repair pathways

It is generally agreed that genetic polymorphisms are associated with, or are even the cause, of most common disorders with a genetic component like cancer. However, the complex metabolism of xenobiotic compounds involving different polymorphic genes could modulate the individual risk factor for cancer (Pavanello & Clonfero, 2000).

DNA repair enzymes continuously monitor chromosomes to correct damaged nucleotide residues generated by exposure to carcinogens and cytotoxic compounds. The damage is partly a consequence of environmental agents such as ultraviolet (UV) light from the sun, inhaled cigarette smoke or deficient dietary habits. However, a large proportion of DNA alterations are caused unavoidably by endogenous mutagens, such as reactive oxygen species and metabolites that can act as alkylating agents. Genome instability caused by the great variety of DNA-damaging agents would be an overwhelming problem for cells and organisms. Thus, DNA repair is a ubiquitous process throughout the living world and defective DNA repair is a risk factor for many types of cancer. Its universality reflects the constant challenge to the integrity of any genome from the inherent instability of DNA, the natural limitations of the accuracy of DNA synthesis and the challenge of the environment.

Recent evidence that some DNA repair functions are haploinsufficient adds weight to the notion that variants in DNA-repair genes constitute part of the spectrum of defects contributing to cancer risk. A coherent understanding of the genomics of human DNA repair genes, especially single nucleotide polymorphisms (SNPs), will greatly facilitate the investigation of the role that these variations play in modulating carcinogenesis.

Several studies have shown that genes directly involved in DNA repair and in the maintenance of genome integrity, or genes indirectly involved in the repair of DNA damage through the regulation of the cell cycle, are critical for protecting against the mutations that lead to cancer (Hakem, 2008). Evidence suggests that the difference in DNA repair capacity among individuals is genetically determined, and that reduced DNA repair capacity constitutes a statistically significant risk factor for development of several cancers, associated with reduced protein function rather than absence of its function. Recently a number of polymorphisms of genes that encode for DNA repair proteins have been described (Wood et al., 2005). However, we will not discuss the mechanisms of these pathways in detail; instead we will focus on how epidemiologic studies contribute to understand the role of genetic polymorphisms present in DNA repair genes and individual susceptibility to breast and thyroid cancers.

2.1 BER – Base excision repair

The base excision repair (BER) pathway is mainly responsible for the lesion-specific removal that arises from endogenous or exogenous agents inducing base damage, which are the most frequent insult in cellular DNA. Reactive oxygen species (ROS) are produced from

endogenous sources, most notably the oxidative metabolism in the mitochondria, and from exogenous sources, such as ionizing radiation. ROS attack DNA readily, generating a variety of DNA lesions, such as strand breaks and oxidized bases. If not properly removed, DNA damage can be potentially devastating to normal cell physiology, leading to mutagenesis and/or cell death, especially in the case of cytotoxic lesions that block the progression of DNA/RNA polymerases (Maynard et al., 2009). BER pathway involves two sub-pathways, the short-patch responsible for the replacement of a single base, and the long-patch, which results in the incorporation of 2-13 nucleotides. The two pathways progress through different major processes that initially involve the removal of the damage base by glycosylases (Li et al., 2010).

2.1.1 BER and breast cancer

Several enzymes of the BER pathway act in concert to keep the DNA intact and maintain genomic integrity. One of the most studied genes of BER pathway has been the *XRCC1* gene. *XRCC1* is a scaffolding protein that is involved in the repair of single-strand breaks, the most common lesion in cellular DNA (Li et al., 2009). Concerning the *XRCC1* gene polymorphisms, several studies suggest a dual effect of these SNPs in cancer risk. In fact, genetic variation in six BER pathway genes (*XRCC1*, *ADPRT*, *APEX1*, *OGG1*, *LIG3*, and *MUTYH*) is associated with breast cancer risk in two large population-based case-control studies in the United States and Poland (Zhang et al., 2006). Additionally, *XRCC1* haplotypes revealed no significant association between Trp194-Arg399 haplotype and risk of breast cancer, neither in Western nor Asian countries, but a recent meta-analysis has indicated that the Arg194-Gln399 haplotype of *XRCC1* might be a risk factor for breast cancer in Asian countries (Saadat, 2010). However, other meta-analysis suggests that polymorphisms Arg280His and Arg399Gln may modify breast cancer risk differently in Caucasian and Asian populations (Li et al., 2009), and a meta-analysis study between the breast cancer and the *XRCC1* polymorphisms Arg194Trp, Arg399Gln and Arg280His in different inheritance models suggested that Arg399Gln was associated with a trend of increased breast cancer risk when using both dominant and recessive models to analyze the data (Huang et al., 2009).

Results published by our group concerning the breast cancer risk and *XRCC1* Arg194Trp and Arg399Gln polymorphisms do not show any association between these polymorphisms and breast cancer risk, but it was observed that menopausal age together with *XRCC1* Arg194Trp and Arg399Gln polymorphisms might be involved in individual susceptibility towards breast cancer (Silva et al., 2007)

DNA polymerase beta (Pol β) provides most of the gap-filling synthesis at abasic sites of damaged DNA in the base excision repair pathway, a polymorphic key gene in BER. A case-control study has shown two polymorphisms in the Pol β protein, the Pro242Arg and Lys289Met, associated with breast cancer risk and cancer progression. In fact, a strong association between breast cancer occurrence and the TT genotype of the Lys289Met (C to G transition) polymorphism and the CG genotype of the Pro242Arg polymorphism was found. Polymorphism-polymorphism interaction between the TT genotype of the Lys289Met and the CG genotype of the Pro242Arg (C to G transition) polymorphisms increased the risk of breast cancer (Sliwinski et al., 2007)

Several DNA BER repair gene polymorphisms have been described, which affect DNA repair capacity and modulate cancer susceptibility, namely with the risk of acute skin

reactions following radiotherapy. In fact, it was reported that *XRCC1* 399Gln or *APEX1* 148Glu alleles may be protective against acute skin reactions following radiotherapy (Chang-Claude et al., 2005). However, it was not confirmed in breast cancer patients (Bartsch et al., 2007). In addition, in a retrospectively evaluation of SNPs in DNA repair genes, it was observed that *XRCC1* Arg399Gln may be predictive of survival outcome in patients with metastasis breast cancer treated with DNA damaging chemotherapy (Bewick et al., 2006). Outcome and survival in anthracycline-based and cyclophosphamide/methotrexate/5-fluorouracil-based chemotherapy of invasive breast cancer are unpredictable. It was observed that, carriers of the *XRCC1* 1196 AA genotype had a reduced risk for recurrence/death and patients treated with chemotherapy but not radiotherapy, suggesting that DNA repair enzyme *XRCC1* is a potential treatment predictor for the outcome and survival of anthracycline and cyclophosphamide/methotrexate/5-fluorouracil-based chemotherapy of invasive breast cancer (Jaremko et al., 2007). However, among incoherent results, recent studies strongly suggest a main role of BER in chemotherapy (Bewick et al., 2006; Goode et al., 2002b; Kelley & Fishel, 2008) in breast cancer.

2.1.2 BER and thyroid cancer

The results obtained by our group, do not reveal a significant involvement of *XRCC1* Arg194Trp and Arg399Gln, *OGG1* Ser326Cys, *APEX1* Asp148Glu, *MUTYH* Gln335His and *PARP1* Val762Ala polymorphisms on the individual susceptibility towards thyroid cancer, since the frequency of the different genotypes are similar in control and cancer patients population (data not published).

Additionally, since thyroid cancer incidence is recurrently reported to be higher in women (which was also the predominant gender in our case group), we compared genotypic frequencies according to sex among thyroid cancer patients (in order to examine for any sex-specific genetic effect), but the frequency of the different genotypes considered did not differ significantly with gender in thyroid cancer patients (data not published).

Concerning the role of *XRCC1* polymorphisms in thyroid cancer, a study reported by (Ho et al., 2009) showed, in white non-Hispanics, that *XRCC1* 194Trp variant allele may be associated with increased risk of differential thyroid carcinoma (DTC), while the *XRCC1* 399Gln variant allele may be associated with decreased risk of DTC. These results are in agreement with the data reported by (Chiang et al., 2008) in Chinese DTC populations. However, Sigurdson and colleagues (Sigurdson et al., 2009) reported that, among residents near Semipalatinsk, Kazakhstan *XRCC1* Arg194Trp is associated with decreased thyroid nodule risks for increasing minor alleles, and a similar patterns of association were observed for a small number of papillary thyroid cancers. The results in our study do not reveal an association between *XRCC1* Arg194Trp genotypes and thyroid cancer risk, and these differences might be due a different risk factors for thyroid tumours (exposure to ionizing radiation (Sigurdson et al., 2009) versus sporadic tumours), and genetic background of different populations.

Concerning *XRCC1* Arg280His polymorphism only a positive association was described in Caucasian populations (Garcia-Quispes et al., 2011). Interestingly, in white non-Hispanics, the *XRCC1* 399Gln variant allele may be associated with decreased risk of DTC, and in Caucasians, who lived in the areas of the Russian Federation and Belarus contaminated with radionuclides from Chernobyl fallout, it was observed that the *XRCC1* Arg399Gln

polymorphism, regardless of radiation exposure, was associated with a decreased risk of Papillary Thyroid Carcinoma (PTC) according to the multiplicative and dominant models of inheritance (Sigurdson et al., 2009). However, we cannot exclude that other *XRCC1* gene polymorphisms may interact, alone or when combined, with other genes such as the ADPRT (Chiang et al., 2008). However, our results do not support an association between thyroid cancer risk and the *XRCC1* Arg399Gln polymorphism, suggesting the need of larger studies and/or a meta-analysis, in order to understand the role of the *XRCC1* polymorphisms in thyroid cancer. Additionally, we can rule out the utility of *XRCC1* haplotypes in predicting DTC risk.

In general, the results reviewed suggest that larger studies are required to define the role of *XRCC1* polymorphisms in susceptibility to well differentiated thyroid cancer. However, the scarcity of data concerning other BER genes creates a gap in knowledge about the effective role of polymorphisms in BER pathway and individual susceptibility to thyroid cancer.

2.2 NER – Nucleotide excision repair

Several DNA repair pathways have evolved to repair DNA adducts and function to prevent genomic instability and promote cell survival. The nucleotide excision repair (NER) pathway is important for DNA repair, removing adducts responsible for the distortion of double helix. Such adducts can be caused by UV irradiation and environmental agents such as tobacco smoke (polycyclic aromatic hydrocarbons). This pathway is also the main mechanism for the repair of bulky DNA adducts generated by breast cancer chemotherapeutic agents, such as platinum drugs and cyclophosphamide and may be involved in the repair of nonbulky DNA lesions that result from oxidative damage (Bewick et al., 2011; Oksenysh & Coin, 2010). Failure to eliminate these lesions can lead to oncogenesis, developmental abnormalities and accelerated ageing.

NER pathway involves several proteins that act in order to restore the normal homeostasis of the cell. There are two sub-pathways of the NER pathway, global-genomic-NER (GG-NER) and transcription-coupled NER (TC-NER), which differ only in the step involving recognition of the DNA lesion (Shuck et al., 2008). The complex XPC-HR23B or CSA and CSB proteins are responsible for the initial step in the GG-NER and in TC-NER, respectively. The recognition of the distortion of double helix is followed by the opening of the DNA by the XPB and XPD ATPases/helicases of the transcription/repair factor (TFIIH). TFIIH is a multisubunit factor composed of 10 subunits which catalyses helix opening during NER (Li et al., 2010). The DNA strand opening favors the recruitment of XPA and RPA, which help to enlarge the opened structure and drive the dissociation of the CDK activating kinase (CAK) complex from TFIIH. The recruitment of the endonucleases XPG and XPF triggers dual incision and excision of the protein-free damaged oligonucleotide. The gap is filled by the resynthesis machinery and the DNA extremities sealed (Oksenysh & Coin, 2010).

Deficiency in NER results in three rare genetic disorders: *Xeroderma pigmentosum* (XP), trichothiodystrophy (TTD) and Cockayne syndrome (CS), characterize by increased cancer frequencies, neurodegeneration and ageing.

2.2.1 Nucleotide excision repair and breast cancer

Genomic instability is a hallmark of all cancers (Hanahan & Weinberg, 2000). At the cellular level, damaged DNA that is not properly repaired can lead to genomic instability, apoptosis, or senescence, which can greatly affect the organism's development and ageing process

(Hakem, 2008). DNA repair pathways are among the mechanisms most frequently deregulated in cancer. These mechanisms allow non-transformed cells to repair their DNA after specific damage or in some circumstance, to induce apoptosis if repair is not possible. This mechanism protects against uncontrolled proliferation in the context of abnormal genetic background. Disruption of these pathways in cancer produces an increase in chromosome breaks and mutagenesis (Amir et al., 2010).

Several polymorphisms in NER genes have been described. However, studies investigating the association of NER genes polymorphisms with breast cancer risk produced controversial results.

Genetic polymorphisms identified in genes encoding DNA repair enzymes are believed to be candidates for associations with several types of cancers, including breast cancer. One of the most studied NER genes is *ERCC2 (XPD)*, which plays a key role in NER pathway. Several polymorphisms in the *ERCC2* gene have been described, including the commonly occurring Asp312Asn and Lys751Gln. However, the published results have been contradictory. Recently, one meta-analysis study revealed no association between both polymorphisms and breast cancer risk (Pabalan et al., 2010). We had also reported similar results previously (Silva et al., 2006a). The main causes for breast cancer remain unclear; however some environmental compounds have been regarded as increasing the risk of breast cancer, especially the ones responsible for generation of DNA adducts (PAHs, aromatic amines). The *ERCC2* polymorphisms have been extensively correlated with high levels of DNA adducts (or lower DNA repair capacity). This reduction in DNA repair capacity is also influenced by these polymorphic variations, being predictive of DNA repair capacity (Crew et al., 2007; Shi et al., 2004).

Other genes of NER pathway have been studied, though not so often, in association studies. For example, the gene *ERCC4 (XPF)* that codes for the subunit of the protein complex ERCC1-ERCC4 responsible for the removal of the damaged single-stranded fragment (Lee et al., 2005), have been studied and the results revealed a significant association of Arg415Gln polymorphism and breast cancer risk (Smith et al., 2003), but not for Ser835Ser polymorphism as described by Lee and colleagues. This research team showed a combined effect between the *ERCC4* synonymous polymorphism and Asp312Asn *ERCC2* polymorphism (Lee et al., 2005). The other sub-unit of protein complex ERCC1-ERCC4, coded by *ERCC1* gene, has also been a target of some epidemiologic studies (Bewick et al., 2011; Crew et al., 2007; Lee et al., 2005; Shin et al., 2008), resulting in contradictory data.

XPG (ERCC5) gene is also required for the removal of the damaged single-stranded nucleotide fragment, together with the complex ERCC1-ERCC4. Rajaraman and colleagues described in their work the relevance of one polymorphism of *ERCC5* gene providing suggestive evidence that variant allele of Asp1104His was associated with increased risk of breast cancer overall, and suggesting further an increased susceptibility to breast cancer in radiologic technologists exposed to low levels of radiation (Rajaraman et al., 2008). However, there is no agreement regarding the role of this gene in breast cancer susceptibility (Crew et al., 2007; Jorgensen et al., 2007; Kumar et al., 2003; Mechanic et al., 2006; Shen et al., 2006).

XPC encodes a basic protein that is essential for damage recognition in sub-pathway GG-NER (Sugasawa, 2008). Several studies have been developed concerning the role of *XPC* gene polymorphisms in breast cancer risk. However, the results have been inconsistent. Early this year a meta-analysis including 11 studies was published revealing no associations between the polymorphisms of *XPC* gene under study and breast cancer risk (Zheng et al., 2011). However, the role of this gene in breast cancer should not be excluded, since it was

shown that *XPC* gene, as other *XP* gene products, interact with and stimulate specific DNA glycosylases (e.g. thymine DNA glycosylase (TDG)), initiators of BER, beyond their functions in NER pathway (Sugasawa, 2008). Moreover, gene-gene and gene-environment interactions should also be considered which are not in the meta-analysis studies.

XPA protein interacts with many of the core repair factors in NER pathway, and without it, no stable pre-incision complex can form, nor can NER occur, making it the limiting factor in damage recognition (Shuck et al., 2008). However, *XPA* gene has not been extensively studied in connection with breast cancer risk (Crew et al., 2007; Jelonek et al., 2010; Shen et al., 2006), and the results published did not describe this gene as potentially related with breast cancer risk.

Many more polymorphisms in the NER genes have been found, however there aren't enough consistent epidemiologic studies into the link between this pathway and breast cancer. More studies are needed to form any reliable conclusions.

The new era in cancer treatment and prevention lies in the ability to treat patients individually according to their genetic constitution and the DNA repair status of their tumours. The nature of DNA lesions caused by therapeutic agents requires complex repair mechanisms, possibly involving simultaneously different repair pathways. DNA damage acquired from these treatments can initiate a number of cellular pathways involved in DNA repair, cell cycle control, metabolism and apoptosis (Bewick et al., 2011). For example, it is well known the importance of NER pathway in repair of bulky DNA adducts, such those caused by tobacco smoke as well as intrastrand cross-links (ICL) caused by chemotherapeutic agents, such as *cis-platinum* or anthracyclines (Latimer et al., 2010; Saffi et al., 2010). If so, SNPs in genes in this pathway may significantly affect DNA repair efficiency, influencing clinical outcome and thus may help identify patients that can benefit from certain treatments.

2.2.2 Nucleotide excision repair and thyroid cancer

The main, and well documented, cause for thyroid cancer is ionizing radiation, although other risk factors have been pointed out as candidates, such as dietary iodine deficiency, hormonal factors, lymphocytic thyroiditis and familial history (Kondo et al., 2006).

In a previous report, we found a significant association between a haplotype of two SNPs (Asp312Asn and Lys751Gln) in *ERCC2* gene, and thyroid cancer risk (Silva et al., 2005) suggesting that this pathway may be relevant for thyroid carcinogenesis. Later we also conducted another study including more SNPs in different genes of NER (*CCNH* Val270Ala, *CDK7* Asn33Asn, *RAD23B* Ala249Val, *ERCC1* Gln504Lys, *ERCC4* Arg415Gln, *ERCC5* Asp1104His, *ERCC5* Cys526Ser, *ERCC6* Arg1230Pro, *ERCC6* Gln1413Arg, *XPC* Ala499Val and *XPC* Lys939Gln) on the individual susceptibility to non-familial thyroid cancer (manuscript in preparation), where we showed that patients carrying at least one variant allele of *CCNH* Val270Ala polymorphism seems to be at increased risk for thyroid cancer. To our knowledge, no other reports have been published trying to find susceptibility alleles in NER genes associating them with risk for thyroid cancer. One possible explanation for this is that the lesions produced by ionizing radiation are more likely to be repaired by BER, NHEJ and HR.

Several authors have described the increased incidence of a second malignancy in patients after diagnosis of thyroid cancer (Brown et al., 2008; Canchola et al., 2006; Garner et al., 2007; Verkooijen et al., 2006), pointing out breast cancer as the most frequent occurrence. However, some authors also considered the opposite, thyroid cancer being subsequent to breast cancer, suggesting the exposure to radiotherapy as the main cause of second malignancy in adjacent organs as a result of scattered radiation (Adjadj et al., 2003; Huang et

al., 2009). Furthermore, the incidence of other tumours might not represent a therapy effect but rather might be due to common risk factors. Therefore, the exposure to ionizing radiation and hormonal factors has been the risk factors well documented for both malignancies, although, there is no consensus in the results.

2.3 MMR – Mismatch repair

The Mismatch Repair (MMR) pathway plays a crucial role in repairing mismatches, which are small bulges in the DNA duplex, caused by small insertions, deletions or nucleotide substitutions in one strand of the duplex. Mismatches can be generated during DNA replication and repair. The failure of MMR leads to high mutation rates, microsatellite instability (MSI), losses of heterozygosity (LOH), reduction in apoptosis processes and increases in cell survival, as well as predisposition for carcinogenesis (Schofield & Hsieh, 2003; Schroering et al., 2007). MMR is also associated with an anti-recombination function, suppressing homologous recombination and plays a role in DNA-damage signaling (Smith et al., 2008).

The main MMR pathway is initiated by the recognition of a mismatch by the heterodimer consisting of the MSH2 and MSH6 proteins (also called MutS α). MutS α is responsible for the recognition of base mismatches and insertion/deletion loop (IDLs) in mono- to tetranucleotide repeats. This complex, MutS α , is able to recognize most base-base mismatches and short IDLs (Hsieh & Yamane, 2008).

Another MMR pathway, consisting of MSH2 and MSH3 heterodimers (MutS β) is primarily responsible for binding to and correcting insertion/deletion mutations, preferentially dinucleotide and larger IDLs. Upon DNA mismatch recognition the repair process proceeds with the participation of the heterodimer consisting of MLH1 and PMS2 (also called MutL α), which acts as an endonuclease. Subsequent DNA excision, directed by strand breaks located either 5' or 3' to the mispair, is carried out by the exonuclease EXO1 (Hsieh & Yamane, 2008; Jiricny, 2006).

2.3.1 Mismatch repair and breast cancer

The *MSH2* gene is central in mismatch recognition and has been the most studied gene of MMR. There are several studies reporting mutations (Murata et al., 2002) and polymorphisms in several *MSH2* variants (Poplawski et al., 2005; Wong et al., 2008). Poplawski and colleagues showed a significant association between Gly322Asp polymorphism of the *MSH2* gene and breast cancer risk (Poplawski et al., 2005). However, another study of several families conducted by Wong and colleagues did not find any association between *MSH2* and breast cancer (Wong et al., 2008). However, the scarcity of data about the involvement of polymorphisms in other MMR genes in breast cancer susceptibility, contributed to our MMR multigene study, which included *MSH3*, *MSH4*, *MSH6*, *MLH1*, *MLH3*, *PMS1* and *MUTYH* genes (Conde et al., 2009). Our results showed the potential involvement of Leu844Pro *MLH3* gene polymorphism in breast cancer susceptibility, as well as some SNP-SNP interactions. Different activities and functions of these genes as well as SNP variations may alter the level of repair, leading to higher rates of mutations and therefore an increase of breast cancer risk or conversely play a protective role in breast carcinogenesis.

2.3.2 Mismatch repair and thyroid cancer

To our knowledge, there are no data reporting the involvement of mismatch repair genes in thyroid cancer susceptibility.

2.4 DSB - Double strand breaks repair

Double-stranded breaks (DSBs) are the most injurious DNA damage. The failure to repair DSBs can result in chromosomal abnormalities, such as DNA translocations, that lead to cancerogenesis and are common in many cancers. DSBs can occur as a consequence of direct exposure to harmful exogenous agents, such as ionizing radiation, or by endogenous by-products of many metabolic processes, such as reactive oxygen species (ROS). They can also be generated during V(D)J recombination and when DNA single-strand breaks are encountered during DNA replication. The termini of chromosomes can also be recognized as DSB due to defective metabolism of telomeres. In order to face the threat of DSBs, prokaryotes and eukaryotes developed two mechanisms of repair, DNA end-joining and homologous repair.

DNA end-joining is the most straightforward repair mechanisms of DSBs, since it simply rejoins the broken ends regardless of the genetic consequences. There are two processes through which DNA end-joining can occur, non-homologous end-joining (NHEJ) and microhomology mediated end-joining (MMEJ). The first one is a Ku-dependent mechanism while the second one is Ku-independent. Both processes can maintain structural integrity, although, do not guarantee genetic integrity, being both error-prone and capable of generating new mutations. In NHEJ pathway, DNA ends are recognized and targeted by a heterodimeric Ku70/80 complex leaving the broken ends accessible for other factors. This DNA binding complex is important for recruitment of additional NHEJ proteins. Once bound to DNA, Ku proteins recruit the large DNA-dependent protein kinase catalytic subunit (DNAPKcs). The association of DNAPKcs-DNA-Ku complex activates the serine/threonine kinase activity of DNAPKcs contributing to the phosphorylation of the histone H2AX at lesion site and other factors such as nucleases. In case of incompatible ends, broken ends are processed by an endonuclease protein, named Artemis, trimming DNA overhangs and hairpins formed at the transition of double to single stranded DNA. Alternatively, the ssDNA tails can be filled in by polymerases (pol μ and pol λ). NHEJ repair is finalized by the complex XRCC4/LigaseIV that rejoins DNA ends. When broken DNA ends are not accessible for Ku proteins, due to other polypeptides covalently attached to DNA ends, the end joining occurs via Ku-independent pathway (MMEJ). The foremost distinguishing property of MMEJ is the use of 5–25 nucleotides sequences during the alignment of broken ends before joining, thereby resulting in larger deletions flanking the original break (Lieber, 2010; McVey & Lee, 2008).

In S-phase of cell cycle, there is a second copy of the genome, thus repair machinery can use this sister chromatid as template in order to achieve an error-free repair. This mechanism is known as homologous recombination repair (HR). In human cells, the early events are not completely understood, however the recruitment of the Mre11-Rad50-Nbs1 (MRN) complex and the phosphorylation of histone H2AX are two probable events, although these are not specific to HR since they can also occur, to a lesser extent, in NHEJ and MMEJ. Unlike end-joining repair, HR requires extensively resected broken ends to generate a 3' single-stranded (ss) DNA tail. Firstly, MRN complex exposes both 3' ends, the 3' ssDNA tail is then stabilized by binding of RPA which facilitates the assembly of RAD51. With the help of RAD52 and BRCA2, a nucleoprotein filament is formed along the ssDNA tail and the search for homology in the sister chromatid by RAD51 is initiated. RAD51 catalyzes strand exchange during which ssDNA invades homologous duplex DNA forming a Holliday junction, which provides a primer to initiate new DNA synthesis, and a displacement loop (D-loop). Once the D-loop is formed, cells may undergo a process termed synthesis-

dependent strand annealing. The 3' end in the D-loop is extended by repair synthesis and then the newly synthesized DNA strand dissociates to anneal to its original second strand to complete the reaction (Li & Heyer, 2008).

2.4.1 DSB and breast cancer

Linkage analysis of families with a high risk of breast cancer has identified two major susceptibility genes, *BRCA1* and *BRCA2*. The *BRCA1* and *BRCA2* genes are numerically the most important susceptibility genes for breast cancer, accounting for more than 80% of incidence in families with six or more cases of early-onset breast cancer. *BRCA1* tumours are typically invasive ductal carcinomas in which there is a high incidence of triple negative phenotype (negative for estrogen receptor, progesterone receptor and HER2). On the contrary, no distinctive histopathological phenotype has been described in *BRCA2* tumours. The main roles of the *BRCA1* and *BRCA2* are well known and are reviewed elsewhere (Gudmundsdottir & Ashworth, 2006).

Hereditary breast cancer only accounts for 5-10% of all cases (Dapic et al., 2005). Although high penetrance genes, such as *BRCA1* and *BRCA2*, can explain some of these cases, the sporadic cases are still not well understood and the search for susceptibility genes continues. The fact that two major hereditary breast cancer genes are involved in DSB repair pathway, point to the relevance of DSBs in sporadic breast cancer risk. Thus, due to the emergence of comprehensive high density maps of SNPs and affordable genotyping platforms, several genes involved in DSB repair have been genotyped in breast cancer patients, in order to find susceptibility alleles. The most studied genes are *NBS1*, *RAD51*, *XRCC2*, *XRCC3*, *BRCA1* and *BRCA2*. To a lesser extent, the NHEJ genes are also studied.

The *NBS1* gene codes for the protein NBS1 that participates in the MRN complex responsible for the DSB recognition and the early stages of the repair, as described above (Stracker & Petrini, 2011). Therefore, many studies were conducted in order to verify if a variant of this gene could be a susceptibility allele for breast cancer (Goode et al., 2002a; Kuschel et al., 2002; Lu et al., 2006; Millikan et al., 2005; Pooley et al., 2008; Silva et al., 2010; Smith et al., 2008; Zhang et al., 2005). The most studied SNPs were Asp399Asp, Glu185Gln, Leu34Leu and Pro672Pro. With the exception of Glu185Gln, all variants are synonymous, thus do not alter the conformational structure of the protein. No correlation was found between these polymorphisms and breast cancer. With the exception of a couple of studies (Lu et al., 2006; Smith et al., 2008), all results from studies in different populations regarding Glu185Gln were negative. Lu et al. found statistically significant association of the SNPs Glu185Gln and 5' UTR 924T>C individually and haplotypes in young non-Hispanic white women in Texas, USA (Lu et al., 2006). Smith et al. showed that there were significant trends in breast cancer risk with increasing numbers of risk genotypes (at least one variant allele) of *NBS1* 185 GluGln/GlnGln in African-Americans (Smith et al., 2008). These discrepancies across studies might be associated with different genetic backgrounds, different risk factors in different populations, and the sample size of these studies. Thus, more studies are required in order to make any statement, although, according to the results obtained until now, *NBS1* is not a probable low penetrance gene.

The *RAD51* protein is responsible for the central activity of the HRR pathway, in which it catalyses the invasion of the broken ends of the DSB into the intact sister chromatid. Among several polymorphisms in *RAD51* gene, a functional SNP at position 135 in 5' UTR, changing a guanine to cytosine, was reported. Indeed, it was stated that this variant allele improves *RAD51* expression (Hasselbach et al., 2005). Consequently, many molecular

epidemiological studies were performed with the purpose of examining an association between this *RAD51* variant and susceptibility to breast cancer. Many inconsistencies were found, even within the same population. Thus, a meta-analysis of all results found until now might bring some more precise estimation of the association of this SNP with susceptibility to breast cancer. Recently, four important meta-analyses (Gao et al., 2011; Sun et al., 2011; Wang et al., 2010; Zhou et al., 2011), covering tens of other studies and thousands of subjects, were unanimous to state that the variant allele of *RAD51* G135C may contribute to increased breast cancer susceptibility, which is in accordance with biological function study, which showed a more aggressive and poor prognosis phenotype (Costa et al., 2008). Zhou et al. also reported that the C variant of this SNP is associated with an augmented breast cancer risk among the *BRCA2* mutation carriers, but not *BRCA1* (Zhou et al., 2011). Therefore, taking into account these meta-analyses, *RAD51* G135C is a good candidate for a low penetrant risk factor for breast cancer. Recently, however, Yu et al. stated that these studies are not convincing since most are biased. According to the authors the populations have no representation of real general breast cancer cases since they took into account all breast cancer cases, including the *BRCA1* and *BRCA2* mutation carriers (Yu et al., 2011). The authors suggest that correct experimental design should be followed, such as subgroup meta-analysis in specific populations.

XRCC2 protein is a *RAD51*-related protein, essential for efficient HRR, and hence for maintenance of chromosome stability, making part of the nucleoprotein filament that acts as a cofactor for the *RAD51* strand invasion and exchange activities, although there are other indications of its involvement in the late stages of the HRR pathway, namely the branch migration and Holliday junction resolution (Dudas & Chovanec, 2004). The *XRCC2* Arg188His polymorphism is by far the most studied in this gene. Many are in disagreement and contradictory, thus, recently, a meta-analysis was published by Yu et al. where all results published until then were analyzed and a more convincing and precise estimation of the association of this SNP and breast cancer was made (Yu et al., 2010). The authors reached the conclusion that *XRCC2* Arg188His is not associated with individual susceptibility for breast cancer. However, they suggested that this SNP can modify the risk for breast cancer in response to exogenous compounds, and stressed the importance of investigating this possibility. Subsequent to this publication, a report made by us (Silva et al., 2010) was published with results concerning this SNP in a Portuguese population. The results were also negative when the SNP alone was considered, although, when the population was stratified according to the breast feeding status, it was observed that individuals that never breast fed and carried one variant allele of this polymorphism have a decreased risk for breast cancer. It is known that women that breast fed for long periods have a reduced risk for breast cancer. This can be explained by the fact that the exfoliation of ductal cells as a consequence of breast feeding might remove a significant number of cells with genetic damage, preventing their transformation into neoplastic cells. In those women that do not breast feed we have found a protective role of the variant allele of *XRCC2* Arg188His that might be related to a more efficient repair of DNA lesions. However, taking into consideration the size of our sample stratification, the small number of cases could act as a limitation factor. Thus, to exclude false positive results, further investigation in larger populations needs to be done.

XRCC3 protein is also a *Rad51*-related protein that participates in homologous recombination repair to maintain chromosome stability. *XRCC3* forms filamentous structures in complex with *Rad51C* that assists *RAD51*-mediated strand invasion (Li &

Heyer, 2008). *XRCC3* is a highly polymorphic gene and many SNPs have been already described. Among them, *XRCC3* Thr241Met, 5'UTR A/G and IVS5-14 A/G are the most studied. As a result of inconclusive data of the several studies reported, four meta-analyses were published, three covering *XRCC3* Thr241Met (Economopoulos & Sergentanis, 2010; Garcia-Closas et al., 2006; Lee et al., 2007) and two the *XRCC3* 5'UTR A/G and IVS5-14 A/G (Garcia-Closas et al., 2006; Qiu et al., 2010a). In a first approach to obtain accurate results García-Closas et al. performed a meta-analysis with two populations, one from USA and other from Poland (Garcia-Closas et al., 2006). The authors concluded that the variant allele of these SNPs has a weak association with breast cancer. Later Lee et al. conducted a case-control with a Korean population and a meta-analysis of other 12 studies (Lee et al., 2007). The results fail to show statistically significant association of *XRCC3* Thr241Met with breast cancer. However the meta-analysis suggests a weak association between *XRCC3* Thr241Met and breast cancer, highlighting the differences between oriental and occidental populations. Recently, two more meta-analysis were published (Economopoulos & Sergentanis, 2010; Qiu et al., 2010a). Economopoulos & Sergentanis performed a meta-analysis concerning *XRCC3* Thr241Met and the results seems to be similar to those reported previously, thus, variant allele is associated with elevated breast cancer risk in non-Chinese subjects (Economopoulos & Sergentanis, 2010). Indeed, the authors have some reservations with regards to the studies with Chinese populations since no consistent data were obtained. Qiu et al., in order to fill a shortage of meta-analysis of *XRCC3* 5'UTR A/G and IVS5-14 A/G, published a report encompassing all eligible data from several studies concerning these two SNPs (Qiu et al., 2010a). The results suggest that the variant allele of *XRCC3* 5'UTR A/G is associated with breast cancer risk while the variant allele of *XRCC3* IVS5-14 A/G has a protective effect on breast cancer. Subsequently to these reports a work conducted by us (Silva et al., 2010) showed that *XRCC3* Thr241Met alone does not confer susceptibility to breast cancer. However, after stratification according to menopausal status, post-menopausal women carrying at least one variant allele seem to have lower risk for breast cancer, although stratifications applied to small populations could act as a limitation factor.

Along with *BRCA2*, *BRCA1* is well documented as a hereditary breast cancer susceptibility gene. However, there are much fewer published data addressing susceptibility alleles of *BRCA1* in sporadic breast cancer (Cox et al., 2005; Freedman et al., 2005; Goode et al., 2002a; Huo et al., 2009). Of the four eligible reports, none showed association of common SNPs in *BRCA1* with breast cancer susceptibility. One report described a haplotype in *BRCA1* gene that may cause an increased risk for breast cancer, precisely, a 20% increment in risk for breast cancerigenesis. However, the authors failed to find out which variant is responsible for the association (Cox et al., 2005). Other study showed a possible association between an interaction of *BRCA1* and *ZNF350* with individual susceptibility for breast cancer (Huo et al., 2009). Similarly, *BRCA2* has few studies what concern sporadic breast cancer. However, a meta-analysis of about 44,903 subjects was recently published about *BRCA2* Asn372His polymorphism (Qiu et al., 2010b). This SNP is the most studied in this gene and according to the authors the variant allele may be a low-penetrant risk factor for breast cancer disease. However, the authors suggest that larger studies need to be done and homogeneous populations should be recruited, including well matched controls. Other meta-analysis also has reported the same results some years earlier (Garcia-Closas et al., 2006). Other study published by Ishitobi et al. reported null results for *BRCA2* Asn372His but not *BRCA2* Met784Val in a Japanese population. Specifically, the *BRCA2* Met784Val variant allele showed a significantly lower survival rate of 63% (Ishitobi et al., 2003).

To a lesser extent NHEJ genes were also studied. An early study found that the combined effect of individual SNPs of NHEJ genes may be significantly associated with breast cancer risk; indeed Fu et al. found interesting results concerning estrogen exposure (Fu et al., 2003). The authors stated that women with greater putative high-risk genotypes have an increased risk for breast cancer disease and the results were even more significant and the risk stronger in women that never had a history of pregnancy. The authors also found statistically significant results for *Ku70* c.-1310 C>G and *XRCC4* Thr1394Gly individually. Later, Willems et al. also found similar results for the *Ku70* c.-1310 C>G SNP in a Belgian population (Willems et al., 2009). García-Closas et al. published a meta-analysis stating negative results for *LIG4* Asp568Asp, Thr9Ile and *XRCC4* IVS7-1A>G. Larger studies must be performed in order to verify these results and other populations should be taken into account (García-Closas et al., 2006).

2.4.2 DSB and thyroid cancer

The only observed risk factor for thyroid cancer is ionizing radiation. However, most of cases do not have a history of ionizing radiation exposure and people exposed to X- or γ -rays, for example, not necessarily develop thyroid cancer. Thus, it is suggested that there might be an individual sensitivity to ionizing radiation due to genetic factors, such as polymorphic genes. Since ionizing radiation causes DSBs, it is plausible to study SNPs in genes that are involved in the DSBs repair. Until now, few studies have been done concerning end-joining and homologous recombination repair. A study conducted by Bastos et al. observed that the coexistence of three or more variant alleles of *XRCC3* Thr241Met and *RAD51* 5'UTR (Ex1-59G>T) genes were associated with a significant higher risk for thyroid cancer (Bastos et al., 2009). However, independently the SNPs show no association with thyroid cancer. This SNP-SNP association might lead to a deficit in the formation of the DNA damage-induced RAD51 foci and consequently a deficient repair by HRR system. Sturgis et al. also reported a possible association of the variant allele of *XRCC3* Thr241Met with thyroid cancer (Sturgis et al., 2005). Indeed, among 10 SNPs studied, which include other SNPs of DSBs repair genes, such as *BRCA1*, *BRCA2*, *RAD51*, *XRCC3* and *XRCC7*, only the *XRCC3* Thr241Met seems to confer susceptibility for thyroid cancer. Further molecular epidemiological and functional studies must be performed in order to assure the accuracy of the results reported by both groups. Other study conducted by Gomes et al. shows a marginal association of NHEJ pathway SNPs with thyroid cancer (Gomes et al., 2010). To be precise, statically significant results were found for association of *Ku80* 3'UTR Ex21-238G>A and Ex21+338T>C SNPs with papillary tumours, after stratification by tumour type (papillary and follicular), showing that different histological types can have different genetic basis. This study seems to be the only one performed until now, concerning NHEJ and thyroid cancer.

3. Conclusion

Several reports have been published associating some SNPs in DNA repair genes with breast and thyroid cancer disease. Although relevant, the modifying effect of the majority of these SNPs in cell phenotype is still not understood and association studies are contradictory. This inconsistency might be due to different populations used, sample sizes, sample selection bias, genetic background and life style. Retrospective studies like these have to be interpreted with care and are difficult to draw meaningful practical conclusions from that can help directly patients with breast and thyroid cancer. In order to acquire more

powerful and accurate results several meta-analysis also have been published reviewing all studies made till publication date about a particular SNP and disease. Again, these reports need to be interpreted with caution, since many authors have study selection bias, including all cases of breast cancer, even the ones with *BRCA1* and *BRCA2* mutations carriers. Prospective studies and adequately selected meta-analysis should give further insight about the relevance of SNPs in breast and thyroid cancerigenesis.

Although the real role of SNPs in cancerigenesis is not well established by the authors, the new era in cancer treatment and prevention lies in the ability to treat patients individually according their genetic constitution and the DNA repair status of their tumours. For that, is crucial to have specific knowledge about the polymorphisms carried by each patient and how these polymorphisms influences response to therapy.

In fact, the existence of inter-individual variation influences response and survival rate following chemotherapy and radiation treatment of cancer. Standard cancer therapy involves the use of agents that themselves damage DNA with the ultimate goal of killing the cell. However, damaging the DNA does not always kill the cell, which is avoided by DNA repair pathways that remove the damage from DNA. Recent studies have suggested that the targeting of repair pathways by specific agents can result in effective killing of tumour cells (Li et al., 2010). DNA damage acquired from these treatments can initiate a number of cellular pathways involved in DNA repair, cell cycle control, metabolism and apoptosis (Bewick et al., 2011). If so, SNPs in genes of DNA repair may significantly affect its efficiency, clinical outcome and thus may help identify patients that can benefit from various treatments. Accordingly, new strategies for individualization of treatment in cancer patients are becoming an emerging issue.

Due to a phenomenon known as linkage disequilibrium, the value of a SNP in the same chromosome could be associated with specific values in other SNPs nearby. Indeed, specific SNPs associations (tagSNPs) related with the toxicity/efficacy of DNA damaging chemotherapy, improve our ability to map SNPs in specific genes associated with chemotherapy resistance and assure a better clinical outcome for cancer patients. This approach seems to be of importance since it can associate haplotype blocks with cancer therapy with lesser resources (Anuniação et al., 2010; Frazer et al., 2007).

Some SNPs in DNA repair genes have been reported as potential markers for individual susceptibility to breast (e.g. *XRCC1*; *XRCC2* and *XRCC3* genes polymorphisms) and thyroid (e.g. *XRCC1*; *XRCC3* and *RAD51* genes polymorphisms) cancers. However, the incoherent results don't have enough strength to demonstrate the real role of those SNPs, at least with regards to breast and thyroid carcinomas. We think that genetic polymorphisms, particularly SNPs, may have low influence in breast and thyroid cancerigenesis, however, SNPs may be much more relevant in acquired resistance to chemotherapeutic agents. In fact, new strategies for individualization of treatment in cancer patients are becoming an emerging issue. This approach must be the best way to find a practical result and clinical use for association studies. The emergence of SNPs with important roles in cancer therapy is now the focus of our work.

4. References

Adjadj, E., Rubino, C., Shamsaldim, A., Le, M. G., Schlumberger, M., & de, Vathaire F. (15-9-2003). The risk of multiple primary breast and thyroid carcinomas. *Cancer*, 98, 6, 1309-1317.

- Amir, E., Seruga, B., Serrano, R., & Ocana, A. (2010). Targeting DNA repair in breast cancer: a clinical and translational update. *Cancer Treat.Rev.*, 36, 7, 557-565.
- Anuniação, O., Gomes, B. C., Vinga, S., Gaspar, J., Oliveira, A. L., & Rueff, J. (2010). Data Mining Approach for the detection of High-Risk Breast Cancer Groups. *Advances in Bioinformatics - Advances in soft computing*, 74, 43-52.
- Bartsch, H., Dally, H., Popanda, O., Risch, A., & Schmezer, P. (2007). Genetic risk profiles for cancer susceptibility and therapy response. *Recent Results Cancer Res.*, 174, 19-36.
- Bastos, H. N., Antao, M. R., Silva, S. N., Azevedo, A. P., Manita, I., Teixeira, V., Pina, J. E., Gil, O. M., Ferreira, T. C., Limbert, E., Rueff, J., & Gaspar, J. F. (2009). Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. *Thyroid*, 19, 10, 1067-1075.
- Bewick, M. A., Conlon, M. S., & Lafrenie, R. M. (2006). Polymorphisms in XRCC1, XRCC3, and CCND1 and survival after treatment for metastatic breast cancer. *J Clin.Oncol.*, 24, 36, 5645-5651.
- Bewick, M. A., Lafrenie, R. M., & Conlon, M. S. (2011). Nucleotide excision repair polymorphisms and survival outcome for patients with metastatic breast cancer. *J Cancer Res.Clin.Oncol.*, 137, 3, 543-550.
- Bonassi, S., El-Zein, R., Bolognesi, C., & Fenech, M. (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis*, 26, 1, 93-100.
- Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W. P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M. P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M. R., Zijno, A., Norppa, H., & Fenech, M. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, 28, 3, 625-631.
- Brown, A. P., Chen, J., Hitchcock, Y. J., Szabo, A., Shrieve, D. C., & Tward, J. D. (2008). The risk of second primary malignancies up to three decades after the treatment of differentiated thyroid cancer. *J Clin.Endocrinol.Metab*, 93, 2, 504-515.
- Canchola, A. J., Horn-Ross, P. L., & Purdie, D. M. (2006). Risk of second primary malignancies in women with papillary thyroid cancer. *Am.J Epidemiol.*, 163, 6, 521-527.
- Chang-Claude, J., Popanda, O., Tan, X. L., Kropp, S., Helmbold, I., von, Fournier D., Haase, W., Sautter-Bihl, M. L., Wenz, F., Schmezer, P., & Ambrosone, C. B. (2005). Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and acute side effects of radiotherapy in breast cancer patients. *Clin.Cancer Res.*, 11, 13, 4802-4809.
- Chiang, F. Y., Wu, C. W., Hsiao, P. J., Kuo, W. R., Lee, K. W., Lin, J. C., Liao, Y. C., & Juo, S. H. (2008). Association between polymorphisms in DNA base excision repair genes XRCC1, APE1, and ADPRT and differentiated thyroid carcinoma. *Clin.Cancer Res.*, 14, 18, 5919-5924.
- Conde, J., Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study. *BMC.Cancer*, 9, 344-
- Costa, S., Pinto, D., Pereira, D., Rodrigues, H., Cameselle-Teijeiro, J., Medeiros, R., & Schmitt, F. (2008). XRCC1 Arg399Gln and RAD51 5'UTR G135C polymorphisms

- and their outcome in tumor aggressiveness and survival of Portuguese breast cancer patients. *Breast Cancer Res.Treat.*, 109, 1, 183-185.
- Cox, D. G., Kraft, P., Hankinson, S. E., & Hunter, D. J. (2005). Haplotype analysis of common variants in the BRCA1 gene and risk of sporadic breast cancer. *Breast Cancer Res.*, 7, 2, R171-R175.
- Crew, K. D., Gammon, M. D., Terry, M. B., Zhang, F. F., Zablotska, L. B., Agrawal, M., Shen, J., Long, C. M., Eng, S. M., Sagiv, S. K., Teitelbaum, S. L., Neugut, A. I., & Santella, R. M. (2007). Polymorphisms in nucleotide excision repair genes, polycyclic aromatic hydrocarbon-DNA adducts, and breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 16, 10, 2033-2041.
- Dapic, V., Carvalho, M. A., & Monteiro, A. N. (2005). Breast cancer susceptibility and the DNA damage response. *Cancer Control*, 12, 2, 127-136.
- Dhillon, V. S., Thomas, P., Iarmarcovai, G., Kirsch-Volders, M., Bonassi, S., & Fenech, M. (2011). Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. *Mutagenesis*, 26, 1, 33-42.
- Dudas, A. & Chovanec, M. (2004). DNA double-strand break repair by homologous recombination. *Mutat.Res.*, 566, 2, 131-167.
- Economopoulos, K. P. & Sergentanis, T. N. (2010). XRCC3 Thr241Met polymorphism and breast cancer risk: a meta-analysis. *Breast Cancer Res.Treat.*, 121, 2, 439-443.
- El-Zein, R., Vral, A., & Etzel, C. J. (2011). Cytokinesis-blocked micronucleus assay and cancer risk assessment. *Mutagenesis*, 26, 1, 101-106.
- Fackenthal, J. D. & Olopade, O. I. (2007). Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations. *Nat.Rev.Cancer*, 7, 12, 937-948.
- Frazer, K. A., Ballinger, D. G., Cox, D. R., Hinds, D. A., Stuve, L. L., Gibbs, R. A., Belmont, J. W., Boudreau, A., Hardenbol, P., Leal, S. M., Pasternak, S., Wheeler, D. A., Willis, T. D., Yu, F., Yang, H., Zeng, C., Gao, Y., Hu, H., Hu, W., Li, C., Lin, W., Liu, S., Pan, H., Tang, X., Wang, J., Wang, W., Yu, J., Zhang, B., Zhang, Q., Zhao, H., Zhao, H., Zhou, J., Gabriel, S. B., Barry, R., Blumenstiel, B., Camargo, A., Defelice, M., Faggart, M., Goyette, M., Gupta, S., Moore, J., Nguyen, H., Onofrio, R. C., Parkin, M., Roy, J., Stahl, E., Winchester, E., Ziaugra, L., Altshuler, D., Shen, Y., Yao, Z., Huang, W., Chu, X., He, Y., Jin, L., Liu, Y., Shen, Y., Sun, W., Wang, H., Wang, Y., Wang, Y., Xiong, X., Xu, L., Waye, M. M., Tsui, S. K., Xue, H., Wong, J. T., Galver, L. M., Fan, J. B., Gunderson, K., Murray, S. S., Oliphant, A. R., Chee, M. S., Montpetit, A., Chagnon, F., Ferretti, V., Leboeuf, M., Olivier, J. F., Phillips, M. S., Roumy, S., Sallee, C., Verner, A., Hudson, T. J., Kwok, P. Y., Cai, D., Koboldt, D. C., Miller, R. D., Pawlikowska, L., Taillon-Miller, P., Xiao, M., Tsui, L. C., Mak, W., Song, Y. Q., Tam, P. K., Nakamura, Y., Kawaguchi, T., Kitamoto, T., Morizono, T., Nagashima, A., Ohnishi, Y., Sekine, A., Tanaka, T., Tsunoda, T., Deloukas, P., Bird, C. P., Delgado, M., Dermitzakis, E. T., Gwilliam, R., Hunt, S., Morrison, J., Powell, D., Stranger, B. E., Whittaker, P., Bentley, D. R., Daly, M. J., de Bakker, P. I., Barrett, J., Chretien, Y. R., Maller, J., McCarroll, S., Patterson, N., Pe'er, I., Price, A., Purcell, S., Richter, D. J., Sabeti, P., Saxena, R., Schaffner, S. F., Sham, P. C., Varilly, P., Altshuler, D., Stein, L. D., Krishnan, L., Smith, A. V., Tello-Ruiz, M. K., Thorisson, G. A., Chakravarti, A., Chen, P. E., Cutler, D. J., Kashuk, C. S., Lin, S., Abecasis, G. R., Guan, W., Li, Y., Munro, H. M., Qin, Z. S., Thomas, D. J., McVean, G., Auton, A.,

- Bottolo, L., Cardin, N., Eyheramendy, S., Freeman, C., Marchini, J., Myers, S., Spencer, C., Stephens, M., Donnelly, P., Cardon, L. R., Clarke, G., Evans, D. M., Morris, A. P., Weir, B. S., Tsunoda, T., Mullikin, J. C., Sherry, S. T., Feolo, M., Skol, A., Zhang, H., Zeng, C., Zhao, H., Matsuda, I., Fukushima, Y., Macer, D. R., Suda, E., Rotimi, C. N., Adebamowo, C. A., Ajayi, I., Aniagwu, T., Marshall, P. A., Nkwodimmah, C., Royal, C. D., Leppert, M. F., Dixon, M., Peiffer, A., Qiu, R., Kent, A., Kato, K., Niikawa, N., Adewole, I. F., Knoppers, B. M., Foster, M. W., Clayton, E. W., Watkin, J., Gibbs, R. A., Belmont, J. W., Muzny, D., Nazareth, L., Sodergren, E., Weinstock, G. M., Wheeler, D. A., Yakub, I., Gabriel, S. B., Onofrio, R. C., Richter, D. J., Ziaugra, L., Birren, B. W., Daly, M. J., Altshuler, D., Wilson, R. K., Fulton, L. L., Rogers, J., Burton, J., Carter, N. P., Clee, C. M., Griffiths, M., Jones, M. C., McLay, K., Plumb, R. W., Ross, M. T., Sims, S. K., Willey, D. L., Chen, Z., Han, H., Kang, L., Godbout, M., Wallenburg, J. C., L'Archeveque, P., Bellemare, G., Saeki, K., Wang, H., An, D., Fu, H., Li, Q., Wang, Z., Wang, R., Holden, A. L., Brooks, L. D., McEwen, J. E., Guyer, M. S., Wang, V. O., Peterson, J. L., Shi, M., Spiegel, J., Sung, L. M., Zacharia, L. F., Collins, F. S., Kennedy, K., Jamieson, R., & Stewart, J. (18-10-2007). A second generation human haplotype map of over 3.1 million SNPs. *Nature*, 449, 7164, 851-861.
- Freedman, M. L., Penney, K. L., Stram, D. O., Riley, S., McKean-Cowdin, R., Le, Marchand L., Altshuler, D., & Haiman, C. A. (2005). A haplotype-based case-control study of BRCA1 and sporadic breast cancer risk. *Cancer Res.*, 65, 16, 7516-7522.
- Fu, Y. P., Yu, J. C., Cheng, T. C., Lou, M. A., Hsu, G. C., Wu, C. Y., Chen, S. T., Wu, H. S., Wu, P. E., & Shen, C. Y. (2003). Breast cancer risk associated with genotypic polymorphism of the nonhomologous end-joining genes: a multigenic study on cancer susceptibility. *Cancer Res.*, 63, 10, 2440-2446.
- Gao, L. B., Pan, X. M., Li, L. J., Liang, W. B., Zhu, Y., Zhang, L. S., Wei, Y. G., Tang, M., & Zhang, L. (2011). RAD51 135G/C polymorphism and breast cancer risk: a meta-analysis from 21 studies. *Breast Cancer Res. Treat.*, 125, 3, 827-835.
- Garcia-Closas, M., Egan, K. M., Newcomb, P. A., Brinton, L. A., Titus-Ernstoff, L., Chanock, S., Welch, R., Lissowska, J., Peplonska, B., Szeszenia-Dabrowska, N., Zatonski, W., Bardin-Mikolajczak, A., & Struewing, J. P. (2006). Polymorphisms in DNA double-strand break repair genes and risk of breast cancer: two population-based studies in USA and Poland, and meta-analyses. *Hum. Genet.*, 119, 4, 376-388.
- Garcia-Quispe, W. A., Perez-Machado, G., Akdi, A., Pastor, S., Galofre, P., Biarnes, F., Castell, J., Velazquez, A., & Marcos, R. (2011). Association studies of OGG1, XRCC1, XRCC2 and XRCC3 polymorphisms with differentiated thyroid cancer. *Mutat. Res.*, 709-710, 67-72.
- Garner, C. N., Ganetzky, R., Brainard, J., Hammel, J. P., Berber, E., Siperstein, A. E., & Milas, M. (2007). Increased prevalence of breast cancer among patients with thyroid and parathyroid disease. *Surgery*, 142, 6, 806-813.
- Gaspar, J., Rodrigues, S., Gil, O. M., Manita, I., Ferreira, T. C., Limbert, E., Goncalves, L., Pina, J. E., & Rueff, J. (2004). Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. *Cancer Genet. Cytogenet.*, 151, 1, 60-67.
- Gomes, B. C., Silva, S. N., Azevedo, A. P., Manita, I., Gil, O. M., Ferreira, T. C., Limbert, E., Rueff, J., & Gaspar, J. F. (2010). The role of common variants of non-homologous

- end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk. *Oncol.Rep.*, 24, 4, 1079-1085.
- Goode, E. L., Dunning, A. M., Kuschel, B., Healey, C. S., Day, N. E., Ponder, B. A., Easton, D. F., & Pharoah, P. P. (2002a). Effect of germ-line genetic variation on breast cancer survival in a population-based study. *Cancer Res.*, 62, 11, 3052-3057.
- Goode, E. L., Ulrich, C. M., & Potter, J. D. (2002b). Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 11, 12, 1513-1530.
- Grubbs, E. G., Rich, T. A., Li, G., Sturgis, E. M., Younes, M. N., Myers, J. N., Edeiken-Monroe, B., Fornage, B. D., Monroe, D. P., Staerke, G. A., Williams, M. D., Waguespack, S. G., Hu, M. I., Cote, G., Gagel, R. F., Cohen, J., Weber, R. S., Anaya, D. A., Holsinger, F. C., Perrier, N. D., Clayman, G. L., & Evans, D. B. (2008). Recent advances in thyroid cancer. *Curr.Probl.Surg.*, 45, 3, 156-250.
- Gudmundsdottir, K. & Ashworth, A. (25-9-2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*, 25, 43, 5864-5874.
- Hakem, R. (2008). DNA-damage repair; the good, the bad, and the ugly. *EMBO J*, 27, 4, 589-605.
- Hanahan, D. & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, 100, 1, 57-70.
- Hasler, J. A. (1999). Pharmacogenetics of cytochromes P450. *Mol.Aspects Med.*, 20, 1-2, 12-137.
- Hasselbach, L., Haase, S., Fischer, D., Kolberg, H. C., & Sturzbecher, H. W. (2005). Characterisation of the promoter region of the human DNA-repair gene Rad51. *Eur.J.Gynaecol.Oncol.*, 26, 6, 589-598.
- Hedenfalk, I., Ringner, M., Ben-Dor, A., Yakhini, Z., Chen, Y., Chebil, G., Ach, R., Loman, N., Olsson, H., Meltzer, P., Borg, A., & Trent, J. (2003). Molecular classification of familial non-BRCA1/BRCA2 breast cancer. *Proc.Natl.Acad.Sci.U.S.A*, 100, 5, 2532-2537.
- Ho, T., Li, G., Lu, J., Zhao, C., Wei, Q., & Sturgis, E. M. (2009). Association of XRCC1 polymorphisms and risk of differentiated thyroid carcinoma: a case-control analysis. *Thyroid*, 19, 2, 129-135.
- Hsieh, P. & Yamane, K. (2008). DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech.Ageing Dev.*, 129, 7-8, 391-407.
- Huang, Y., Li, L., & Yu, L. (2009). XRCC1 Arg399Gln, Arg194Trp and Arg280His polymorphisms in breast cancer risk: a meta-analysis. *Mutagenesis*, 24, 4, 331-339.
- Hunter, D. J., Riboli, E., Haiman, C. A., Albanes, D., Altshuler, D., Chanock, S. J., Haynes, R. B., Henderson, B. E., Kaaks, R., Stram, D. O., Thomas, G., Thun, M. J., Blanche, H., Buring, J. E., Burt, N. P., Calle, E. E., Cann, H., Canzian, F., Chen, Y. C., Colditz, G. A., Cox, D. G., Dunning, A. M., Feigelson, H. S., Freedman, M. L., Gaziano, J. M., Giovannucci, E., Hankinson, S. E., Hirschhorn, J. N., Hoover, R. N., Key, T., Kolonel, L. N., Kraft, P., Le, Marchand L., Liu, S., Ma, J., Melnick, S., Pharaoh, P., Pike, M. C., Rodriguez, C., Setiawan, V. W., Stampfer, M. J., Trapido, E., Travis, R., Virtamo, J., Wacholder, S., & Willett, W. C. (2005). A candidate gene approach to searching for low-penetrance breast and prostate cancer genes. *Nat.Rev.Cancer*, 5, 12, 977-985.
- Huo, X., Lu, C., Huang, X., Hu, Z., Jin, G., Ma, H., Wang, X., Qin, J., Wang, X., Shen, H., & Tang, J. (2009). Polymorphisms in BRCA1, BRCA1-interacting genes and

- susceptibility of breast cancer in Chinese women. *J.Cancer Res.Clin.Oncol.*, 135, 11, 1569-1575.
- Inskip, P. D. (2001). Thyroid cancer after radiotherapy for childhood cancer. *Med.Pediatr.Oncol.*, 36, 5, 568-573.
- Ishitobi, M., Miyoshi, Y., Ando, A., Hasegawa, S., Egawa, C., Tamaki, Y., Monden, M., & Noguchi, S. (2003). Association of BRCA2 polymorphism at codon 784 (Met/Val) with breast cancer risk and prognosis. *Clin.Cancer Res.*, 9, 4, 1376-1380.
- Jaremko, M., Justenhoven, C., Schroth, W., Abraham, B. K., Fritz, P., Vollmert, C., Illig, T., Simon, W., Schwab, M., & Brauch, H. (2007). Polymorphism of the DNA repair enzyme XRCC1 is associated with treatment prediction in anthracycline and cyclophosphamide/methotrexate/5-fluorouracil-based chemotherapy of patients with primary invasive breast cancer. *Pharmacogenet.Genomics*, 17, 7, 529-538.
- Jelonek, K., Gdowicz-Klosok, A., Pietrowska, M., Borkowska, M., Korfanty, J., Rzeszowska-Wolny, J., & Widlak, P. (2010). Association between single-nucleotide polymorphisms of selected genes involved in the response to DNA damage and risk of colon, head and neck, and breast cancers in a Polish population. *J Appl.Genet.*, 51, 3, 343-352.
- Jiricny, J. (2006). The multifaceted mismatch-repair system. *Nat.Rev.Mol.Cell Biol.*, 7, 5, 335-346.
- Jorgensen, T. J., Visvanathan, K., Ruczinski, I., Thuita, L., Hoffman, S., & Helzlsouer, K. J. (2007). Breast cancer risk is not associated with polymorphic forms of xeroderma pigmentosum genes in a cohort of women from Washington County, Maryland. *Breast Cancer Res.Treat.*, 101, 1, 65-71.
- Kawajiri, K., Nakachi, K., Imai, K., Yoshii, A., Shinoda, N., & Watanabe, J. (1990). Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P450IA1 gene. *FEBS Lett.*, 263, 1, 131-133.
- Kelley, M. R. & Fishel, M. L. (2008). DNA repair proteins as molecular targets for cancer therapeutics. *Anticancer Agents Med.Chem.*, 8, 4, 417-425.
- Kondo, T., Ezzat, S., & Asa, S. L. (2006). Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat.Rev.Cancer*, 6, 4, 292-306.
- Kumar, R., Hoglund, L., Zhao, C., Forsti, A., Snellman, E., & Hemminki, K. (2003). Single nucleotide polymorphisms in the XPG gene: determination of role in DNA repair and breast cancer risk. *Int.J Cancer*, 103, 5, 671-675.
- Kuschel, B., Auranen, A., McBride, S., Novik, K. L., Antoniou, A., Lipscombe, J. M., Day, N. E., Easton, D. F., Ponder, B. A., Pharoah, P. D., & Dunning, A. (2002). Variants in DNA double-strand break repair genes and breast cancer susceptibility. *Hum.Mol.Genet.*, 11, 12, 1399-1407.
- Latimer, J. J., Johnson, J. M., Kelly, C. M., Miles, T. D., Beaudry-Rodgers, K. A., Lalanne, N. A., Vogel, V. G., Kanbour-Shakir, A., Kelley, J. L., Johnson, R. R., & Grant, S. G. (2010). Nucleotide excision repair deficiency is intrinsic in sporadic stage I breast cancer. *Proc.Natl.Acad.Sci.U.S.A*, 107, 50, 21725-21730.
- Lee, S. A., Lee, K. M., Park, S. K., Choi, J. Y., Kim, B., Nam, J., Yoo, K. Y., Noh, D. Y., Ahn, S. H., & Kang, D. (2007). Genetic polymorphism of XRCC3 Thr241Met and breast cancer risk: case-control study in Korean women and meta-analysis of 12 studies. *Breast Cancer Res.Treat.*, 103, 1, 71-76.

- Lee, S. A., Lee, K. M., Park, W. Y., Kim, B., Nam, J., Yoo, K. Y., Noh, D. Y., Ahn, S. H., Hirvonen, A., & Kang, D. (2005). Obesity and genetic polymorphism of ERCC2 and ERCC4 as modifiers of risk of breast cancer. *Exp.Mol.Med.*, 37, 2, 86-90.
- Li, H., Ha, T. C., & Tai, B. C. (2009). XRCC1 gene polymorphisms and breast cancer risk in different populations: a meta-analysis. *Breast*, 18, 3, 183-191.
- Li, S. X., Sjolund, A., Harris, L., & Sweasy, J. B. (2010). DNA repair and personalized breast cancer therapy. *Environ.Mol.Mutagen.*, 51, 8-9, 897-908.
- Li, X. & Heyer, W. D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res.*, 18, 1, 99-113.
- Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu.Rev.Biochem.*, 79, 181-211.
- Lu, J., Wei, Q., Bondy, M. L., Li, D., Brewster, A., Shete, S., Yu, T. K., Sahin, A., Meric-Bernstam, F., Hunt, K. K., Singletary, S. E., Ross, M. I., & Wang, L. E. (2006). Polymorphisms and haplotypes of the NBS1 gene are associated with risk of sporadic breast cancer in non-Hispanic white women <or=55 years. *Carcinogenesis*, 27, 11, 2209-2216.
- Maynard, S., Schurman, S. H., Harboe, C., de Souza-Pinto, N. C., & Bohr, V. A. (2009). Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis*, 30, 1, 2-10.
- McVey, M. & Lee, S. E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet.*, 24, 11, 529-538.
- Mechanic, L. E., Millikan, R. C., Player, J., de Cotret, A. R., Winkel, S., Worley, K., Heard, K., Heard, K., Tse, C. K., & Keku, T. (2006). Polymorphisms in nucleotide excision repair genes, smoking and breast cancer in African Americans and whites: a population-based case-control study. *Carcinogenesis*, 27, 7, 1377-1385.
- Millikan, R. C., Player, J. S., Decotret, A. R., Tse, C. K., & Keku, T. (2005). Polymorphisms in DNA repair genes, medical exposure to ionizing radiation, and breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 14, 10, 2326-2334.
- Mollerup, S., Ryberg, D., Hewer, A., Phillips, D. H., & Haugen, A. (1999). Sex differences in lung CYP1A1 expression and DNA adduct levels among lung cancer patients. *Cancer Res.*, 59, 14, 3317-3320.
- Murata, H., Khattar, N. H., Kang, Y., Gu, L., & Li, G. M. (22-8-2002). Genetic and epigenetic modification of mismatch repair genes hMSH2 and hMLH1 in sporadic breast cancer with microsatellite instability. *Oncogene*, 21, 37, 5696-5703.
- Narod, S. A. & Foulkes, W. D. (2004). BRCA1 and BRCA2: 1994 and beyond. *Nat.Rev.Cancer*, 4, 9, 665-676.
- Oksenych, V. & Coin, F. (2010). The long unwinding road: XPB and XPD helicases in damaged DNA opening. *Cell Cycle*, 9, 1, 90-96.
- Pabalan, N., Francisco-Pabalan, O., Sung, L., Jarjanazi, H., & Ozcelik, H. (2010). Meta-analysis of two ERCC2 (XPD) polymorphisms, Asp312Asn and Lys751Gln, in breast cancer. *Breast Cancer Res.Treat.*, 124, 2, 531-541.
- Pavanello, S. & Clonfero, E. (2000). [Biomarkers of gentotoxic risk and metabolic polymorphism]. *Med.Lav.*, 91, 5, 431-469.
- Peng, S., Lu, B., Ruan, W., Zhu, Y., Sheng, H., & Lai, M. (2011). Genetic polymorphisms and breast cancer risk: evidence from meta-analyses, pooled analyses, and genome-wide association studies. *Breast Cancer Res.Treat.*,

- Perera, F. P. & Weinstein, I. B. (2000). Molecular epidemiology: recent advances and future directions. *Carcinogenesis*, 21, 3, 517-524.
- Pooley, K. A., Baynes, C., Driver, K. E., Tyrer, J., Azzato, E. M., Pharoah, P. D., Easton, D. F., Ponder, B. A., & Dunning, A. M. (2008). Common single-nucleotide polymorphisms in DNA double-strand break repair genes and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, 17, 12, 3482-3489.
- Poplawski, T., Zadrozny, M., Kolacinska, A., Rykala, J., Morawiec, Z., & Blasiak, J. (2005). Polymorphisms of the DNA mismatch repair gene HMSH2 in breast cancer occurrence and progression. *Breast Cancer Res. Treat.*, 94, 3, 199-204.
- Qiu, L. X., Mao, C., Yao, L., Yu, K. D., Zhan, P., Chen, B., Liu, H. G., Yuan, H., Zhang, J., Xue, K., & Hu, X. C. (2010a). XRCC3 5'-UTR and IVS5-14 polymorphisms and breast cancer susceptibility: a meta-analysis. *Breast Cancer Res. Treat.*, 122, 2, 489-493.
- Qiu, L. X., Yao, L., Xue, K., Zhang, J., Mao, C., Chen, B., Zhan, P., Yuan, H., & Hu, X. C. (2010b). BRCA2 N372H polymorphism and breast cancer susceptibility: a meta-analysis involving 44,903 subjects. *Breast Cancer Res. Treat.*, 123, 2, 487-490.
- Rajaraman, P., Bhatti, P., Doody, M. M., Simon, S. L., Weinstock, R. M., Linet, M. S., Rosenstein, M., Stovall, M., Alexander, B. H., Preston, D. L., & Sigurdson, A. J. (1-12-2008). Nucleotide excision repair polymorphisms may modify ionizing radiation-related breast cancer risk in US radiologic technologists. *Int. J. Cancer*, 123, 11, 2713-2716.
- Rueff, J., Gaspar, J., & Kranendonk, M. (2002). DNA polymorphisms as modulators of genotoxicity and cancer. *Biol. Chem.*, 383, 6, 923-932.
- Rydberg, P., Magnusson, A. L., Zorcec, V., Granath, F., & Tornqvist, M. (1996). Adducts to N-terminal valines in hemoglobin from butadiene metabolites. *Chem. Biol. Interact.*, 101, 3, 193-205.
- Saadat, M. (2010). Haplotype analysis of XRCC1 (at codons 194 and 399) and susceptibility to breast cancer, a meta-analysis of the literatures. *Breast Cancer Res. Treat.*, 124, 3, 785-791.
- Saffi, J., Agnoletto, M. H., Guecheva, T. N., Batista, L. F., Carvalho, H., Henriques, J. A., Sary, A., Menck, C. F., & Sarasin, A. (2010). Effect of the anti-neoplastic drug doxorubicin on XPD-mutated DNA repair-deficient human cells. *DNA Repair (Amst)*, 9, 1, 40-47.
- Sarasin, A., Bounacer, A., Lepage, F., Schlumberger, M., & Suarez, H. G. (1999). Mechanisms of mutagenesis in mammalian cells. Application to human thyroid tumours. *C.R. Acad. Sci. III*, 322, 2-3, 143-149.
- Schofield, M. J. & Hsieh, P. (2003). DNA mismatch repair: molecular mechanisms and biological function. *Annu. Rev. Microbiol.*, 57, 579-608.
- Schroering, A. G., Edelbrock, M. A., Richards, T. J., & Williams, K. J. (2007). The cell cycle and DNA mismatch repair. *Exp. Cell Res.*, 313, 2, 292-304.
- Scully, R. & Puget, N. (2002). BRCA1 and BRCA2 in hereditary breast cancer. *Biochimie*, 84, 1, 95-102.
- Shen, J., Desai, M., Agrawal, M., Kennedy, D. O., Senie, R. T., Santella, R. M., & Terry, M. B. (2006). Polymorphisms in nucleotide excision repair genes and DNA repair capacity phenotype in sisters discordant for breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, 15, 9, 1614-1619.

- Shi, Q., Wang, L. E., Bondy, M. L., Brewster, A., Singletary, S. E., & Wei, Q. (2004). Reduced DNA repair of benzo[a]pyrene diol epoxide-induced adducts and common XPD polymorphisms in breast cancer patients. *Carcinogenesis*, 25, 9, 1695-1700.
- Shin, A., Lee, K. M., Ahn, B., Park, C. G., Noh, S. K., Park, D. Y., Ahn, S. H., Yoo, K. Y., & Kang, D. (2008). Genotype-phenotype relationship between DNA repair gene genetic polymorphisms and DNA repair capacity. *Asian Pac.J Cancer Prev.*, 9, 3, 501-505.
- Shuck, S. C., Short, E. A., & Turchi, J. J. (2008). Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. *Cell Res.*, 18, 1, 64-72.
- Sigurdson, A. J., Land, C. E., Bhatti, P., Pineda, M., Brenner, A., Carr, Z., Gusev, B. I., Zhumadilov, Z., Simon, S. L., Bouville, A., Rutter, J. L., Ron, E., & Struewing, J. P. (2009). Thyroid nodules, polymorphic variants in DNA repair and RET-related genes, and interaction with ionizing radiation exposure from nuclear tests in Kazakhstan. *Radiat.Res.*, 171, 1, 77-88.
- Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population. *Oncol.Rep.*, 22, 3, 593-598.
- Silva, S. N., Bezerra de, Castro G., Faber, A., Pires, M., Oliveira, V. C., Azevedo, A. P., Cabral, M. N., Manita, I., Pina, J. E., Rueff, J., & Gaspar, J. (2006a). The role of ERCC2 polymorphisms in breast cancer risk. *Cancer Genet.Cytogenet.*, 170, 1, 86-88.
- Silva, S. N., Cabral, M. N., Bezerra de, Castro G., Pires, M., Azevedo, A. P., Manita, I., Pina, J. E., Rueff, J., & Gaspar, J. (2006b). Breast cancer risk and polymorphisms in genes involved in metabolism of estrogens (CYP17, HSD17beta1, COMT and MnSOD): possible protective role of MnSOD gene polymorphism Val/Ala and Ala/Ala in women that never breast fed. *Oncol.Rep.*, 16, 4, 781-788.
- Silva, S. N., Gil, O. M., Oliveira, V. C., Cabral, M. N., Azevedo, A. P., Faber, A., Manita, I., Ferreira, T. C., Limbert, E., Pina, J. E., Rueff, J., & Gaspar, J. (2005). Association of polymorphisms in ERCC2 gene with non-familial thyroid cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 14, 10, 2407-2412.
- Silva, S. N., Moita, R., Azevedo, A. P., Gouveia, R., Manita, I., Pina, J. E., Rueff, J., & Gaspar, J. (2007). Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk. *Cancer Detect.Prev.*, 31, 4, 303-309.
- Silva, S. N., Tomar, M., Paulo, C., Gomes, B. C., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2010). Breast cancer risk and common single nucleotide polymorphisms in homologous recombination DNA repair pathway genes XRCC2, XRCC3, NBS1 and RAD51. *Cancer Epidemiol.*, 34, 1, 85-92.
- Sliwinski, T., Ziemba, P., Morawiec, Z., Kowalski, M., Zadrozny, M., & Blasiak, J. (2007). Polymorphisms of the DNA polymerase beta gene in breast cancer. *Breast Cancer Res.Treat.*, 103, 2, 161-166.
- Smith, T. R., Levine, E. A., Freimanis, R. I., Akman, S. A., Allen, G. O., Hoang, K. N., Liu-Mares, W., & Hu, J. J. (2008). Polygenic model of DNA repair genetic polymorphisms in human breast cancer risk. *Carcinogenesis*, 29, 11, 2132-2138.
- Smith, T. R., Levine, E. A., Perrier, N. D., Miller, M. S., Freimanis, R. I., Lohman, K., Case, L. D., Xu, J., Mohrenweiser, H. W., & Hu, J. J. (2003). DNA-repair genetic polymorphisms and breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 12, 11 Pt 1, 1200-1204.

- Stracker, T. H. & Petrini, J. H. (2011). The MRE11 complex: starting from the ends. *Nat.Rev.Mol.Cell Biol.*, 12, 2, 90-103.
- Sturgis, E. M., Zhao, C., Zheng, R., & Wei, Q. (2005). Radiation response genotype and risk of differentiated thyroid cancer: a case-control analysis. *Laryngoscope*, 115, 6, 938-945.
- Sugasawa, K. (2008). Xeroderma pigmentosum genes: functions inside and outside DNA repair. *Carcinogenesis*, 29, 3, 455-465.
- Sun, H., Bai, J., Chen, F., Jin, Y., Yu, Y., Jin, L., & Fu, S. (2011). RAD51 G135C polymorphism is associated with breast cancer susceptibility: a meta-analysis involving 22,399 subjects. *Breast Cancer Res.Treat.*, 125, 1, 157-161.
- Taioli, E., Sram, R. J., Binkova, B., Kalina, I., Popov, T. A., Garte, S., & Farmer, P. B. (2007). Biomarkers of exposure to carcinogenic PAHs and their relationship with environmental factors. *Mutat.Res.*, 620, 1-2, 16-21.
- Teixeira, J. P., Gaspar, J., Martinho, G., Silva, S., Rodrigues, S., Mayan, O., Martin, E., Farmer, P. B., & Rueff, J. (2002). Aromatic DNA adduct levels in coke oven workers: correlation with polymorphisms in genes GSTP1, GSTM1, GSTT1 and CYP1A1. *Mutat.Res.*, 517, 1-2, 147-155.
- Venkitaraman, A. R. (25-1-2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell*, 108, 2, 171-182.
- Verkooijen, R. B., Smit, J. W., Romijn, J. A., & Stokkel, M. P. (2006). The incidence of second primary tumors in thyroid cancer patients is increased, but not related to treatment of thyroid cancer. *Eur.J Endocrinol.*, 155, 6, 801-806.
- Wang, Z., Dong, H., Fu, Y., & Ding, H. (2010). RAD51 135G>C polymorphism contributes to breast cancer susceptibility: a meta-analysis involving 26,444 subjects. *Breast Cancer Res.Treat.*, 124, 3, 765-769.
- Willems, P., De Ruyck K., Van den Broecke, R., Makar, A., Perletti, G., Thierens, H., & Vral, A. (2009). A polymorphism in the promoter region of Ku70/XRCC6, associated with breast cancer risk and oestrogen exposure. *J.Cancer Res.Clin.Oncol.*, 135, 9, 1159-1168.
- Wong, E. M., Tesoriero, A. A., Pupo, G. M., McCredie, M. R., Giles, G. G., Hopper, J. L., Mann, G. J., Goldgar, D. E., & Southey, M. C. (2008). Is MSH2 a breast cancer susceptibility gene? *Fam.Cancer*, 7, 2, 151-155.
- Wood, R. D., Mitchell, M., & Lindahl, T. (2005). Human DNA repair genes, 2005. *Mutat.Res.*, 577, 1-2, 275-283.
- Wu, M. T., Simpson, C. D., Christiani, D. C., & Hecht, S. S. (2002). Relationship of exposure to coke-oven emissions and urinary metabolites of benzo(a)pyrene and pyrene in coke-oven workers. *Cancer Epidemiol.Biomarkers Prev.*, 11, 3, 311-314.
- Xu, X., Kelsey, K. T., Wiencke, J. K., Wain, J. C., & Christiani, D. C. (1996). Cytochrome P450 CYP1A1 MspI polymorphism and lung cancer susceptibility. *Cancer Epidemiol.Biomarkers Prev.*, 5, 9, 687-692.
- Yu, K. D., Chen, A. X., Qiu, L. X., Fan, L., Yang, C., & Shao, Z. M. (2010). XRCC2 Arg188His polymorphism is not directly associated with breast cancer risk: evidence from 37,369 subjects. *Breast Cancer Res.Treat.*, 123, 1, 219-225.
- Yu, K. D., Li, B., Zhou, Y., & Shao, Z. M. (2011). Is RAD51 135G>C polymorphism really associated with breast cancer in general population? Biased design and results lead to inappropriate conclusion. *Breast Cancer Res.Treat.*,

- Zhang, L., Zhang, Z., & Yan, W. (2005). Single nucleotide polymorphisms for DNA repair genes in breast cancer patients. *Clin.Chim.Acta*, 359, 1-2, 150-155.
- Zhang, Y., Newcomb, P. A., Egan, K. M., Titus-Ernstoff, L., Chanock, S., Welch, R., Brinton, L. A., Lissowska, J., Bardin-Mikolajczak, A., Peplonska, B., Szeszenia-Dabrowska, N., Zatonski, W., & Garcia-Closas, M. (2006). Genetic polymorphisms in base-excision repair pathway genes and risk of breast cancer. *Cancer Epidemiol.Biomarkers Prev.*, 15, 2, 353-358.
- Zheng, W., Cong, X. F., Cai, W. H., Yang, S., Mao, C., & Zou, H. W. (2011). Current evidences on XPC polymorphisms and breast cancer susceptibility: a meta-analysis. *Breast Cancer Res.Treat.*,
- Zhou, G. W., Hu, J., Peng, X. D., & Li, Q. (2011). RAD51 135G>C polymorphism and breast cancer risk: a meta-analysis. *Breast Cancer Res.Treat.*, 125, 2, 529-535.

DNA Mismatch Repair (MMR) Genes and Endometrial Cancer

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1. Introduction

The incidence of endometrial cancer among malignant gynecological tumors has increased with lifestyle and environmental changes. In the US, 40,000 patients are diagnosed with endometrial cancer annually, and 7,500 patients die of this disease (Jemal et al., 2009).

The number and prevalence of cases of endometrial cancer have increased worldwide and control of this cancer is urgently required. However, many aspects of the mechanism of carcinogenesis and pattern of advancement are unclear. Environmental factors such as obesity and a high estrogen level are thought to play important carcinogenic roles, but a close association with hereditary disposition has also been suggested, since double cancer and an increased incidence of cancer in relatives are common in patients with endometrial cancer.

Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC), is a hereditary disease in which there is frequent development of colorectal, endometrial, and ovarian cancers. The cause is thought to be mutation of the DNA mismatch repair (MMR) gene in germ cells. However, the conventional explanation of the mechanism involving genetic changes - mutations of cancer-related genes - is inadequate and epigenetic changes in endometrial cancer are now being examined. In particular, aberrant DNA methylation is thought to play a key role in endometrial carcinogenesis. Breakdown of the DNA mismatch repair mechanism due to DNA hypermethylation plays a particularly important role in the development of endometrial cancer.

2. Lynch syndrome

Lynch syndrome is a hereditary disease that includes frequent development of colorectal, endometrial, and ovarian cancers, and which is inherited in an autosomal dominant manner. Lynch syndrome is caused by a hereditary defect in the DNA mismatch repair (MMR) gene and the incidences in colorectal and endometrial cancers are 2-3% and 1-2%, respectively (Hampel et al., 2006). This syndrome was initially reported by Wartin et al. in 1913 in a family with a high risk of development of colorectal cancer. Subsequent analysis of

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this family led Lynch to propose the disease concept of cancer family syndrome in 1971 (Lynch HT et al., 1971, 2000).

Six MMR genes, the causative genes in Lynch syndrome, have been cloned: *hMSH2*, *hMLH1*, *hMSH3*, *hMSH6*, *hPMS1* and *hPMS2*. An aberration in one of these genes prevents accurate repair of base mismatches produced during DNA replication and repair. In Lynch syndrome with a *hMLH1* or *hMSH2* mutation, the frequencies of colorectal and endometrial cancers are 68% and 62%, respectively, and the lifetime risk of developing endometrial cancer is higher than that for colorectal cancer in women (Resnick et al., 2009).

Diagnosis of Lynch syndrome is based on clinical criteria. In 1990, the International Collaborative Group (ICG)-HNPCC established the following diagnostic criteria for HNPCC, which are referred to as the classical Amsterdam Criteria: 1) HNPCC is diagnosed when 3 or more patients with histologically confirmed colorectal cancer are present in a family line and one is a first relative of the other two; 2) colorectal cancer develops over two generations; and 3) one case is diagnosed at younger than 50 years old (Vasen et al., 1991). In 1999, the new Amsterdam Criteria (Amsterdam II) (Vansen et al., 1999) (Table 1) were published.

Amsterdam Minimum Criteria (1990)

1. At least 3 cases of colorectal cancer in relatives (verified pathologically)
2. One is a first degree relative of the other two
3. At least two successive generations should be affected
4. One case of colorectal cancer diagnosed before the age of 50 years old
5. FAP should be excluded

Revised Amsterdam Criteria II (1998)

1. At least 3 relatives with an HNPCC-associated cancer (cancer of the colorectum, endometrium, small bowel, ureter or renal pelvis)
- 2-5: As for the minimum criteria

Table 1. Clinical Diagnostic Criteria for HNPCC (FAP, familial adenomatous polyposis)

These criteria address endometrial cancer, small intestinal cancer, urethral cancer, and kidney cancer, in addition to the colorectal cancer included in the classic criteria.

3. DNA mismatch repair (MMR) gene and endometrial cancer

DNA mismatch repair (MMR) system corrects DNA base pairing errors in newly replicated DNA. Mismatched nucleotides may be present after DNA replication, along with small insertion/deletion mutations that tend to occur at repetitive sequences. The MMR system is an excision/resynthesis system that can be divided into 4 phases: (i) recognition of a mismatch, (ii) recruitment of repair enzymes, (iii) excision of the incorrect sequence, and (iv) resynthesis by DNA polymerase using the parental strand as a template. This system is conserved through evolution from bacteria to human (Jascur & Boland., 2006).

An aberration in one of MMR genes prevents accurate repair of base mismatches produced during DNA replication, resulting in production of a DNA chain of altered length, particularly in highly repeated sequences (microsatellites). This phenomenon is called

microsatellite instability (MSI) and can lead to an increased frequency of errors in target genes involved in carcinogenesis, resulting in cancerization of the cell. Among the MMR genes, germline mutations of *hMLH1* on chromosome 3 and *hMSH2* on chromosome 2 are thought to cause most cases of HNPCC. Mutation of *hMSH6* has also been proposed to be important for development of HNPCC-associated endometrial cancer, but the details are unclear (Fig. 1) (Banno et al., 2009).

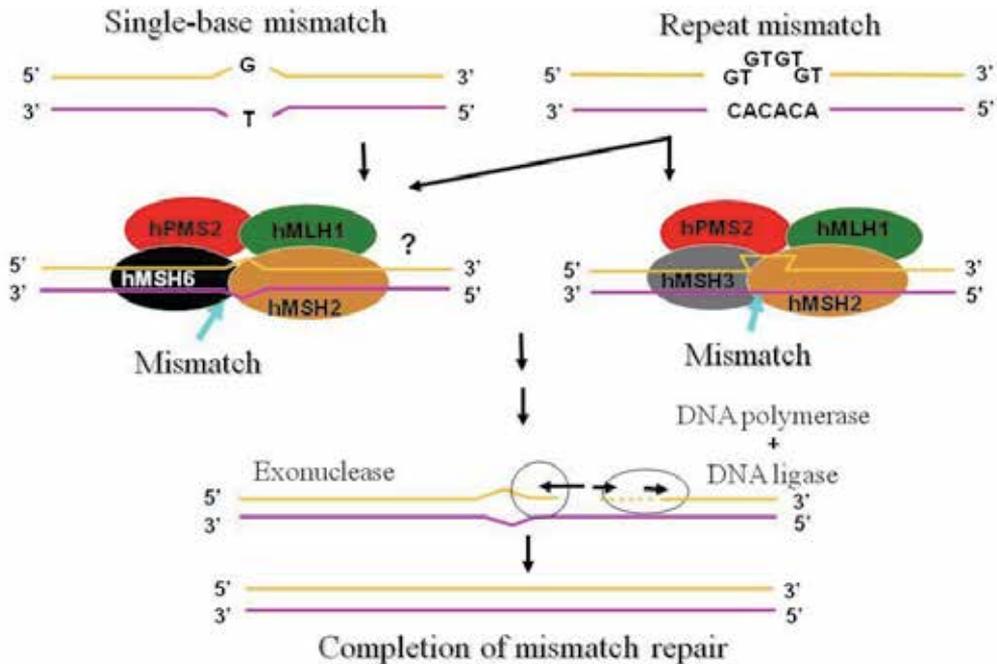


Fig. 1. The DNA mismatch repair mechanism in humans

4. Differences in the risk for endometrial cancer between carriers of various MMR gene mutations

Differences in phenotypes and cancer risks between DNA mismatch repair gene mutations in patients with Lynch syndrome have been widely investigated. Early studies comparing colorectal cancer (CRC) with other cancers indicated that patients with a *hMSH2* mutation had a higher risk for cancer other than CRC compared to patients with a *hMLH1* mutation (Vasen et al., 1996). Further studies in Germany, Finland and France showed that families with a *hMSH2* mutation had a higher risk for endometrial cancer compared to those with a *hMLH1* mutation (Parc, 2003; Peltomaki, 2001; Vasen, 2001). However, most recent studies have found no difference in the risk for endometrial cancer between genotypes. Thus, Goecke et al. compared 435 and 553 patients in Germany with confirmed or probable *hMLH1* and *hMSH2* mutations, respectively, and found significant differences in the risk for colorectal, gastric and prostate cancers, but not in the risk for endometrial cancer (Goecke et al., 2006). Kastrinos et al. conducted a large cross-sectional study in 112 unrelated patients with a *hMLH1* mutation and 173 with a *hMSH2* mutation in the United States and showed that the *hMLH1* carriers had a higher prevalence of colorectal cancer than the *hMSH2*

carriers, whereas the prevalence of endometrial cancer was similar in the two groups. Extracolonic Lynch syndrome-associated tumors, other than endometrial cancer, predominate in *hMSH2* carriers, with a higher tumor burden among family members (Kastrinos et al., 2008).

Therefore, it is very important to clarify the MMR mutations associated with a high cancer risk for management of patients and family members. Based on the results of studies to date, there is no significant difference in the risk of endometrial cancer for patients with different MMR mutations.

5. Clinical diagnostic criteria for Lynch syndrome

Since the Amsterdam Criteria for Lynch syndrome were proposed in 1991, several other diagnostic criteria, including the Japanese Criteria and the Bethesda Criteria, have been published. The confusion caused by the different criteria was resolved by revision of the Amsterdam Criteria by the ICG-HNPCC in 1998, to give the new Amsterdam Criteria (Vasen et al., 1999) (Table 1). These criteria address endometrial cancer, small intestinal cancer, urethral cancer, kidney cancer, and colorectal cancer. Cases not meeting the classical Amsterdam Criteria may meet the new Amsterdam Criteria, and this has resulted in an increased number of cases diagnosed as Lynch syndrome. In addition, discovery of Lynch syndrome is now possible through investigation of familial histories of endometrial cancer patients. The revision also recognized the importance of cooperation among gynecologists for identification of Lynch syndrome. However, one concern with the new criteria is the omission of ovarian, breast and stomach cancer, which may also be associated with Lynch syndrome.

The 1999 revised Amsterdam criteria II include endometrial cancer as a Lynch syndrome-related tumor, but women who develop endometrial cancer as the initial cancer and patients with a family tree with insufficient details are not included; thus, a high false negative rate has been reported based on these criteria (Resnick et al., 2009). For colorectal cancer, the Bethesda criteria require MSI testing, but this is not applicable for patients who develop endometrial cancer as the initial cancer. Thus, there is a need to establish criteria for selection of patients with endometrial cancer who should undergo screening (Garg & Soslow, 2009).

6. Carcinoma of the lower uterine segment (LUS) and Lynch syndrome

Endometrial cancer arises from the uterine body and fundus in many cases, but can also originate from the lower region of the uterine body through the upper region of the cervix. Such tumors are referred to as carcinoma of the lower uterine segment (LUS) or isthmus, and account for 3-6.3% of all cases of endometrial cancer. The association of carcinoma of the LUS with Lynch syndrome has attracted recent attention. The frequency of Lynch syndrome in general endometrial cancer is 1-2% (Hampel et al., 2006). In contrast, Lynch syndrome has a high frequency in cases of carcinoma of the LUS, with one report in the US suggesting that 29% of such cases could also be diagnosed with Lynch syndrome and that the *hMSH2* mutation was present at a high frequency in these cases (Westin et al., 2008). Demonstration of an association between carcinoma of the LUS and Lynch syndrome in a large-scale survey would allow patients with carcinoma of the LUS to be classified as a high-risk group for Lynch syndrome (Masuda et al., 2011).

7. Screening for endometrial cancer and prophylactic hysterectomy in Lynch syndrome

Women with Lynch syndrome have a high risk for endometrial cancer, with a life-long incidence of 40% to 60%, which is similar to or greater than that of colon cancer (Aarno et al., 1999). Therefore, a woman diagnosed with Lynch syndrome should undergo screening or prophylactic hysterectomy.

Potential screening methods include transvaginal ultrasound and endometrial biopsy. Transvaginal or transabdominal sonography is used to evaluate endometrial conditions and thickness. Some studies have shown a high false-positive rate and poor efficacy (Rijcken, 2003; Dove-Edwin, 2002), while others have shown high sensitivity and negative predictive values (Lécuru et al, 2010); therefore, the effect of this approach is unclear. Endometrial biopsy is not used for general screening, but may be useful for patients with Lynch syndrome with a high risk for endometrial cancer. Thus, women who have a DNA mismatch repair gene mutation or a family history of this mutation should undergo a biopsy every year at the age of 30-35 (Lindor et al, 2006).

Prophylactic hysterectomy has not been thought to reduce the cancer risk in women with Lynch syndrome. In 1997, the Cancer Genetics Studies Consortium suggested that there was insufficient evidence to recommend that women with Lynch syndrome should have prophylactic surgery to reduce the risk of gynecologic cancer (Burke et al, 1997). However, prophylactic hysterectomy has been realistically conducted in some institutions. The effects of prophylactic hysterectomy are of interest. Schmeler et al. (Schmeler et al., 2006) showed that prophylactic hysterectomy had a cancer-protective effect based on a retrospective cohort analysis in 315 women with a detected *hMLH1*, *hMSH2* or *hMSH6* germline mutation from 1973 to 2004. Outcomes were compared between 61 patients who underwent hysterectomy for prophylaxis or benign disease and 210 patients who did not undergo prophylactic hysterectomy. None of the 61 patients in the hysterectomy group developed endometrial cancer, whereas 69 (33%) in the non-hysterectomy group had endometrial cancer. These results indicate that prophylactic hysterectomy significantly decreased the development of endometrial cancer.

These results suggest that further studies should be conducted to compare the morbidity and mortality between screening using sonography or endometrial biopsy and prophylactic surgery.

8. Microsatellite instability (MSI) and endometrial cancer

Microsatellite instability occurs when the mismatch repair system is damaged. Microsatellites are DNA sequences of repeating units of 1 to 5 base pairs. Abnormalities in the mismatch repair system may cause replication errors in the repeating unit, leading to changes in length that are referred to as MSI. MSI caused by MMR gene aberration is detectable by PCR using microsatellite markers. In screening for Lynch syndrome, use of 5 microsatellite markers, two mononucleotide repeats (BAT26 and BAT25) and three dinucleotide repeats (D5S346, D2S123, and D17S250), is recommended (Boland et al., 1998). MSI is observed in certain types of cancer, including 20 to 30% of cases of endometrial cancer (Kanaya et al., 2003). These results suggest that MMR gene abnormalities occur frequently in endometrial cancer.

To investigate the status and characteristics of familial endometrial cancer, Banno et al. (Banno et al., 2004a) surveyed the familial and medical histories of 385 patients who underwent treatment for endometrial cancer. MSI analysis was performed in 38 of these patients. The familial histories showed that 2 of the 385 cases met the new Amsterdam Criteria for Lynch syndrome, giving a rate of Lynch syndrome of about 0.5%. Investigation of familial accumulation of cancer in 890 relatives (439 men and 451 women) of the 38 endometrial cancer patients who underwent MSI analysis revealed high incidences of endometrial cancer, colorectal cancer and ovarian cancer, suggesting that a hereditary factor common to Lynch syndrome is also involved in endometrial cancer. MSI analysis detected at least one of 5 microsatellite markers (D2S123, D3S1284, D5S404, D9S162: microsatellite loci containing CA repeats and hMSH2 intron 12: a polyA-sequence-containing microsatellite locus) in 12 of the 38 cases (31.6%). This rate is very high compared to MSI in cancers of other organs, demonstrating that abnormal DNA mismatch repair plays an important role in endometrial cancer. The patients with MSI showed a tendency to have double cancer (such as ovarian cancer) compared with patients with microsatellite stability (MSS), although the difference was not significant (27% vs. 15%). Regarding prognosis, none of the MSI-positive cases were fatal (0/11, 0%), while 5 MSI-negative (MSS) cases were fatal (5/27, 19%). The difference was not significant, but this tendency is similar to that for Lynch syndrome-associated colorectal cancer. The incidences of moderately differentiated adenocarcinoma G2 (36%) and poorly differentiated adenocarcinoma G3 (18%) tended to be higher in MSI-positive endometrial cancer, although again the difference was not significant. These findings appear contradictory with the favorable prognosis, but interestingly they may reflect the biological characteristics of endometrial cancer induced by abnormal DNA mismatch repair (Banno et al., 2004b).

9. DNA hypermethylation and endometrial cancer

Epigenetics refers to the information stored after somatic cell division that is not contained within the DNA base sequence. Recent findings have shown that epigenetic changes - selective abnormalities in gene function that are not due to DNA base sequence abnormalities - play a significant role in carcinogenesis in various organs. In particular, the relationship between cancer and aberrant hypermethylation of specific genome regions has attracted attention. A completely new model for the mechanism of carcinogenesis has been proposed in which hypermethylation of unmethylated CpG islands in the promoter regions of cancer-related genes in normal cells silences these genes and leads to the cell becoming cancerous (Figure 2).

The main difference between epigenetic abnormalities and genetic abnormalities such as gene mutations is that epigenetic changes are reversible and do not involve changes in base sequence. This suggests that restoration of gene expression is possible and that epigenetic mechanisms may constitute important molecular targets for treatment. Attempts have begun to detect aberrant DNA methylation in cancer cells present in minute quantities in biological samples and to apply the results to cancer diagnosis, prediction of the risk of carcinogenesis, and definition of the properties of a particular cancer. The MMR gene *hMLH1* is a typical gene that is silenced by DNA methylation. In endometrial cancer, *hMLH1* silencing is found in approximately 40% of cases and is an important step in the early stages of carcinogenesis, with the loss of DNA mismatch repair function proposed to lead to mutation of genes such as PTEN. In patients with endometrial cancer, Banno et al. found

aberrant hypermethylation of *hMLH1*, APC, E-cadherin, and CHFR in 40.4%, 22.0%, 14.0%, and 13.3% of cases, respectively. A significant decrease in protein expression was found in patients with aberrant methylation of *hMLH1* ($P < 0.01$) and E-cadherin ($P < 0.05$), and aberrant methylation of *hMLH1* was also found in 14.3% of patients with atypical endometrial hyperplasia (AEH).

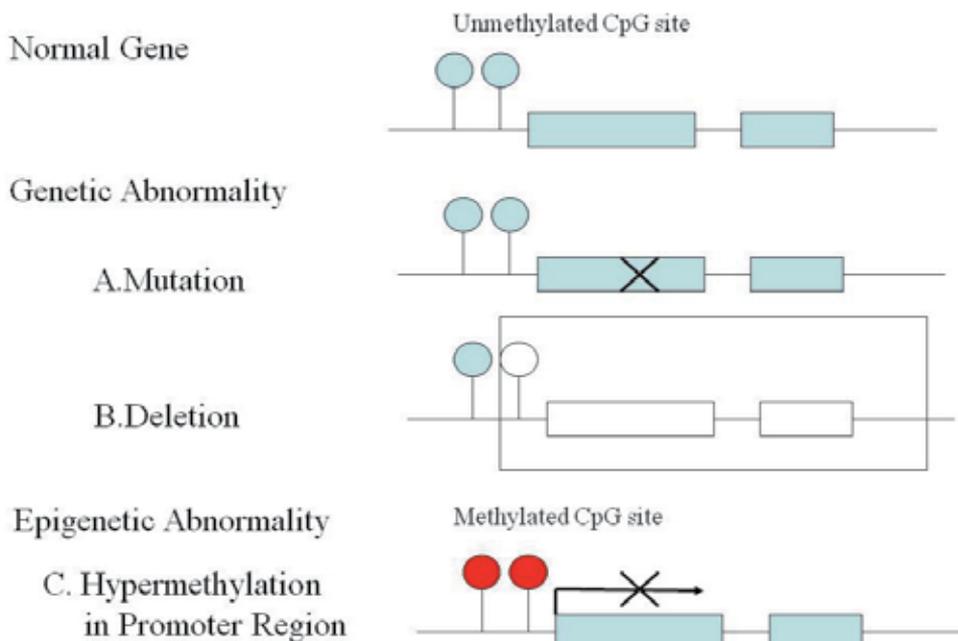


Fig. 2. Inactivation mechanism of cancer genes

However, no aberrant methylation of the four cancer-related genes was found in patients with a normal endometrium. These results indicate that aberrant methylation of specific genes associated with carcinogenesis in endometrial cancer does not occur in a normal endometrium. Aberrant methylation of *hMLH1* was most frequent, and the observation of this phenomenon in AEH, which is found in the first stage of endometrial cancer, supports the hypothesis that aberrant methylation of *hMLH1* is an important event in carcinogenesis in endometrial cancer (Banno, 2006; Muraki, 2009).

10. Conclusion

The DNA mismatch repair pathway is important in carcinogenesis of endometrial cancer. Recent analyses have shown that the MMR pathway can be impaired via both genetic and epigenetic mechanisms. Genetically, Lynch syndrome in cases of endometrial cancer is caused by hereditary defects in the MMR genes. However, there have been fewer studies on endometrial cancer compared to colorectal cancer in patients with Lynch syndrome. Clarification of the pathology and development of screening and genetic tests are required for further progress in this area. Epigenetic research in endometrial cancer suggests that damage to the mismatch repair system plays a significant role in

carcinogenesis and that DNA hypermethylation is important in this mechanism. Many attempts are currently being made to use epigenetic abnormalities as new methods of diagnosis and treatment based on control of methylation. Further studies of the genetic and epigenetic mechanisms may have potential for diagnosis, risk assessment, and treatment of endometrial cancer.

11. References

- Aarnio, M., Sankila, R. & Järvinen, HJ. (1999). Cancer risk in mutation carriers of DNA-mismatch-repair genes. *Int J Cancer*, Vol. 81, p.p. 214-218
- Banno, K., Susumu, N. & Nozawa, S. (2004a). Two Japanese kindreds occurring endometrial cancer meeting new clinical criteria for hereditary non-polyposis colorectal cancer (HNPCC), Amsterdam Criteria II. *J Obstet Gynaecol Res*, Vol.30, p.p.287-292
- Banno, K., Susumu, N. & Nozawa, S. (2004b). Association of HNPCC and endometrial cancer. *Int J Clin Oncol*, Vol.9, p.p. 262-269
- Banno, K., Yanokura, M. & Aoki, D. (2006). Relationship of aberrant DNA hypermethylation of cancer-related genes with carcinogenesis of endometrial cancer. *Oncol Rep*, Vol.16, p.p.1189-1196
- Banno, K., Yanokura, M. & Aoki, D. (2009). Endometrial Cancer as a Familial Tumor: Pathology and Molecular Carcinogenesis. *Curr Genomics*, Vol.10, p.p.127-132
- Boland, CR., Thibodeau, SN. & Srivastava, S. (1998). A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination Microsatellite Instability in Colorectal Cancer. *Cancer Res*, Vol. 58, p.p. 5248-5257
- Burke, W., Petersen, G. & Varricchio, C. (1997). Recommendations for follow-up care of individuals with an inherited predisposition to cancer. I. Hereditary nonpolyposis colon cancer. Cancer Genetics Studies Consortium. *JAMA*, Vol.277, p.p.915-919
- Dove-Edwin, I., Boks D. & Thomas, HJ. (2002). The outcome of endometrial carcinoma surveillance by ultrasound scan in women at risk of hereditary nonpolyposis colorectal carcinoma and familial colorectal carcinoma. *Cancer*, Vol.94, p.p.1708-1712
- Garg, K. & Soslow, RA. (2009). Lynch syndrome (hereditary non-polyposis colorectal cancer) and endometrial carcinoma. *J Clin Pathol*, Vol. 62, p.p. 679-684
- Goecke, T., Schulmann, K. & Moeslein, G. (2006). German HNPCC Consortium. Genotype-Phenotype Comparison of German MLH1 and MSH2 Mutation Carriers Clinically Affected With Lynch Syndrome: A Report by the German HNPCC Consortium. *J Clin Oncol*, Vol. 24, p.p. 4285-4292
- Hampel, H., Frankel, W. & de la Chapelle A. (2006). Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial carcinoma patients. *Cancer Res*, Vol. 66, p.p. 7810-7817
- Jascur, T. & Boland, CR. (2006). Structure and function of the components of the human DNA mismatch repair system. *Int J Cancer*, Vol. 119, p.p. 2030-2035
- Kanaya, T., Kyo S. & Inoue, M (2003). Frequent hypermethylation of MLH1 promoter in normal endometrium of patients with endometrial cancers. *Oncogene*, Vol.22, p.p. 2352-2360

- Kastrinos, F., Stoffel, EM. & Syngal, S. (2008). Phenotype Comparison of MLH1 and MSH2 Mutation Carriers in a Cohort of 1,914 Individuals Undergoing Clinical Genetic Testing in the United States. *Cancer Epidemiol Biomarkers Prev*, Vol. 17, p.p. 2044-2051
- Lécuru, F., Huchon, C. & Puig, PL. (2010). Contribution of ultrasonography to endometrial cancer screening in patients with hereditary nonpolyposis colorectal cancer/Lynch syndrome. *Int J Gynecol Cancer*, Vol.20, p.p.583-587
- Lindor, NM., Petersen, GM. & Press, N. (2006). Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: a systematic review. *JAMA*, Vol.296, p.p.1507-1517
- Lynch, HT. & Krush, AJ. (1971). The cancer family syndrome and cancer control. *Surg Gynecol Obstet*, Vol. 132, p.p. 247-250
- Lynch, HT. & Lynch, JF. (2000). Hereditary nonpolyposis colorectal cancer. *Semin Surg Oncol*, Vol.18, p.p.305-313
- Masuda, K., Banno, K. & Aoki, D. (2011). Carcinoma of the lower uterine segment (LUS): Clinicopathological characteristics and association with Lynch syndrome. *Curr Genomics*, Vol.12, p.p.25-29
- Muraki, Y., Banno, K. & Aoki, D. (2009). Epigenetic DNA hypermethylation: Clinical applications in endometrial cancer. *Oncol Rep*, Vol.22, p.p.967-972
- Parc, Y., Boisson, C. & Olschwang, S. (2003). Cancer Risk in 348 French MSH2 or MLH1 gene carriers. *J Med Genet*, Vol. 40, p.p. 208-213
- Peltomaki, P., Gao, X. & Mecklin, JP. (2001). Genotype and phenotype in hereditary nonpolyposis colon cancer: a study of families with different vs. shared predisposing mutations. *Fam Cancer*, Vol.1, p.p.9-15
- Resnick, KE. Hampel, H. & Cohn, DE. (2009). Current and emerging trends in Lynch syndrome identification in women with endometrial cancer. *Gynecol Oncol*, Vol.18, p.p.128-134
- Rijcken, FE., Mourits, MJ. & van der Zee AG. (2003). Gynecologic screening in hereditary nonpolyposis colorectal cancer. *Gynecol Oncol*, Vol. 91, p.p. 74-80
- Schmeler, KM., Lynch, HT. & Lu, KH. (2006). Prophylactic surgery to reduce the risk of gynecologic cancers in the Lynch syndrome. *N Engl J Med*, Vol. 354, p.p. 261-269
- Vasen, HF., Mecklin, JP. & Lynch, HT. (1991). The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Dis Colon Rectum*, Vol. 34, p.p. 424-425
- Vasen, HF., Wijnen, JT. & Khan, PM. (1996). Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. *Gastroenterology*, Vol.110, p.p.1020-1027
- Vasen, HF., Watson, P. & Lynch, HT. (1999). New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaboratory Group on HNPCC. *Gastroenterology*, Vol. 116, p.p. 1453-1456
- Vasen, HF., Stormorken, A. & Wijnen, JT. (2001). MSH2 mutation carriers are at higher risk of cancer than MLH1 mutation carriers: a study of hereditary nonpolyposis colorectal cancer families. *J Clin Oncol*, Vol.19, p.p.4074-4080

- Westin, S., Lacour, R. & Broaddus, R. (2008). Carcinoma of the lower uterine segment: A newly described association with Lynch syndrome. *J Clin Oncol*, Vol. 26, p.p. 5965-5971
- Wijnen, J., de Leeuw, W. & Fodde, R. (1999). Familial endometrial cancer in female carriers of MSH6 germline mutations. *Nat Genet*, Vol. 23, p.p. 142-144

Human Apurinic/Apyrimidinic Endonuclease is a Novel Drug Target in Cancer

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1. Introduction

Genomic DNA possesses an inherent instability, at risk from damage by spontaneous base lesions, metabolic by-products, and exogenous sources such as ultraviolet light, ionising radiation and chemical agents. Unrepaired, this damage could result in non-canonical base pairing during replication, leading to the propagation of potentially mutagenic lesions. A number of DNA repair mechanisms have evolved to ensure genomic integrity can be preserved. So critical are these repair pathways that mutations within constituent genes are associated with several cancer predisposition syndromes such as hereditary non-polyposis carcinoma coli (HNPCC) or BRCA-deficient breast and ovarian cancer syndromes (Sweasy, Lang, and DiMaio 2006).

Cancer therapies commonly rely upon the induction of DNA damage to exert their effects. Upregulation of DNA repair pathways in cancer is common and may impact upon response to therapy and contribute to development of treatment resistance. Inhibition of DNA repair offers exciting possibilities for the future treatment of cancer. DNA repair constituents may also be used as biomarkers to predict tumour response to treatment and improve outcome prognostication. Pharmacological inhibition of DNA repair might potentiate the effects of anticancer agents, improving response rates, overcoming resistance, and improving outcomes. Furthermore, there may be scope to specifically target tumour cells using DNA repair inhibitors by exploiting genetic differences with normal tissue.

Base excision repair (BER) is critical for the repair of damage induced by alkylating chemotherapy agents such as temozolomide and dacarbazine. Targeting BER has shown considerable promise in the form of poly (ADP-ribose) polymerase (PARP) inhibitors, and a number of groups are now focusing on other BER targets. This chapter will provide an overview of the BER pathway, with specific consideration of the compelling evidence base for targeting the critical enzyme apurinic/apyrimidinic endonuclease I (APE1) for cancer therapy.

2. Base excision repair

Base excision repair (BER) is responsible for detection and repair of damage caused by a number of mechanisms, including alkylation, oxidation, ring saturation, single strand breaks and base deamination. Although complex, with at least two sub-pathways (see figure 1), BER generally proceeds via: a) recognition and removal of a damaged base by a DNA

glycosylase to form an abasic site intermediate; b) cleavage of the phosphodiester backbone 5' to the abasic site by apurinic/apyrimidinic endonuclease 1 (APE1); c) removal of the 5' sugar fragment; d) incorporation of the correct base by a DNA polymerase; and e) sealing of the strand break by a DNA ligase (Figure 1) (Fortini et al. 2003; Nilsen and Krokan 2001; Izumi et al. 2003; Dianov et al. 2003; Sancar et al. 2004; Barnes and Lindahl 2004; Robertson et al. 2009; Abbotts and Madhusudan 2010).

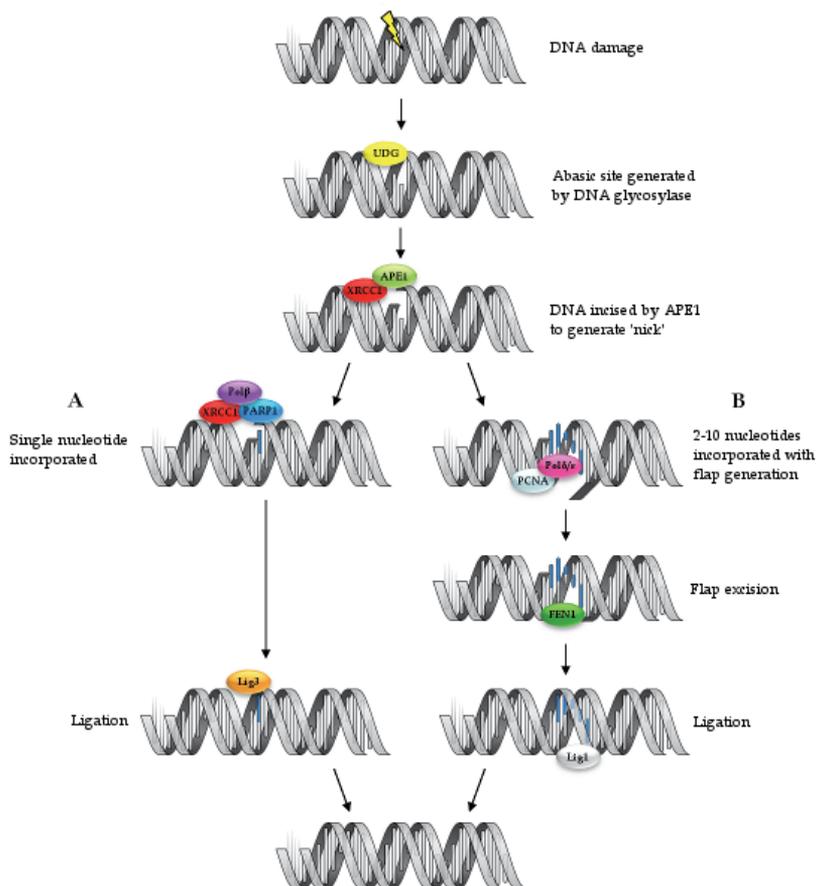


Fig. 1. Short-patch (A) and long-patch (B) base excision repair ¹

DNA glycosylases are a family of damage-specific enzymes which excise the damaged base via cleavage of the *N*-glycosidic bond linking it to the deoxyribose moiety. They induce localised DNA distortion to 'flip out' the damaged base into the binding site for processing. Some have dual functionality, also possessing the ability to cleave the DNA phosphodiester backbone to create a single strand break 3' to the abasic site. More commonly, APE1 incises

¹ 1 UDG = uracil DNA glycosylase, APE1 = apurinic/apyrimidinic endonuclease 1, XRCC1 = X-ray repair cross-complementing group 1, PARP1 = poly(ADP-ribose) polymerase 1, dRP = deoxyribose phosphate, Polβ/δ/ε = DNA polymerase β/δ/ε, PCNA = proliferating cell nuclear antigen, Lig3 = DNA ligase III, FEN1 = flap endonuclease I, Lig1 = DNA ligase I

the DNA backbone 5' to the abasic site, creating a nick bordered by 5'-deoxyribose phosphate (dRP) and 3'-hydroxyl groups. These groups act as blocking moieties, requiring further processing for BER to proceed. A number of enzymes possess this ability, including APE1, DNA polymerases β , λ and ι , and PNKP (polynucleotide kinase 3'-phosphatase).

Repair synthesis proceeds via a DNA polymerase. In the short-patch pathway, DNA Pol β incorporates a single nucleotide 'patch' into the processed abasic site. This pathway requires high concentrations of ATP for completion. When ATP concentrations are low or the oxidative state of the abasic lesion is altered, repair proceeds via the long-patch pathway. This involves the incorporation of 2-10 nucleotides by DNA polymerases δ and ϵ in conjunction with the sliding clamp protein PCNA (proliferating cell nuclear antigen), or alternatively, a Pol β /Rad9-Rad1-Hus1 complex, which bears structural similarity to PCNA. Long-patch nucleotide incorporation displaces the existing 5' DNA in a flap intermediate which is removed by flap endonuclease I (FEN1) (Pascucci et al. 1999; Balakrishnan et al. 2009). The repair is then completed by sealing of the DNA strand break by a DNA ligase. In the short-patch pathway, this primarily occurs via the DNA ligase III-XRCC1 (X-ray cross-complementation group I) scaffold protein heterodimer, whereas the long-patch pathway is completed by DNA ligase I.

Coordination of the BER pathway relies upon members of the poly (ADP-ribose) polymerase (PARP) family, which bind to DNA strand breaks and stabilise the DNA strand until repair can be effected. Once bound, PARPs also catalyse the addition of poly (ADP-ribose) polymers to target proteins, affecting protein-protein interactions and catalytic activities (D'Amours et al. 1999). 90% of PARP poly(ADP-ribosylation) is automodification, leading to recruitment of other BER constituents such as XRCC1, DNA Pol β and DNA LigIII (Megnin-Chanet, Bollet, and Hall 2010). This automodification also stimulates the release of PARP from DNA, allowing access to BER proteins to proceed with repair. Inhibition of PARP leads to persistence of single strand breaks, causing stalling of replication forks and formation of lethal double strand breaks (Durkacz et al. 1980). PARP inhibitors are currently showing promise in clinical trials (Helleday et al. 2008; Jones and Plummer 2008; Chalmers 2009; O'Shaughnessy et al. 2009) (see 6. Targeting APE1 for therapy).

3. Apurinic/aprimidinic endonuclease (APE1)

3.1 Abasic site formation

Abasic site formation occurs at a rate of ~50000 sites per cell per day, through the action of DNA glycosylases in the BER pathway and by spontaneous depurination (Lindahl 1993; Nakamura and Swenberg 1999; Atamna, Cheung, and Ames 2000). Abasic sites can also be induced by exogenous agents such as ionising radiation or alkylating and oxidizing drugs including temozolomide or bleomycin. Without repair, abasic sites cause stalling of replication forks, leading to strand breaks that are cytotoxic in high number (Wilson 2003).

3.2 Apurinic/aprimidinic endonucleases

Apurinic/aprimidinic (AP) endonucleases are critical for the recognition and processing of abasic sites during base excision repair. Two classes of AP endonucleases exist: Class I AP lyases, and Class II AP endonucleases. Class II AP endonucleases can be classified further into two families which are structurally distinct but catalyse reactions with identical products. These families are defined by their structural homology to the two endonucleases

expressed in *E. coli*, exonuclease III (exoIII, encoded by *xth* gene) and endonuclease IV (endoIV, encoded by *nfo*) (Figure 2).

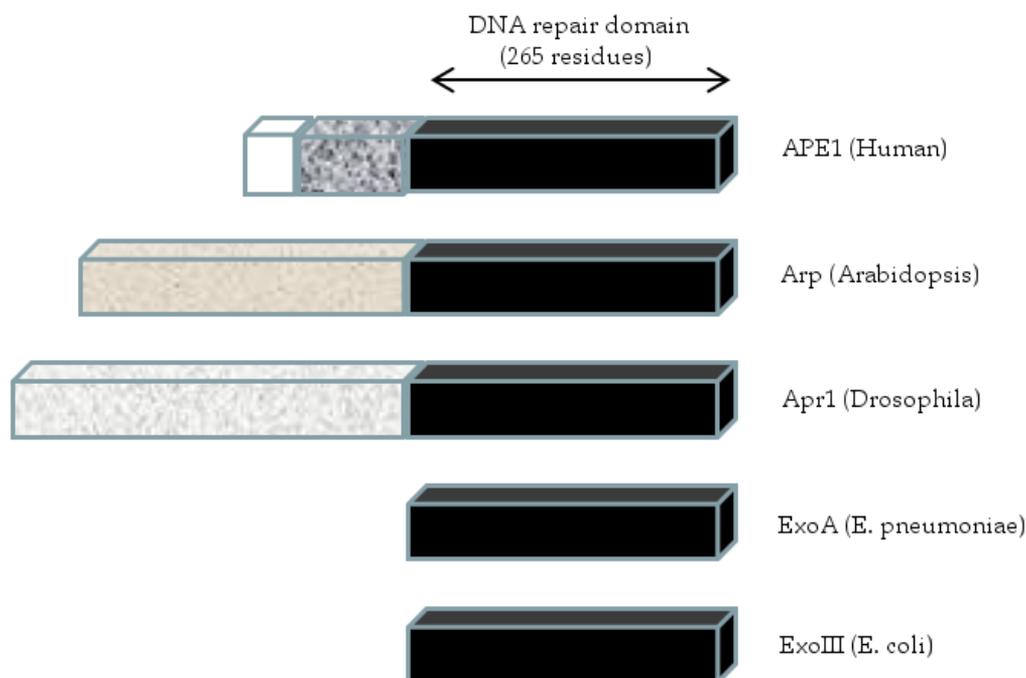


Fig. 2. The Exonuclease III family of AP Endonucleases in various species

The exoIII human homologue, APE1, accounts for 95% of AP endonuclease activity in human cells (Robson and Hickson 1991; Demple, Herman, and Chen 1991; Chen, Herman, and Demple 1991; Robson et al. 1992). A second exonuclease III-like protein, APE2, has recently been identified but is not yet fully characterised (Hadi and Wilson 2000; Hadi et al. 2002; Burkovics et al. 2006). Also known as HAP1 or Ref-1, APE1 is a ubiquitous multifunctional protein, with 350,000 – 7,000,000 units per cell (Chen, Herman, and Demple 1991). The 2.6kb APE1 gene is localised to 14q11.2-12. It consists of four introns and five exons that encompass a 954 nucleotide coding region, encoding a 318-amino acid protein of 35kDa. The C-terminal domain is essential for DNA repair activity, while the N-terminal domain possesses redox regulatory activity and also contains a nuclear localisation sequence. Preservation of APE1 function requires at least 10 evolutionarily conserved amino acids (Asp70, Asp90, Glu96, Tyr171, Asp210, Asn212, Asp219, Asp283, Asp308 and His309) (Figure 3).

APE1 is a globular protein arranged in a four-layered α/β sandwich, sharing significant structural homology with exoIII despite limited sequence homology (Gorman et al. 1997) (Figure 3B). The DNA repair active site is situated within a hydrophobic pocket on top of the α/β sandwich (Figure 3C). During DNA binding, the active site undergoes little conformation change, instead significantly distorting the DNA substrate, probably to displace the bound DNA glycosylase (Mol et al. 2000). Within the active site, highly conserved His309 and Thr283 are vital for catalytic activity. His309 is believed to act as a

general base to abstract a proton from water, forming a hydroxide which attacks the scissile bond of the phosphate group 5' to the abasic site. Also essential is a Glu96-bound magnesium ion which, along with Thr283, may help stabilise the reaction intermediate (Gorman et al. 1997; Lipton et al. 2008).

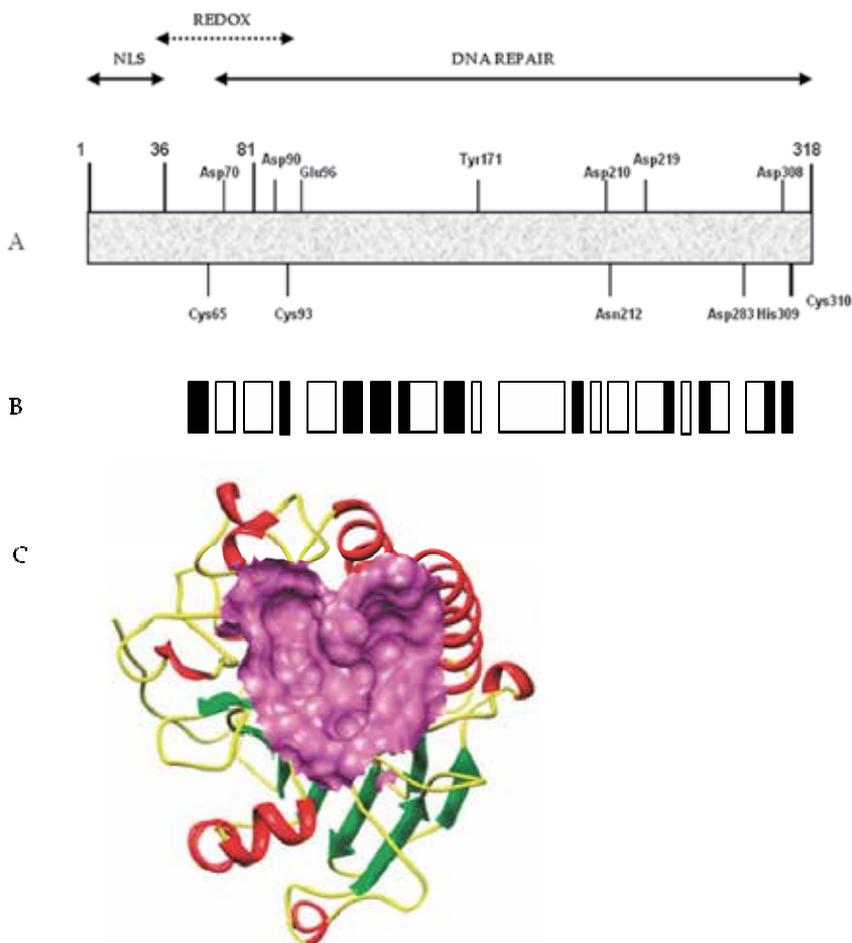


Fig. 3. A. Schematic representation of APE1 protein with critical residues (NLS = nuclear localization sequence). B. APE1 protein structure. White rectangles represent α -helical regions and shaded rectangles represent β -strands. C. Ribbon diagram of APE1 protein structure including DNA repair active site.

3.3 Roles of APE1 in DNA repair

APE1 endonuclease activity catalyses Mg^{2+} -dependent hydrolytic cleavage of the phosphodiester bond of the DNA backbone 5' to an abasic site, producing a nick flanked by a 3'-hydroxyl and a 5'-deoxyribose phosphate group. Like exonuclease III, APE1 has a number of other DNA repair roles mediated through the same highly-conserved active site. APE1 possesses 3'-phosphodiesterase activity, catalysing removal of fragmented sugar moieties 3' to single strand breaks induced by bleomycin or ionising radiation (Parsons,

Dianova, and Dianov 2004). It has exonuclease activity, which may facilitate the removal of mis-paired nucleotides (or nucleoside analogues such as troxacitabine), including those incorrectly inserted by Pol β , thus preserving BER fidelity (Chou, Kukhanova, and Cheng 2000; Chou and Cheng 2002). It has weak 3'-phosphatase activity that removes 3'-phosphate blocking groups left after bifunctional DNA glycosylase base excision (Wiederhold et al. 2004). APE1 also appears to have a role in the nucleotide incision repair pathway, where an endonuclease recognises and processes oxidatively-damaged DNA in a glycosylase-independent manner (Gros et al. 2004; Ishchenko et al. 2006). A mitochondrial targeting signal in the C-terminal has been identified (Li et al. 2010), supporting evidence that APE1 has a role in the repair of oxidatively-damaged mitochondrial DNA (Frossi et al. 2002; Shokolenko et al. 2009).

3.4 Other functions of APE1

APE1 redox regulatory activity includes modulation of the activity of a number of transcription factors, including those involved in proliferation, downregulation of apoptosis and angiogenesis. These findings suggest a role for APE1 redox function in tumourigenesis, cancer progression and treatment resistance (Bapat, Fishel, and Kelley 2008; Luo et al. 2008; Luo et al. 2010). A number of other roles have also been ascribed to APE1, including acetylation-mediated gene regulation, RNA quality control, and involvement in NK-cell-mediated killing. Further discussion is beyond the scope of this chapter, and is reviewed at length elsewhere (Kelley, Georgiadis, and Fishel 2011).

4. Functional preclinical studies of APE1

4.1 APE1 DNA repair activity is critical for cell viability

Complete absence of APE1 is associated with embryonic lethality in mice (Xanthoudakis et al. 1996; Ludwig et al. 1998; Meira et al. 2001). Expression of floxed human APE1 transgene in APE1^{-/-} mouse models by Izumi *et al.* was unable to counter early embryonic loss, but did allow culture of viable nullizygous mouse embryonic fibroblasts (MEFs). Inactivation of transgenic APE1 rapidly induced apoptosis, which could be overcome by transient transfection with wild-type APE1, but not by mutants lacking either DNA repair or acetylation-mediated gene regulatory functions, suggesting the essentiality of both functions. Conversely, transfection with a Cys65Ser mutant reported to be deficient in redox activity was able to prevent apoptosis, suggesting nonessentiality of APE1 redox activity (Izumi et al. 2005). Similarly, knock-in of a cysteine-to-alanine point mutation at Cys64 (Cys65 in hAPE1) is non-lethal in murine models – although the mutation was found to be associated with normal Fos- and Jun-reducing activity, raising doubts regarding the redox role of Cys64 in mice (Ordway, Eberhart, and Curran 2003). Elsewhere, siRNA downregulation of APE1 has been demonstrated to be associated with sequelae of BER inhibition such as AP site accumulation and apoptosis, and can be reversed by yeast Apn1 expression, which lacks redox activity (Fung and Demple 2005). Taken together, these results demonstrate the essentiality of the APE1 DNA repair function, but not redox activity, in cell viability.

4.2 APE1 depletion hypersensitises cells to DNA base damage

Induction of apoptosis in response to APE1 downregulation has been confirmed in numerous cell types and *in vivo* in rats and mice (Robertson et al. 1997; Evans, Limp-Foster,

and Kelley 2000). However, response to knockdown may vary between cell types. In ovarian cancer cells, APE1 knockdown causes S phase prolongation rather than apoptosis, reflected in xenografts as a reduced tumour growth rate that is associated with impaired glucose metabolism suggestive of reduced cellular proliferation (Fishel et al. 2008). Heterozygosity for APE1 in mice is associated with hypersensitivity to oxidative stress (Meira et al. 2001). Antisense depletion of APE1 hypersensitises HeLa cells to the alkylating agent methyl methanesulphonate (MMS), H₂O₂, menadione and paraquat (Walker et al. 1994). Antisense APE1 downregulation also increases sensitivity of lung cancer cells to ionising radiation (Chen and Olkowski 1994), pancreatic cancer cells to gemcitabine (Lau et al. 2004), and glioma cells to MMS, temozolomide and nitrosurea (Ono et al. 1994; Silber et al. 2002). SiRNA-mediated APE1 downregulation enhances cytotoxicity to alkylating agents and hydrogen peroxide in osteosarcoma cells (Wang, Luo, and Kelley 2004), cisplatin in non-small cell lung cancer cells (Wang et al. 2009), and ionising radiation in glioma cells (Naidu et al. 2010). Double negative APE1 mutation expression is associated with enhanced cytotoxicity to antimetabolites and alkylating agents (McNeill et al. 2009). Downregulation of APE1 using an adenoviral vector in a colon cancer mouse model successfully reduced APE1 expression levels and was associated with an increased response to ionising radiation (Xiang et al. 2008). This evidence highlights the therapeutic potential of targeting APE1 with small molecular inhibitors to improve radio- and chemotherapeutic efficiency.

4.3 APE1 overexpression protects cells from DNA damage and is implicated in treatment resistance

Upregulation of APE1 has a protective effect against agents causing DNA damage. Transfection of hAPE1 into AP endonuclease-mutant *E. coli* and Apn1-deficient yeast restored resistance to the effects of DNA damaging agents (Robson and Hickson 1991; Demple, Herman, and Chen 1991; Wilson et al. 1995). Co-expression of hAPE1 as a chimeric protein with MGMT conferred resistance to hydrogen peroxide and MMS in AP endonuclease-deficient *E. coli*. Overexpression of the chimeric protein in HeLa cells had a similar protective effect (Hansen et al. 1998). In human teratocarcinoma cells (Robertson et al. 2001) and melanocytes (Yang et al. 2005), overexpression of APE1 conferred resistance of radiation and chemotherapy, although this effect was not replicated in cells from other mammalian species (Herring et al. 1999; Tomicic, Eschbach, and Kaina 1997). Modulation of APE1 activity may offer a strategy to improve treatment response in tumours with high levels of APE1 expression.

Chemotherapy agents can induce APE1 upregulation, contributing to treatment resistance. In non small cell lung cancer cells, cisplatin treatment induces a dose-dependent increase in APE1 expression. When APE1 is downregulated using antisense methods, cisplatin cytotoxicity is significantly increased (Wang et al. 2009), suggesting that combining inhibitors of APE1 with chemotherapeutic agents may overcome treatment resistance.

5. APE1 and human cancer

5.1 APE1 overexpression in human cancers

APE1 expression is cell cycle dependent, with highest levels not surprisingly seen during early and middle S-phase (Fung, Bennett, and Demple 2001). Immunohistochemical analysis of many human cancers has demonstrated elevated levels of APE1 (see table). For example, APE1 protein expression is increased in human gliomas, and is positively correlated with

AP endonuclease activity. AP endonuclease activity was also found to be positively correlated with tumour grade, and with the fraction of S-phase cells, suggesting that APE1 activity is related to level of proliferation (Bobola et al. 2001). APE1 is also elevated in prostate cancer, with immunohistochemical staining levels increasing from low in benign prostatic hypertrophy to intense in prostatic carcinoma (Kelley et al. 2001).

Tumour site	APE1 expression	Preclinical findings	Clinical findings
Breast ²	Nuclear in normal Mixed localisation in ductal carcinoma <i>in situ</i>		Nuclear expression associated with negative lymph node status, low angiogenesis Asp148Glu associated with reduced rate of early radiotherapy toxicity
Cervical ³	Overexpression with nuclear localisation		Increased expression associated with radioresistance
Colon ⁴	Predominantly cytoplasmic	siRNA inhibition sensitises LOVO cells and xenografts to ionising radiation (IR)	
Head & neck ⁵	Overexpression with nuclear, cytoplasmic, or mixed localisation		Overexpression associated with earlier relapse, reduced survival Nuclear localisation associated with nodal positivity, treatment resistance
Gastro- oesophageal ⁶	Mixed or nuclear expression		Nuclear expression associated with poor survival Cytoplasmic expression associated with differentiation
Germ cell ⁷	Overexpression with nuclear localisation	Overexpression confers bleomycin resistance	
Glio- blastoma ⁸	APE1 overexpression	Upregulation by oxidative stress associated with resistance to MMS, temozolomide (TMZ), IR Antisense downregulation sensitises cells to TMZ siRNA, CRT0044876 and lucanthone sensitise cells to IR	

Table 1. Summary of preclinical and clinical evidence for APE1 as a predictive and prognostic biomarker

2 (Kakolyris et al. 1998; Chang-Claude, Pongda, et al. 2005; Andreassen et al. 2005; Andreassen et al. 2006)

3 (Xu et al. 1997; Herring et al. 1998)

4 (Kakolyris et al. 1997; Xiang et al. 2008)

5 (Koukourakis et al. 2001)

6 (Al-Attar et al. 2010)

7 (Robertson et al. 2001)

8 (Bobola et al. 2001; Silber et al. 2002; Naidu et al. 2010)

Tumour site	APE1 expression	Experimental findings	Clinical findings
Lung ⁹	Overexpression with mixed localisation (nuclear in normal tissue)	APE1 upregulated by cisplatin treatment siRNA inhibition reduces cell growth and induces apoptosis	Asp148Glu associated with sensitivity to IR, improved response to chemo-radiotherapy Low expression associated with cisplatin sensitivity, improved survival Overexpression associated with chemoresistance
Medulloblastoma ¹⁰	Predominantly nuclear Increased in expression in women, younger patients	siRNA inhibition sensitises cells to carmustine and TMZ	Overexpression associated with early relapse
Melanoma ¹¹	Predominantly nuclear	Potential APE1 inhibitor resveratrol sensitises cells to dacarbazine	
Osteosarcoma ¹²	Overexpression	Reduced HO5 cell viability following antisense depletion Antisense downregulation sensitises cells to MMS, H ₂ O ₂ , thiotepa, etoposide, IR	Overexpression associated with poor survival
Ovarian ¹³	Nuclear/cytoplasmic	siRNA inhibition reduces SKOV-3x growth	Nuclear expression associated with poor survival
Pancreaticobiliary ¹⁴	Nuclear localisation	APE1 upregulated by gemcitabine treatment Antisense downregulation sensitises cell to gemcitabine	Absence of cytoplasmic staining associated with high risk features
Prostate ¹⁵	Increasing overexpression from benign prostatic hypertrophy to prostatic intraepithelial neoplasia to cancer Predominantly cytoplasmic		

Table 1. (cont.). Summary of preclinical and clinical evidence for APE1 as a predictive and prognostic biomarker5.4. APE1 polymorphisms and cancer susceptibility

9 (Hu et al. 2001; Su et al. 2007; Wang et al. 2009)

10 (Bobola et al. 2005)

11 (Yang et al. 2005)

12 (Wang, Luo, and Kelley 2004)

13 (Moore et al. 2000; Fishel et al. 2008; Al-Attar et al. 2010)

14 (Lau et al. 2004; Al-Attar et al. 2010)

15 (Kelley et al. 2001)

5.2 APE1 subcellular localisation in cancer

APE1 expression demonstrates complex and heterogenous localisation patterns that vary between tissue types. Nuclear localisation is common, and is thought to reflect functions in DNA repair. Cytoplasmic localisation is commonly seen in cell types exhibiting rapid metabolic or proliferative rates, particularly when under high oxidative stress. Cytoplasmic localisation is predominantly within the mitochondria and endoplasmic reticulum, in keeping with the role of mitochondria in cellular response to oxidative stress, and may relate to APE1's role in mitochondrial DNA repair and redox regulation of transcription factors (Tell et al. 2005). In non-small cell lung cancer, APE1 dysregulation is common, with upregulation in the cytoplasm contributing to global overexpression and associated with increased superoxide production and lipid peroxidation (Yoo et al. 2008).

Alterations in subcellular distribution of APE1 compared to normal tissue have been seen in a number of human tumours. Normal colorectal mucosa features nuclear staining in the less differentiated cells in the lower parts of the crypts, with cytoplasmic staining in the superficial epithelium. In both adenomas and carcinomas, subcellular restriction is lost and a mixed localisation pattern develops, with a predominance of cytoplasmic staining (Kakolyris et al. 1997). Increased cytoplasmic staining is also seen in thyroid (Tell et al. 2000), hepatocellular (Di Maso et al. 2007), epithelial ovarian (Moore et al. 2000) and prostate carcinomas (Kelley et al. 2001). In contrast, melanomas display an increased level of APE1 expression which is predominantly localised to the nucleus, compared to cytoplasmic staining in normal skin (Yang et al. 2005). Increased levels of APE1 expression with nuclear-specific localisation are also seen in cervical carcinomas (Xu et al. 1997), bladder cancers (Sak et al. 2005), rhabdomyosarcomas (Thomson et al. 2001), and squamous cell head and neck cancers (Koukourakis et al. 2001). Some tumour types have shown variance of APE1 localisation between studies. There is consensus regarding elevated levels of APE1 in non-small cell lung cancer, but different groups have found predominantly cytoplasmic (Wang et al. 2009) or nuclear (Puglisi et al. 2001; Kakolyris et al. 1999) localisation.

5.3 APE1 expression and localisation as a marker

Alterations in APE1 expression may be of prognostic significance. In non-small cell lung cancer, elevated APE1 expression is an independent poor prognostic factor, associated with reduced disease-free and overall survival (Puglisi et al. 2001). Similarly, elevated APE1 is suggestive of poor prognosis in medulloblastoma (Bobola et al. 2005), ovarian, gastro-oesophageal and pancreatico-biliary cancers (Al-Attar et al. 2010). Alterations in APE1 localisation may also be of prognostic significance. Breast cancers display heterogenous localisation, compared to predominantly nuclear distribution in normal breast tissue. Localisation appears to be correlated to patient outcomes, with nuclear localisation being associated with better prognostic features such as differentiation, reduced angiogenesis and negative lymph node status (Kakolyris et al. 1998; Puglisi et al. 2002). Similar prognostic correlations are also seen in osteosarcoma (Wang, Luo, and Kelley 2004), where cytoplasmic staining is associated with poor survival outcomes. Conversely, in ovarian and pancreatico-biliary cancers, nuclear APE1 expression is associated with aggressive tumour biology and poor overall survival (Al-Attar et al. 2010).

Preclinical evidence suggests that increased APE1 expression may be associated with chemo- and radio-resistance due to efficient repair of therapeutically-induced DNA damage. In tumour samples, APE1 expression levels and subcellular localisation patterns may therefore have potential as a predictive marker for response to treatment. In non-small cell

lung cancers, increased APE1 expression is associated with resistance to cisplatin (Wang et al. 2009), and in cervical cancers elevated expression correlates to radio-resistance (Herring et al. 1998). In germ cell tumours (Robertson et al. 2001) and medulloblastoma (Bobola et al. 2005), increased APE1 levels are associated with both chemo- and radio-resistance. Similarly, increased nuclear localisation of APE1 in head and neck cancers is associated with chemo- and radio-resistance (Koukourakis et al. 2001). In contrast, however, high levels of APE1 expression in bladder tumours are associated with radiosensitivity and improved survival rates (Sak et al. 2005). The authors hypothesise that this may be because repair of irradiation-induced base damage requires phosphodiester backbone cleavage as an intermediary step, leading to the increased acquisition of potentially lethal double strand breaks – hence, efficient BER may actually increase radiotherapy-induced lethality. Alternatively, increased APE1 redox activity might lead to an increased stress response and hence a greater response to radiotherapy. While further study is required to clearly elucidate the prognostic significance of APE1 expression in different tumour types, the current body of evidence suggests the potential of APE1 as a prognostic biomarker.

5.4 APE1 polymorphisms and cancer susceptibility

Polymorphic APE1 variants have been reported and correlated to cancer susceptibility. In an analysis of seven APE1 polymorphisms, reduced repair activity was noted in four (Hadi et al. 2000). In a Chinese study, a Thr141Gly promoter polymorphism was associated with reduced levels of APE1 mRNA in blood and lung tissue, possibly due to reduced affinity for Oct-1 transcription factor, thought to be activated by DNA damage. Homozygosity for the Gly allele was associated with a 40% reduction in lung cancer risk (Lu et al. 2009). Asp148Glu, a variant which exhibits normal repair activity, is associated with an increased risk of cancer development (Gu et al. 2009), including melanoma (Li et al. 2006), pancreatic (Li et al. 2007), cervical (Farkasova et al. 2008) and lung cancer susceptibility (Agachan et al. 2009). The Asp148Glu variant may also be associated with hypersensitivity to ionising radiation (Hu et al. 2001), although it has also been demonstrated to have a protective effect against acute radiation toxicity reactions in normal skin (Chang-Claude, Popanda, et al. 2005). Further study is required to further elucidate how APE1 polymorphisms might impact response to treatment.

5.5 APE1 as a predictive and prognostic biomarker

A growing body of evidence suggests that alterations in APE1 expression levels and subcellular localisation may have predictive or prognostic significance in many human cancers. Similarly, a number of APE1 single-nucleotide polymorphisms (SNPs) have been identified and correlated to APE1 activity and tumour risk. Recent patent applications have been filed to utilise tissue APE1 as a biomarker in lung, breast and ovarian cancers (Deutsch 2003; Hagmann et al. 2008). Incorporating biomarker studies into future clinical trials offers the opportunity to corroborate and expand upon current knowledge to develop APE1 as a clinically relevant biomarker.

6. Targeting APE1 for therapy

The early promise of PARP inhibitors highlights the potential of BER proteins as therapeutic targets (Lord and Ashworth 2008; Fong et al. 2009). PARP inhibitors have shown particular promise in the setting of BRCA-deficient breast cancers, highlighting an important

therapeutic concept that may be applicable to inhibitors of APE1. Synthetic lethality exploits inter-gene relationships where the loss of function of either of two related genes is non-lethal, but loss of both causes cell death. This offers the potential to specifically target cancer cells through inhibition of a gene known to be in a synthetic lethal relationship with a mutated tumour suppressor gene (Rehman, Lord, and Ashworth 2010). BRCA-1 and -2 have long been identified as tumour suppressors, being mutated in an inherited cancer predisposition that increases susceptibility to breast and ovarian tumours (Miki et al. 1994). Both gene products have a role in the homologous recombination (HR) DNA repair pathway, which repairs double strand DNA breaks (DSBs) (Venkitaraman 2002). A degree of redundancy exists between the BER and HR pathways, allowing cells to compensate for the loss of one pathway. The BER enzyme PARP1, which binds to single strand DNA breaks and recruits other repair proteins, can be successfully targeted for inhibition, leading to failure of the BER pathway, replication fork stalling, and acquisition of double strand breaks. In normal cells, these double strand breaks are repaired via HR. In BRCA-deficient cells, however, loss of effective HR leads to DSB persistence and cell death. As heterozygosity at a BRCA allele is associated with effective HR, PARP inhibition specifically targets only tumour cells with acquired BRCA^{-/-} homozygosity (Bryant et al. 2005; Farmer et al. 2005). Phase I and II trials of PARP inhibitors have demonstrated favourable efficacy and limited toxicity in BRCA-related breast and ovarian cancers (reviewed in (Rehman, Lord, and Ashworth 2010)) and phase III trials are underway.

Other potential synthetic lethal relationships in PARP inhibition are currently being explored. 'BRCAness' refers to a subset of breast cancers, including 'triple negative' (oestrogen-, progesterone- and HER2-negative) and 'basal phenotype' cancers, that possess molecular and histopathological similarity to BRCA-deficient tumours, that may successfully be targeted by PARP inhibition (Turner, Tutt, and Ashworth 2004; Giorgetti et al. 2007). There is also developing interest in PTEN (phosphatase and tensin homolog), which is mutated in many sporadic cancers and, like BRCA mutations, causes a defect in homologous recombination (Shen et al. 2007; Mendes-Pereira et al. 2009).

Recent evidence suggests that other BER factors may also be targeted by a synthetic lethality approach. Mismatch repair (MMR) is responsible for the repair of DNA damage occurring during replication, and shares a degree of overlap with BER function. For example, 8-oxoguanine base lesions, which are induced by metabolic ROS and can cause mutagenic GC→TA transversions if unrepaired, may be processed by both BER and MMR. Mutations in the mismatch repair (MMR) genes *MLH1* or *MSH2* are implicated in human non-polyposis carcinoma coli (HNPCC) and some sporadic colorectal cancers. siRNA inhibition of the BER constituent DNA polymerase β/γ is selectively lethal in *MLH1/MSH2* mutant cell lines, suggesting a synthetic lethality relationship. Given the potential for BER inhibitors as a synthetic lethality target, it remains to be established if additional factors such as APE1 may have a role in this capacity.

7. APE1 inhibitors: Progress to date

7.1 APE1 DNA repair domain inhibitors

The first reported inhibitor of the APE1 repair domain, CRT0044876, was identified following high-throughput fluorescence-based screening of a small molecular chemical library. Applied alone, this compound increased the rate of AP site accumulation in tumour cell lines without evidence of intrinsic cytotoxicity. In combination with various base

damaging agents, including the alkylators MMS and temozolomide (TMZ), a synergistic increase in AP site accumulation was noted, associated with essentially complete (>99%) cell death. This effect was not replicated when the DNA damaging agents used induced damage repaired by mechanisms other than BER, suggesting BER-specific inhibition (Madhusudan et al. 2005). CRT0044876-induced BER inhibition has been reproduced elsewhere (Guikema et al. 2007; Seo and Kinsella 2009), including cytotoxicity potentiation with ionising radiation (Koll et al. 2008), although another group was not able to replicate potentiation (Fishel and Kelley 2007).

CRT0044876 contains an indole ring which is thought to interact with the APE1 active site, while the compound's carboxylate group coordinates towards the active site catalytic magnesium atom. Utilising knowledge gained from the identification and development of CRT0044876, molecular modelling techniques were utilised to design inhibitor templates to screen a computer database of 2.6m chemical compounds. Promising hits were chosen for their active site 'fit' and drug-like properties, then screened in the laboratory for specific inhibitory activity. A number of highly-potent, highly-specific non-competitive inhibitors of APE1 DNA repair were identified. Like CRT0044876, these compounds had low intrinsic toxicity, but are able to potentiate the cytotoxicity of alkylating agents in various cancer cell lines. Interestingly, the inhibitory effect was significantly increased in the Asp148Glu APE1 polymorph, previously implicated in cancer predisposition (see Section 5.4. APE1 polymorphisms and cancer susceptibility) (Mohammed et al. 2011).

A number of other groups have been working on the development of APE1 DNA repair inhibitors. Seiple et al. have identified the potential of arylstibonic acids, which possess inherent inhibitory activity on an initial fluorescence-based high-throughput screen (Seiple et al. 2008). Simeonov et al. utilised a similar fluorescence screening assay to identify three compounds (6-hydroxy-DL-DOPA, Reactive Blue 2 and myricetin) which potentiate MMS cytotoxicity in HeLa cells associated with a quantifiable increase in AP site accumulation. Modelling studies of 6-hydroxy-DL-DOPA suggest that it docks to the APE1 active site in a similar manner to CRT0044876 (Simeonov et al. 2009). Bapat *et al.* modified the previously-described fluorescence assay to identify the novel inhibitor AR02, which is able to selectively block APE1 DNA repair function in glioma cells and potentiate cytotoxicity of alkylating agents (Bapat et al. 2010). Zawahir et al. performed an *in silico* pharmacophore model-based screen to identify 21 potent and specific inhibitors. The most potent of the inhibitors identified to date share common features of two carboxylate groups arranged around a hydrophobic core, bearing structural similarity to the 3'- and 5'- deoxyribosephosphate groups on abasic DNA (Seiple et al. 2008; Zawahir et al. 2009).

The topoisomerase II inhibitor lucanthone has also been identified as a potential inhibitor of APE1 DNA repair activity, without impact on redox function (Bases and Mendez 1997; Luo and Kelley 2004). Lucanthone treatment in cell culture induces an increase in abasic site accumulation (Mendez, Goldman, and Bases 2002), and potentiates the cytotoxic effects of MMS and temozolomide (Luo and Kelley 2004). Clinically, lucanthone treatment accelerates regression of brain metastases following whole brain radiotherapy (Del Rowe et al. 1999). However, it is unclear whether this effect is mediated via APE1 inhibition, or as a function of lucanthone's effect on topoisomerase (Fishel and Kelley 2007).

Methoxyamine is a small molecular inhibitor of BER which irreversibly binds to abasic DNA sites, preventing processing by APE1 (Liu and Gerson 2004). Methoxyamine potentiates temozolomide *in vitro* (Taverna et al. 2001; Fishel et al. 2007) and in tumour xenografts (Liu,

Nakatsuru, and Gerson 2002; Yan et al. 2007). It also potentiates radiotherapy cytotoxicity in combination with the potent radiosensitiser 5-iodo-2'-deoxyuridine (Taverna et al. 2003). Methoxyamine has undergone phase I clinical trials in combination with pemetrexed (Anthony et al. 2009) and temozolomide (Sawides et al. 2010) in patients with advanced refractory cancer.

7.2 APE1 redox domain inhibitors

Through redox-mediated transcription factor activation, APE1 has cytoprotective and angiogenic influence in response to cellular stresses. The APE1 redox domain has therefore also been evaluated as a possible target for small molecule inhibition.

The naturally-occurring compound resveratrol, found in grapes and red wine, has been suggested as an inhibitor of tumorigenesis (Bhat and Pezzuto 2002; Aziz, Kumar, and Ahmad 2003). Computer modelling suggests it may bind the APE1 redox domain (Yang et al. 2005). Resveratrol exposure has been shown to inhibit activation of the APE-1 dependent antiapoptotic transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NFκB), and is also able to sensitise melanoma cells *in vitro* to dacarbazine treatment (Yang et al. 2005). However, these results have not been substantiated elsewhere (Fishel and Kelley 2007; Luo et al. 2008).

Soy isoflavanes are under investigation for a possible role in the treatment of prostate cancer. Prostate cell survival is significantly decreased when co-treated with soy isoflavanes in combination with radiation, compared to radiation alone. This is associated with downregulation in APE1 levels, and therefore a reduced level of NFκB and the proangiogenic transcription factor hypoxia-inducible factor 1α (HIF-1α) binding that may mediate the increase in radiosensitivity (Raffoul et al. 2007; Singh-Gupta et al. 2008). However, it is unclear how APE1 downregulation is mediated by soy isoflavanes, and whether associated downregulation of BER also has an impact on treatment sensitivity (Kelley, Georgiadis, and Fishel 2011).

APX3330 (also known as E3330) is a small molecule inhibitor of APE1 redox function. It specifically and selectively blocks APE1-mediated reduction of various transcription factors to their activated state (Zou and Maitra 2008; Luo et al. 2008; Zou et al. 2009; Nyland et al. 2010). APX3330 exposure has been demonstrated to reduce tumour cell growth (Saitou et al. 2005), migration (Zou and Maitra 2008) and angiogenesis (Zou et al. 2009). A number of APX3330 analogues with improved potency are currently under evaluation (Kelley, Georgiadis, and Fishel 2011).

7.3 APE1 inhibition and toxicity

Inhibition of APE1 offers exciting therapeutic potential. Given that APE1 is ubiquitously expressed in normal and malignant cells, concerns exist regarding the risk of inhibitor toxicity in normal tissue, particularly when used in combination with systemic chemotherapy treatments. This risk could be reduced by targeting inhibitor use in cancers with high levels of APE1 overexpression, or combining inhibitor use with targeted treatment such as radiotherapy. Long term toxicity is also of concern, as inhibition of APE1 in normal cells may result in propagation of potentially mutagenic DNA damage, leading to secondary malignancies. However, it is likely that the target population for APE1 inhibition will be patients with advanced cancer, where risk of secondary malignancy will not be of clinical significance (Abbotts and Madhusudan 2010).

8. Conclusion

Genomic integrity is constantly challenged by damage inflicted from a variety of endogenous and exogenous sources, including spontaneous deamination, reactive oxygen species, ionising radiation, ultraviolet light and chemical agents. Highly conserved pathways of DNA repair have evolved to maintain stability within the genome. Base excision repair (BER) processes and repairs damage to individual bases induced by alkylation, oxidation or ring saturation. Human apurinic/apyrimidinic endonuclease 1 (APE1) is a critical BER enzyme that recognises and processes the site of excised base damage (an 'abasic' site). APE1 is of considerable interest as a potential predictive and prognostic biomarker in cancer. Polymorphisms causing variable APE1 activity may alter cancer susceptibility and treatment response. High levels of oxidative stress in the tumour microenvironment may induce an increased rate of DNA damage acquisition, leading to an upregulation of BER that contributes to the dysregulation of APE1 expression commonly observed in solid tumours. Furthermore, APE1 expression is upregulated in response to treatment with DNA damaging agents such as chemotherapy and ionising radiation, and is frequently associated with resistance to treatment and poor prognostic outcomes. Further characterisation of APE1 polymorphisms and expression levels in human cancer will allow development of APE1 as a predictive and prognostic biomarker.

Preclinical study has confirmed APE1 as an emerging therapeutic target in cancer. Overexpression of APE1 is induced by DNA damaging agents and is associated with treatment resistance. Constitutional or engineered downregulation of APE1 confers sensitivity to treatment, and can overcome chemoresistance. A number of inhibitors of the APE1 DNA repair domain are currently under development, showing promise *in vitro* in their ability to potentiate the actions of agents causing alkylating or oxidation damage and overcome treatment resistance. Further development of these inhibitors into clinically-relevant compounds is an important and expanding area of cancer therapeutics.

9. References

- Abbotts, R., and S. Madhusudan. 2010. Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. *Cancer Treat Rev* 36 (5):425-35.
- Agachan, B., O. Kucukhuseyin, P. Aksoy, A. Turna, I. Yaylim, U. Gormus, A. Ergen, U. Zeybek, B. Dalan, and T. Isbir. 2009. Apurinic/apyrimidinic endonuclease (APE1) gene polymorphisms and lung cancer risk in relation to tobacco smoking. *Anticancer Res* 29 (6):2417-20.
- Al-Attar, A., L. Gossage, K. R. Fareed, M. Shehata, M. Mohammed, A. M. Zaitoun, I. Soomro, D. N. Lobo, R. Abbotts, S. Chan, and S. Madhusudan. 2010. Human apurinic/apyrimidinic endonuclease (APE1) is a prognostic factor in ovarian, gastro-oesophageal and pancreatico-biliary cancers. *Br J Cancer* 102 (4):704-9.
- Andreassen, C. N., J. Alsner, J. Overgaard, C. Herskind, J. Haviland, R. Owen, J. Homewood, J. Bliss, and J. Yarnold. 2005. TGF β 1 polymorphisms are associated with risk of late normal tissue complications in the breast after radiotherapy for early breast cancer. *Radiotherapy and Oncology* 75 (1):18-21.
- Andreassen, C. N., J. Alsner, M. Overgaard, F. B. Sorensen, and J. Overgaard. 2006. Risk of radiation-induced subcutaneous fibrosis in relation to single nucleotide

- polymorphisms in TGFB1, SOD2, XRCC1, XRCC3, APEX and ATM--a study based on DNA from formalin fixed paraffin embedded tissue samples. *Int J Radiat Biol* 82 (8):577-86.
- Anthony, S. P., L. S. Rosen, G. J. Weiss, M. S. Gordon, B. J. Adams, S. L. Gerson, D. Alvarez, C. P. Theuer, and B. R. Leigh. 2009. A phase I study of daily oral TRC102 (methoxyamine) to enhance the therapeutic effects of pemetrexed in patients with advanced refractory cancer. *J Clin Oncol (Meeting Abstracts)* 27 (15S):2552.
- Atamna, H., I. Cheung, and B. N. Ames. 2000. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. *Proc Natl Acad Sci U S A* 97 (2):686-91.
- Aziz, M. H., R. Kumar, and N. Ahmad. 2003. Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review). *Int J Oncol* 23 (1):17-28.
- Balakrishnan, L., P. D. Brandt, L. A. Lindsey-Boltz, A. Sancar, and R. A. Bambara. 2009. Long patch base excision repair proceeds via coordinated stimulation of the multienzyme DNA repair complex. *J Biol Chem* 284 (22):15158-72.
- Bapat, A., M. Fishel, and M. R. Kelley. 2008. Going Ape as an Approach to Cancer Therapeutics. *Antioxid Redox Signal*.
- Bapat, A., L. S. Glass, M. H. Luo, M. L. Fishel, E. C. Long, M. M. Georgiadis, and M. R. Kelley. 2010. Novel Small-Molecule Inhibitor of Apurinic/Apyrimidinic Endonuclease 1 Blocks Proliferation and Reduces Viability of Glioblastoma Cells. *Journal of Pharmacology and Experimental Therapeutics* 334 (3):988-998.
- Barnes, D. E., and T. Lindahl. 2004. Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu Rev Genet* 38:445-76.
- Bases, R. E., and F. Mendez. 1997. Topoisomerase inhibition by lucanthone, an adjuvant in radiation therapy. *Int J Radiat Oncol Biol Phys* 37 (5):1133-7.
- Bhat, K. P., and J. M. Pezzuto. 2002. Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci* 957:210-29.
- Bobola, M. S., A. Blank, M. S. Berger, B. A. Stevens, and J. R. Silber. 2001. Apurinic/Apyrimidinic endonuclease activity is elevated in human adult gliomas. *Clin Cancer Res* 7 (11):3510-8.
- Bobola, M. S., L. S. Finn, R. G. Ellenbogen, J. R. Geyer, M. S. Berger, J. M. Braga, E. H. Meade, M. E. Gross, and J. R. Silber. 2005. Apurinic/apurimidinic endonuclease activity is associated with response to radiation and chemotherapy in medulloblastoma and primitive neuroectodermal tumors. *Clin Cancer Res* 11 (20):7405-14.
- Bryant, H. E., N. Schultz, H. D. Thomas, K. M. Parker, D. Flower, E. Lopez, S. Kyle, M. Meuth, N. J. Curtin, and T. Helleday. 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434 (7035):913-7.
- Burkovics, P., V. Szukacsov, I. Unk, and L. Haracska. 2006. Human Ape2 protein has a 3'-5' exonuclease activity that acts preferentially on mismatched base pairs. *Nucleic Acids Research* 34 (9):2508-2515.
- Chalmers, A. J. 2009. The potential role and application of PARP inhibitors in cancer treatment. *Br Med Bull* 89:23-40.
- Chang-Claude, J., O. Popanda, X. L. Tan, S. Kropp, I. Helmbold, D. von Fournier, W. Haase, M. L. Sautter-Bihl, F. Wenz, P. Schmezer, and C. B. Ambrosone. 2005. Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and

- acute side effects of radiotherapy in breast cancer patients. *Clinical Cancer Research* 11 (13):4802-4809.
- Chang-Claude, J., O. Poponda, X. L. Tan, D. von Fournier, W. Haase, M. L. Sautter-Bihl, F. Wenz, P. Schmezer, and C. Ambrosone. 2005. Effect of polymorphisms in the DNA repair genes XRCC1, APE1 and XPD on acute side effects of radiotherapy (RT) in breast cancer patients. *Strahlentherapie Und Onkologie* 181:94-95.
- Chen, D. S., T. Herman, and B. Demple. 1991. Two distinct human DNA diesterases that hydrolyze 3'-blocking deoxyribose fragments from oxidized DNA. *Nucleic Acids Res* 19 (21):5907-14.
- Chen, D. S., and Z. L. Olkowski. 1994. Biological responses of human apurinic endonuclease to radiation-induced DNA damage. *Ann N Y Acad Sci* 726:306-8.
- Chou, K. M., and Y. C. Cheng. 2002. An exonucleolytic activity of human apurinic/aprimidinic endonuclease on 3' mispaired DNA. *Nature* 415 (6872):655-9.
- Chou, K. M., M. Kukhanova, and Y. C. Cheng. 2000. A novel action of human apurinic/aprimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3' termini of DNA. *J Biol Chem* 275 (40): 31009-15.
- D'Amours, D., S. Desnoyers, I. D'Silva, and G. G. Poirier. 1999. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* 342 (Pt 2):249-68.
- Del Rowe, J. D., J. Bello, R. Mitnick, B. Sood, C. Filippi, J. Moran, K. Freeman, F. Mendez, and R. Bases. 1999. Accelerated regression of brain metastases in patients receiving whole brain radiation and the topoisomerase II inhibitor, luanthone. *Int J Radiat Oncol Biol Phys* 43 (1):89-93.
- Demple, B., T. Herman, and D. S. Chen. 1991. Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. *Proc Natl Acad Sci U S A* 88 (24):11450-4.
- Deutsch, W. A. 2003. Marker for diagnosing breast cancers and ovarian cancers. US Pat. 20030286338.
- Di Maso, V., C. Avellini, L. S. Croce, N. Rosso, F. Quadrifoglio, L. Cesaratto, E. Codarin, G. Bedogni, C. A. Beltrami, G. Tell, and C. Tiribelli. 2007. Subcellular localization of APE1/Ref-1 in human hepatocellular carcinoma: possible prognostic significance. *Mol Med* 13 (1-2):89-96.
- Dianov, G., S. L. Allinson, H. Budworth, and K. M. Sleeth. 2003. Mammalian Base Excision Repair. In *Eukaryotic DNA Damage Surveillance and Repair*, edited by K. W. Caldecott: Kluwer Academic/Plenum Publishers.
- Durkacz, B. W., O. Omidiji, D. A. Gray, and S. Shall. 1980. (ADP-ribose)_n participates in DNA excision repair. *Nature* 283 (5747):593-6.
- Evans, A. R., M. Limp-Foster, and M. R. Kelley. 2000. Going APE over ref-1. *Mutat Res* 461 (2):83-108.
- Farkasova, T., S. Gurska, V. Witkovsky, and A. Gabelova. 2008. Significance of amino acid substitution variants of DNA repair genes in radiosusceptibility of cervical cancer patients; a pilot study. *Neoplasma* 55 (4):330-7.
- Farmer, H., N. McCabe, C. J. Lord, A. N. J. Tutt, D. A. Johnson, T. B. Richardson, M. Santarosa, K. J. Dillon, I. Hickson, C. Knights, N. M. B. Martin, S. P. Jackson, G. C. M. Smith, and A. Ashworth. 2005. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434 (7035):917-921.

- Fishel, M. L., Y. He, A. M. Reed, H. Chin-Sinex, G. D. Hutchins, M. S. Mendonca, and M. R. Kelley. 2008. Knockdown of the DNA repair and redox signaling protein Ape1/Ref-1 blocks ovarian cancer cell and tumor growth. *DNA Repair (Amst)* 7 (2):177-86.
- Fishel, M. L., Y. He, M. L. Smith, and M. R. Kelley. 2007. Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. *Clin Cancer Res* 13 (1):260-7.
- Fishel, M. L., and M. R. Kelley. 2007. The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. *Mol Aspects Med* 28 (3-4):375-95.
- Fong, P. C., D. S. Boss, T. A. Yap, A. Tutt, P. J. Wu, M. Mergui-Roelvink, P. Mortimer, H. Swaisland, A. Lau, M. J. O'Connor, A. Ashworth, J. Carmichael, S. B. Kaye, J. H. M. Schellens, and J. S. de Bono. 2009. Inhibition of Poly(ADP-Ribose) Polymerase in Tumors from BRCA Mutation Carriers. *New England Journal of Medicine* 361 (2):123-134.
- Fortini, P., B. Pascucci, E. Parlanti, M. D'Errico, V. Simonelli, and E. Dogliotti. 2003. The base excision repair: mechanisms and its relevance for cancer susceptibility. *Biochimie* 85 (11):1053-71.
- Frossi, B., G. Tell, P. Spessotto, A. Colombatti, G. Vitale, and C. Pucillo. 2002. H(2)O(2) induces translocation of APE/Ref-1 to mitochondria in the Raji B-cell line. *J Cell Physiol* 193 (2):180-6.
- Fung, H., R. A. O. Bennett, and B. Demple. 2001. Key role of a downstream specificity protein 1 site in cell cycle-regulated transcription of the AP endonuclease gene APE1/APEX in NIH3T3 cells. *Journal of Biological Chemistry* 276 (45):42011-42017.
- Fung, H., and B. Demple. 2005. A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. *Mol Cell* 17 (3):463-70.
- Giorgetti, G., E. Galizia, F. Bianchi, G. Piccinini, C. Loretelli, L. Belvederesi, R. Catalani, D. Gargliardini, C. Ferretti, F. Corradini, R. Bracci, A. Santinelli, and R. Cellerino. 2007. Brcaness phenotype and methylation of BRCA1 promoter in sporadic breast cancers. *Annals of Oncology* 18:52-52.
- Gorman, M. A., S. Morera, D. G. Rothwell, E. de La Fortelle, C. D. Mol, J. A. Tainer, I. D. Hickson, and P. S. Freemont. 1997. The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. *Embo J* 16 (21):6548-58.
- Gros, L., A. A. Ishchenko, H. Ide, R. H. Elder, and M. K. Saparbaev. 2004. The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. *Nucleic Acids Res* 32 (1):73-81.
- Gu, D. Y., M. L. Wang, M. M. Wang, Z. D. Zhang, and J. F. Chen. 2009. The DNA repair gene APE1 T1349G polymorphism and cancer risk: a meta-analysis of 27 case-control studies. *Mutagenesis* 24 (6):507-512.
- Guikema, J. E., E. K. Linehan, D. Tsuchimoto, Y. Nakabeppu, P. R. Strauss, J. Stavnezer, and C. E. Schrader. 2007. APE1- and APE2-dependent DNA breaks in immunoglobulin class switch recombination. *J Exp Med* 204 (12):3017-26.
- Hadi, M. Z., M. A. Coleman, K. Fidelis, H. W. Mohrenweiser, and Iii Dm Wilson. 2000. Functional characterization of Ape1 variants identified in the human population. *Nucleic Acids Res* 28 (20):3871-9.

- Hadi, M. Z., K. Ginalski, L. H. Nguyen, and D. M. Wilson, 3rd. 2002. Determinants in nuclease specificity of Ape1 and Ape2, human homologues of Escherichia coli exonuclease III. *J Mol Biol* 316 (3):853-66.
- Hadi, M. Z., and D. M. Wilson, 3rd. 2000. Second human protein with homology to the Escherichia coli abasic endonuclease exonuclease III. *Environ Mol Mutagen* 36 (4):312-24.
- Hagmann, M-L., J. Karl, J. Kloeckner, M. Roessler, M. Tacke, and M. Theirolf. 2008. APEX as a marker for lung cancer. WIPO WO/2008/116592: F. Hoffmann-La Roche Ltd.
- Hansen, W. K., W. A. Deutsch, A. Yacoub, Y. Xu, D. A. Williams, and M. R. Kelley. 1998. Creation of a fully functional human chimeric DNA repair protein. Combining O6-methylguanine DNA methyltransferase (MGMT) and AP endonuclease (APE/redox effector factor 1 (Ref 1)) DNA repair proteins. *J Biol Chem* 273 (2):756-62.
- Helleday, T., E. Petermann, C. Lundin, B. Hodgson, and R. A. Sharma. 2008. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* 8 (3):193-204.
- Herring, C. J., B. Deans, R. H. Elder, J. A. Rafferty, J. MacKinnon, G. Barzilay, I. D. Hickson, J. H. Hendry, and G. P. Margison. 1999. Expression levels of the DNA repair enzyme HAP1 do not correlate with the radiosensitivities of human or HAP1-transfected rat cell lines. *Br J Cancer* 80 (7):940-5.
- Herring, C. J., C. M. West, D. P. Wilks, S. E. Davidson, R. D. Hunter, P. Berry, G. Forster, J. MacKinnon, J. A. Rafferty, R. H. Elder, J. H. Hendry, and G. P. Margison. 1998. Levels of the DNA repair enzyme human apurinic/apyrimidinic endonuclease (APE1, APEX, Ref-1) are associated with the intrinsic radiosensitivity of cervical cancers. *Br J Cancer* 78 (9):1128-33.
- Hu, J. J., T. R. Smith, M. S. Miller, H. W. Mohrenweiser, A. Golden, and L. D. Case. 2001. Amino acid substitution variants of APE1 and XRCC1 genes associated with ionizing radiation sensitivity. *Carcinogenesis* 22 (6):917-22.
- Ishchenko, A. A., E. Deprez, A. Maksimenko, J. C. Brochon, P. Tauc, and M. K. Saparbaev. 2006. Uncoupling of the base excision and nucleotide incision repair pathways reveals their respective biological roles. *Proc Natl Acad Sci U S A* 103 (8):2564-9.
- Izumi, T., D. B. Brown, C. V. Naidu, K. K. Bhakat, M. A. Macinnes, H. Saito, D. J. Chen, and S. Mitra. 2005. Two essential but distinct functions of the mammalian abasic endonuclease. *Proc Natl Acad Sci U S A* 102 (16):5739-43.
- Izumi, T., L. R. Wiederhold, G. Roy, R. Roy, A. Jaiswal, K. K. Bhakat, S. Mitra, and T. K. Hazra. 2003. Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage. *Toxicology* 193 (1-2):43-65.
- Jones, C., and E. R. Plummer. 2008. PARP inhibitors and cancer therapy - early results and potential applications. *Br J Radiol* 81 Spec No 1:S2-5.
- Kakolyris, S., A. Giatromanolaki, M. Koukourakis, L. Kaklamanis, P. Kanavaros, I. D. Hickson, G. Barzilay, V. Georgoulis, K. C. Gatter, and A. L. Harris. 1999. Nuclear localization of human AP endonuclease 1 (HAP1/Ref-1) associates with prognosis in early operable non-small cell lung cancer (NSCLC). *J Pathol* 189 (3):351-7.
- Kakolyris, S., L. Kaklamanis, K. Engels, S. B. Fox, M. Taylor, I. D. Hickson, K. C. Gatter, and A. L. Harris. 1998. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. *Br J Cancer* 77 (7):1169-73.

- Kakolyris, S., L. Kaklamanis, K. Engels, H. Turley, I. D. Hickson, K. C. Gatter, and A. L. Harris. 1997. Human apurinic endonuclease 1 expression in a colorectal adenoma-carcinoma sequence. *Cancer Res* 57 (9):1794-7.
- Kelley, M. R., L. Cheng, R. Foster, R. Tritt, J. Jiang, J. Broshears, and M. Koch. 2001. Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Ape1/ref-1 in prostate cancer. *Clin Cancer Res* 7 (4):824-30.
- Kelley, M. R., M. M. Georgiadis, and M. L. Fishel. 2011. APE/Ref-1 Role in Redox Signaling: Translational Applications of Targeting the Redox Function of the DNA Repair/Redox Protein APE1/Ref-1. *Current Molecular Pharmacology (under submission)*.
- Koll, T. T., S. S. Feis, M. H. Wright, M. M. Teniola, M. M. Richardson, A. I. Robles, J. Bradsher, J. Capala, and L. Varticovski. 2008. HSP90 inhibitor, DMAG, synergizes with radiation of lung cancer cells by interfering with base excision and ATM-mediated DNA repair. *Mol Cancer Ther* 7 (7):1985-92.
- Koukourakis, M. I., A. Giatromanolaki, S. Kakolyris, E. Sivridis, V. Georgoulas, G. Funtzilas, I. D. Hickson, K. C. Gatter, and A. L. Harris. 2001. Nuclear expression of human apurinic/apyrimidinic endonuclease (HAP1/Ref-1) in head-and-neck cancer is associated with resistance to chemoradiotherapy and poor outcome. *Int J Radiat Oncol Biol Phys* 50 (1):27-36.
- Lau, J. P., K. L. Weatherdon, V. Skalski, and D. W. Hedley. 2004. Effects of gemcitabine on APE/ref-1 endonuclease activity in pancreatic cancer cells, and the therapeutic potential of antisense oligonucleotides. *Br J Cancer* 91 (6):1166-73.
- Li, C., Z. Liu, L. E. Wang, S. S. Strom, J. E. Lee, J. E. Gershenwald, M. I. Ross, P. F. Mansfield, J. N. Cormier, V. G. Prieto, M. Duvic, E. A. Grimm, and Q. Wei. 2006. Genetic variants of the ADPRT, XRCC1 and APE1 genes and risk of cutaneous melanoma. *Carcinogenesis* 27 (9):1894-901.
- Li, D. H., Y. N. Li, L. Jiao, D. Z. Chang, G. Beinart, R. A. Wolff, D. B. Evans, M. M. Hassan, and J. L. Abbruzzese. 2007. Effects of base excision repair gene polymorphisms on pancreatic cancer survival. *International Journal of Cancer* 120 (8):1748-1754.
- Li, M. X., Z. Y. Zhong, J. W. Zhu, D. B. Xiang, N. Dai, X. J. Cao, Y. Qing, Z. Z. Yang, J. Y. Xie, Z. P. Li, L. Baugh, G. Wang, and D. Wang. 2010. Identification and Characterization of Mitochondrial Targeting Sequence of Human Apurinic/Apyrimidinic Endonuclease 1. *Journal of Biological Chemistry* 285 (20):14871-14881.
- Lindahl, T. 1993. Instability and decay of the primary structure of DNA. *Nature* 362 (6422):709-15.
- Lipton, A. S., R. W. Heck, S. Primak, D. R. McNeill, D. M. Wilson, 3rd, and P. D. Ellis. 2008. Characterization of Mg(2+) Binding to the DNA Repair Protein Apurinic/Apyrimidic Endonuclease 1 via Solid-State (25)Mg NMR Spectroscopy. *J Am Chem Soc*.
- Liu, L., and S. L. Gerson. 2004. Therapeutic impact of methoxyamine: blocking repair of abasic sites in the base excision repair pathway. *Curr Opin Investig Drugs* 5 (6):623-7.
- Liu, L., Y. Nakatsuru, and S. L. Gerson. 2002. Base excision repair as a therapeutic target in colon cancer. *Clin Cancer Res* 8 (9):2985-91.
- Lord, C. J., and A. Ashworth. 2008. Targeted therapy for cancer using PARP inhibitors. *Current Opinion in Pharmacology* 8 (4):363-369.

- Lu, J., S. Zhang, D. Chen, H. Wang, W. Wu, X. Wang, Y. Lei, J. Wang, J. Qian, W. Fan, Z. Hu, L. Jin, H. Shen, W. Huang, Q. Wei, and D. Lu. 2009. Functional characterization of a promoter polymorphism in APE1/Ref-1 that contributes to reduced lung cancer susceptibility. *FASEB J*.
- Ludwig, D. L., M. A. MacInnes, Y. Takiguchi, P. E. Purtymun, M. Henrie, M. Flannery, J. Meneses, R. A. Pedersen, and D. J. Chen. 1998. A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity. *Mutat Res* 409 (1):17-29.
- Luo, M., S. Delaplane, A. Jiang, A. Reed, Y. He, M. Fishel, R. L. Nyland Ii, R. F. Borch, X. Qiao, M. M. Georgiadis, and M. R. Kelley. 2008. Role of the Multifunctional DNA Repair and Redox Signaling Protein Ape1/Ref-1 in Cancer and Endothelial Cells: Small-Molecule Inhibition of the Redox Function of Ape1. *Antioxid Redox Signal*.
- Luo, M., H. He, M. R. Kelley, and M. M. Georgiadis. 2010. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. *Antioxid Redox Signal* 12 (11):1247-69.
- Luo, M., and M. R. Kelley. 2004. Inhibition of the human apurinic/apurimidinic endonuclease (APE1) repair activity and sensitization of breast cancer cells to DNA alkylating agents with lucanthone. *Anticancer Res* 24 (4):2127-34.
- Madhusudan, S., F. Smart, P. Shrimpton, J. L. Parsons, L. Gardiner, S. Houlbrook, D. C. Talbot, T. Hammonds, P. A. Freemont, M. J. Sternberg, G. L. Dianov, and I. D. Hickson. 2005. Isolation of a small molecule inhibitor of DNA base excision repair. *Nucleic Acids Res* 33 (15):4711-24.
- McNeill, D. R., W. Lam, T. L. DeWeese, Y. C. Cheng, and D. M. Wilson, 3rd. 2009. Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. *Mol Cancer Res* 7 (6):897-906.
- Megnin-Chanet, F., M. A. Bollet, and J. Hall. 2010. Targeting poly(ADP-ribose) polymerase activity for cancer therapy. *Cell Mol Life Sci* 67 (21):3649-62.
- Meira, L. B., S. Devaraj, G. E. Kisby, D. K. Burns, R. L. Daniel, R. E. Hammer, S. Grundy, I. Jialal, and E. C. Friedberg. 2001. Heterozygosity for the mouse Apex gene results in phenotypes associated with oxidative stress. *Cancer Res* 61 (14):5552-7.
- Mendes-Pereira, A. M., S. A. Martin, R. Brough, A. McCarthy, J. R. Taylor, J. S. Kim, T. Waldman, C. J. Lord, and A. Ashworth. 2009. Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. *Embo Molecular Medicine* 1 (6-7):315-322.
- Mendez, F., J. D. Goldman, and R. E. Bases. 2002. Abasic sites in DNA of HeLa cells induced by lucanthone. *Cancer Invest* 20 (7-8):983-91.
- Miki, Y., J. Swensen, D. Shattuckeids, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Y. Liu, C. Cochran, L. M. Bennett, W. Ding, R. Bell, J. Rosenthal, C. Hussey, T. Tran, M. McClure, C. Frye, T. Hattier, R. Phelps, A. Haugenstrano, H. Katcher, K. Yakumo, Z. Gholami, D. Shaffer, S. Stone, S. Bayer, C. Wray, R. Bogden, P. Dayananth, J. Ward, P. Tonin, S. Narod, P. K. Bristow, F. H. Norris, L. Helvering, P. Morrison, P. Rostek, M. Lai, J. C. Barrett, C. Lewis, S. Neuhausen, L. Cannonalbright, D. Goldgar, R. Wiseman, A. Kamb, and M. H. Skolnick. 1994. A Strong Candidate for the Breast and Ovarian-Cancer Susceptibility Gene Brca1. *Science* 266 (5182):66-71.

- Mohammed, M. Z., V. N. Vyjayanti, C. A. Laughton, L. V. Dekker, P. M. Fischer, D. M. Wilson, 3rd, R. Abbotts, S. Shah, P. M. Patel, I. D. Hickson, and S. Madhusudan. 2011. Development and evaluation of human AP endonuclease inhibitors in melanoma and glioma cell lines. *Br J Cancer*.
- Mol, C. D., T. Izumi, S. Mitra, and J. A. Tainer. 2000. DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination [corrected]. *Nature* 403 (6768):451-6.
- Moore, D. H., H. Michael, R. Tritt, S. H. Parsons, and M. R. Kelley. 2000. Alterations in the expression of the DNA repair/redox enzyme APE/ref-1 in epithelial ovarian cancers. *Clin Cancer Res* 6 (2):602-9.
- Naidu, M. D., J. M. Mason, R. V. Pica, H. Fung, and L. A. Pena. 2010. Radiation Resistance in Glioma Cells Determined by DNA Damage Repair Activity of Ape1/Ref-1. *Journal of Radiation Research* 51 (4):393-404.
- Nakamura, J., and J. A. Swenberg. 1999. Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. *Cancer Res* 59 (11):2522-6.
- Nilsen, H., and H. E. Krokan. 2001. Base excision repair in a network of defence and tolerance. *Carcinogenesis* 22 (7):987-98.
- Nyland, R. L., M. H. Luo, M. R. Kelley, and R. F. Borch. 2010. Design and Synthesis of Novel Quinone Inhibitors Targeted to the Redox Function of Apurinic/Apyrimidinic Endonuclease 1/Redox Enhancing Factor-1 (Ape1/Ref-1). *Journal of Medicinal Chemistry* 53 (3):1200-1210.
- O'Shaughnessy, J., C. Osborne, J. Pippen, M. Yoffe, D. Patt, G. Monaghan, C. Rocha, V. Ossovskaya, B. Sherman, and C. Bradley. 2009. Efficacy of BSI-201, a poly (ADP-ribose) polymerase-1 (PARP1) inhibitor, in combination with gemcitabine/carboplatin (G/C) in patients with metastatic triple-negative breast cancer (TNBC): Results of a randomized phase II trial. *J Clin Oncol* 27 (18 suppl):abstr 3.
- Ono, Y., T. Furuta, T. Ohmoto, K. Akiyama, and S. Seki. 1994. Stable expression in rat glioma cells of sense and antisense nucleic acids to a human multifunctional DNA repair enzyme, APEX nuclease. *Mutat Res* 315 (1):55-63.
- Ordway, J. M., D. Eberhart, and T. Curran. 2003. Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding. *Mol Cell Biol* 23 (12):4257-66.
- Parsons, J. L., Dianova, II, and G. L. Dianov. 2004. APE1 is the major 3'-phosphoglycolate activity in human cell extracts. *Nucleic Acids Res* 32 (12):3531-6.
- Pascucci, B., M. Stucki, Z. O. Jonsson, E. Dogliotti, and U. Hubscher. 1999. Long patch base excision repair with purified human proteins. DNA ligase I as patch size mediator for DNA polymerases delta and epsilon. *J Biol Chem* 274 (47):33696-702.
- Puglisi, F., G. Aprile, A. M. Minisini, F. Barbone, P. Cataldi, G. Tell, M. R. Kelley, G. Damante, C. A. Beltrami, and C. Di Loreto. 2001. Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas. *Anticancer Res* 21 (6A):4041-9.
- Puglisi, F., F. Barbone, G. Tell, G. Aprile, B. Pertoldi, C. Raiti, M. R. Kelley, G. Damante, A. Sobrero, C. A. Beltrami, and C. Di Loreto. 2002. Prognostic role of Ape/Ref-1 subcellular expression in stage I-III breast carcinomas. *Oncol Rep* 9 (1):11-7.
- Raffoul, J. J., S. Banerjee, V. Singh-Gupta, Z. E. Knoll, A. Fite, H. Zhang, J. Abrams, F. H. Sarkar, and G. G. Hillman. 2007. Down-regulation of apurinic/apyrimidinic

- endonuclease 1/redox factor-1 expression by soy isoflavones enhances prostate cancer radiotherapy in vitro and in vivo. *Cancer Res* 67 (5):2141-9.
- Rehman, F. L., C. J. Lord, and A. Ashworth. 2010. Synthetic lethal approaches to breast cancer therapy. *Nature Reviews Clinical Oncology* 7 (12):718-724.
- Robertson, A. B., A. Klungland, T. Rognes, and I. Leiros. 2009. Base excision repair: the long and short of it. *Cellular and Molecular Life Sciences* 66:981-993.
- Robertson, K. A., H. A. Bullock, Y. Xu, R. Tritt, E. Zimmerman, T. M. Ulbright, R. S. Foster, L. H. Einhorn, and M. R. Kelley. 2001. Altered expression of Ape1/ref-1 in germ cell tumors and overexpression in NT2 cells confers resistance to bleomycin and radiation. *Cancer Res* 61 (5):2220-5.
- Robertson, K. A., D. P. Hill, Y. Xu, L. Liu, S. Van Epps, D. M. Hockenbery, J. R. Park, T. M. Wilson, and M. R. Kelley. 1997. Down-regulation of apurinic/aprimidinic endonuclease expression is associated with the induction of apoptosis in differentiating myeloid leukemia cells. *Cell Growth Differ* 8 (4):443-9.
- Robson, C. N., and I. D. Hickson. 1991. Isolation of cDNA clones encoding a human apurinic/aprimidinic endonuclease that corrects DNA repair and mutagenesis defects in *E. coli* xth (exonuclease III) mutants. *Nucleic Acids Res* 19 (20):5519-23.
- Robson, C. N., D. Hochhauser, R. Craig, K. Rack, V. J. Buckle, and I. D. Hickson. 1992. Structure of the human DNA repair gene HAP1 and its localisation to chromosome 14q 11.2-12. *Nucleic Acids Res* 20 (17):4417-21.
- Saitou, Y., K. Shiraki, T. Yamanaka, K. Miyashita, T. Inoue, Y. Yamanaka, Y. Yamaguchi, N. Enokimura, N. Yamamoto, K. Itou, K. Sugimoto, and T. Nakano. 2005. Augmentation of tumor necrosis factor family-induced apoptosis by E3330 in human hepatocellular carcinoma cell lines via inhibition of NF kappa B. *World J Gastroenterol* 11 (40):6258-61.
- Sak, S. C., P. Harnden, C. F. Johnston, A. B. Paul, and A. E. Kiltie. 2005. APE1 and XRCC1 protein expression levels predict cancer-specific survival following radical radiotherapy in bladder cancer. *Clin Cancer Res* 11 (17):6205-11.
- Sancar, A., L. A. Lindsey-Boltz, K. Unsal-Kacmaz, and S. Linn. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 73:39-85.
- Sawides, P., Y. Xu, L. Liu, J. A. Bokar, P. Silverman, A. Dowlati, and S. L. Gerson. 2010. Pharmacokinetic profile of the base-excision repair inhibitor methoxyamine-HCl (TRC102; MX) given as an one-hour intravenous infusion with temozolomide (TMZ) in the first-in-human phase I clinical trial. *J Clin Oncol (Meeting Abstracts)* 28 (15S):e13662.
- Seiple, L. A., J. H. Cardellina, 2nd, R. Akee, and J. T. Stivers. 2008. Potent inhibition of human apurinic/aprimidinic endonuclease 1 by arylstibonic acids. *Mol Pharmacol* 73 (3):669-77.
- Seo, Y. J., and T. J. Kinsella. 2009. Essential Role of DNA Base Excision Repair on Survival in an Acidic Tumor Microenvironment. *Cancer Research* 69 (18):7285-7293.
- Shen, W. H., A. S. Balajee, J. L. Wang, H. Wu, C. Eng, P. P. Pandolfi, and Y. X. Yin. 2007. Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* 128 (1):157-170.

- Shokolenko, I., N. Venediktova, A. Bochkareva, G. L. Wilson, and M. F. Alexeyev. 2009. Oxidative stress induces degradation of mitochondrial DNA. *Nucleic Acids Research* 37 (8):2539-2548.
- Silber, J. R., M. S. Bobola, A. Blank, K. D. Schoeler, P. D. Haroldson, M. B. Huynh, and D. D. Kolstoe. 2002. The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress. *Clin Cancer Res* 8 (9):3008-18.
- Simeonov, A., A. Kulkarni, D. Dorjsuren, A. Jadhav, M. Shen, D. R. McNeill, C. P. Austin, and D. M. Wilson, 3rd. 2009. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. *PLoS One* 4 (6):e5740.
- Singh-Gupta, V., H. Zhang, S. Banerjee, D. Kong, J. J. Raffoul, F. H. Sarkar, and G. G. Hillman. 2008. Radiation-induced HIF-1alpha cell survival pathway is inhibited by soy isoflavones in prostate cancer cells. *Int J Cancer*.
- Su, D., S. Ma, P. Liu, Z. Jiang, W. Lv, Y. Zhang, Q. Deng, S. Smith, and H. Yu. 2007. Genetic polymorphisms and treatment response in advanced non-small cell lung cancer. *Lung Cancer* 56 (2):281-8.
- Sweasy, J. B., T. Lang, and D. DiMaio. 2006. Is base excision repair a tumor suppressor mechanism? *Cell Cycle* 5 (3):250-9.
- Taverna, P., H. S. Hwang, J. E. Schupp, T. Radivoyevitch, N. N. Session, G. Reddy, D. A. Zarling, and T. J. Kinsella. 2003. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. *Cancer Res* 63 (4):838-46.
- Taverna, P., L. Liu, H. S. Hwang, A. J. Hanson, T. J. Kinsella, and S. L. Gerson. 2001. Methoxyamine potentiates DNA single strand breaks and double strand breaks induced by temozolomide in colon cancer cells. *Mutat Res* 485 (4):269-81.
- Tell, G., G. Damante, D. Caldwell, and M. R. Kelley. 2005. The Intracellular Localization of APE1/Ref-1: More than a Passive Phenomenon? *Antioxid Redox Signal* 7 (3-4):367-84.
- Tell, G., L. Pellizzari, C. Pucillo, F. Puglisi, D. Cesselli, M. R. Kelley, C. Di Loreto, and G. Damante. 2000. TSH controls Ref-1 nuclear translocation in thyroid cells. *J Mol Endocrinol* 24 (3):383-90.
- Thomson, B., R. Tritt, M. Davis, and M. R. Kelley. 2001. Histology-specific expression of a DNA repair protein in pediatric rhabdomyosarcomas. *J Pediatr Hematol Oncol* 23 (4):234-9.
- Tomicic, M., E. Eschbach, and B. Kaina. 1997. Expression of yeast but not human apurinic/apyrimidinic endonuclease renders Chinese hamster cells more resistant to DNA damaging agents. *Mutat Res* 383 (2):155-65.
- Turner, N., A. Tutt, and A. Ashworth. 2004. Hallmarks of 'BRCAness' in sporadic cancers. *Nature Reviews Cancer* 4 (10):814-819.
- Venkitaraman, A. R. 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108 (2):171-82.
- Walker, L. J., R. B. Craig, A. L. Harris, and I. D. Hickson. 1994. A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. *Nucleic Acids Res* 22 (23):4884-9.
- Wang, D., M. Luo, and M. R. Kelley. 2004. Human apurinic endonuclease 1 (APE1) expression and prognostic significance in osteosarcoma: enhanced sensitivity of

- osteosarcoma to DNA damaging agents using silencing RNA APE1 expression inhibition. *Mol Cancer Ther* 3 (6):679-86.
- Wang, D., D. B. Xiang, X. Q. Yang, L. S. Chen, M. X. Li, Z. Y. Zhong, and Y. S. Zhang. 2009. APE1 overexpression is associated with cisplatin resistance in non-small cell lung cancer and targeted inhibition of APE1 enhances the activity of cisplatin in A549 cells. *Lung Cancer*.
- Wiederhold, L., J. B. Leppard, P. Kedar, F. Karimi-Busheri, A. Rasouli-Nia, M. Weinfeld, A. E. Tomkinson, T. Izumi, R. Prasad, S. H. Wilson, S. Mitra, and T. K. Hazra. 2004. AP Endonuclease-Independent DNA Base Excision Repair in Human Cells. *Mol Cell* 15 (2):209-20.
- Wilson, D. M., 3rd. 2003. Properties of and substrate determinants for the exonuclease activity of human apurinic endonuclease Ape1. *J Mol Biol* 330 (5):1027-37.
- Wilson, D. M., 3rd, R. A. Bennett, J. C. Marquis, P. Ansari, and B. Demple. 1995. Trans-complementation by human apurinic endonuclease (Ape) of hypersensitivity to DNA damage and spontaneous mutator phenotype in *apn1*-yeast. *Nucleic Acids Res* 23 (24):5027-33.
- Xanthoudakis, S., R. J. Smeyne, J. D. Wallace, and T. Curran. 1996. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. *Proc Natl Acad Sci U S A* 93 (17):8919-23.
- Xiang, D. B., Z. T. Chen, D. Wang, M. X. Li, J. Y. Xie, Y. S. Zhang, Y. Qing, Z. P. Li, and J. Xie. 2008. Chimeric adenoviral vector Ad5/F35-mediated APE1 siRNA enhances sensitivity of human colorectal cancer cells to radiotherapy in vitro and in vivo. *Cancer Gene Ther*.
- Xu, Y., D. H. Moore, J. Broshears, L. Liu, T. M. Wilson, and M. R. Kelley. 1997. The apurinic/apyrimidinic endonuclease (APE/ref-1) DNA repair enzyme is elevated in premalignant and malignant cervical cancer. *Anticancer Res* 17 (5B):3713-19.
- Yan, L., A. Bulgar, Y. L. Miao, V. Mahajan, J. R. Donze, S. L. Gerson, and L. L. Liu. 2007. Combined treatment with temozolomide and methoxyamine: Blocking apurinic/pyrimidinic site repair coupled with targeting topoisomerase II alpha. *Clinical Cancer Research* 13 (5):1532-1539.
- Yang, S., K. Irani, S. E. Heffron, F. Jurnak, and F. L. Meyskens, Jr. 2005. Alterations in the expression of the apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1) in human melanoma and identification of the therapeutic potential of resveratrol as an APE/Ref-1 inhibitor. *Mol Cancer Ther* 4 (12):1923-35.
- Yoo, D. G., Y. J. Song, E. J. Cho, S. K. Lee, J. B. Park, J. H. Yu, S. P. Lim, J. M. Kim, and B. H. Jeon. 2008. Alteration of APE1/ref-1 expression in non-small cell lung cancer: The implications of impaired extracellular superoxide dismutase and catalase antioxidant systems. *Lung Cancer* 60 (2):277-284.
- Zawahir, Z., R. Dayam, J. Deng, C. Pereira, and N. Neamati. 2009. Pharmacophore guided discovery of small-molecule human apurinic/apyrimidinic endonuclease 1 inhibitors. *J Med Chem* 52 (1):20-32.
- Zou, G. M., C. Karikari, Y. Kabe, H. Handa, R. A. Anders, and A. Maitra. 2009. The Ape-1/Ref-1 redox antagonist E3330 inhibits the growth of tumor endothelium and endothelial progenitor cells: therapeutic implications in tumor angiogenesis. *J Cell Physiol* 219 (1):209-18.

Zou, G. M., and A. Maitra. 2008. Small-molecule inhibitor of the AP endonuclease 1/REF-1 E3330 inhibits pancreatic cancer cell growth and migration. *Mol Cancer Ther* 7 (7):2012-21.

Disruption of Protein–DNA Interactions: An Opportunity for Cancer Chemotherapy

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1. Introduction

The use of inhibitors, both natural and synthetic has been a mainstay in the biochemical analysis of cellular pathways from glycolysis, the TCA cycle and the electron transport chain to DNA replication, cell signaling and apoptosis. With advents in screening technology, robotics and combinatorial chemistry, the field of chemical genetics was born. The development and use of small molecule inhibitors (SMIs) to modulate the activities of proteins has provided a wealth of knowledge on a variety of pathways and enhanced drug development targeting novel proteins and activities. The effectiveness of targeting enzyme-substrate interactions is well established and only more recently have protein-protein interactions been effectively targeted with SMIs (Saha et al., 2010; Huang et al., 2008; De et al., 2009; Ballatore et al., 2010; Yang et al., 2010; Weber, 2010). While targeting protein-DNA interactions has been considered by some to be “undruggable interactions” we and others have succeeded in developing SMIs capable of inhibiting these often complex interactions. The ability to develop inhibitors of protein-DNA complex formation capable of *in vivo* activity opens up an entire new class of targetable molecule interactions for potential therapeutic benefit. One could envision inhibiting proteins involved in transcription in addition to DNA replication, DNA repair and recombination. This review will summarize the recent successes in targeting protein-DNA interactions and draw the distinction between those agents that inhibit these interactions directly versus those that reduce protein-DNA interactions via indirect mechanisms. We also highlight the development of DNA repair inhibitors focusing on the clinical utility of targeting DNA repair for cancer therapy.

2. Inhibitors of DNA replication, repair and recombination

Numerous DNA repair pathways are required for genomic stability and chromosome maintenance. Defects or deficiencies in DNA repair proteins, machinery and activity contribute to mutation driven carcinogenesis and the development of cancer. The importance of DNA repair has also been well established in the treatment response in numerous cancer types including testicular, ovarian and lung where defects in DNA repair increase the effectiveness of DNA damaging therapies. Exploiting the inherent genome instability in certain cancers via synthetic lethality targeting novel DNA repair proteins has progressed to clinical trials and holds the potential to significantly impact cancer treatment. First demonstrated in BRCA1/2-mutant breast cancer, targeting DNA repair with SMIs of

poly-ADP-ribose polymerase (PARP) proved clinically effective. While the exact mechanisms of PARP inhibition have not been completely elucidated, the idea of targeting DNA repair is gaining traction in both the laboratory and clinic. Supporting this is the recent editorial from Bruce Alberts in *Science* suggesting that all cancers will display some defect in DNA repair (Alberts, 2009). Some success in early phase trials has propelled this avenue of research though the number of targets is still limited.

Target	Inhibitor	Reference
PARP-1	NU1085	Griffin et al., 1996; Delaney et al., 2000
	NU1064	Bowman et al., 1998
	NU1025	Bowman et al., 1998; Delaney et al., 2000
	PD128763	Delaney et al., 2000
	AG14361	Calabrese et al., 2004
	ABT-888	Donawho et al., 2007
	AG14447	Thomas et al., 2007
RPA	MCI	Anciano et al., 2010
	TDRL-505	Shuck et al., 2010
XPA	TDRL-X80	Neher et al., 2010
DNA-PK	NU7441	Hardcastle et al., 2005; Leahy et al., 2004
	NU7026	Veuger et al., 2003
ATM	KU-55933	Hickson et al., 2004
C-Met	MP470	Qi et al., 2009
Stat3	Galiellalactone	Hellsten et al., 2008; Weidler, et al., 2000
	Falvopiridol	Lee et al., 2006
	IS3295	Turkson et al., 2005
	CPA-1, CPA-7	Turkson et al., 2004
	Platinum (V) tetrachloride	Turkson et al., 2004
NF-kB	Pristimerin	Lu et al., 2010
	KINK-1	Schon et al., 2008
	DHMEQ	Ariga et al., 2002
NOTCH	SAHM1	Moellering et al., 2009
HOXA13	lactam carboxamide	Ng et al., 2007

Table 1. Small molecule inhibitors of DNA repair proteins and transcription factors.

2.1 PARP1 inhibitor – targeting single strand break repair

Poly ADP-ribose polymerase (PARP) is a nuclear protein which binds to single-strand breaks (SSBs) in the DNA and signals downstream repair proteins to the site of damage, initiating DNA repair. PARPs direct involvement in SSB repair is the synthesis of ADP-

ribose polymers, utilizing NAD⁺ as a substrate, which aids in DNA repair and cellular signaling (Calabrese et al., 2004). PARP also plays a role in the homologous recombination (HR) repair pathway (Helleday, 2010) and has been suggested to play a role in the alternative non-homologous end joining pathway (A-NHEJ) (Wang et al., 2006). The indirect role PARP plays in the HR pathway is through its ability to recognize SSBs. In the absence of PARP SSBs accumulate resulting in an increased number of collapsed replication forks which require DNA repair. Therefore, chemotherapeutics which elicit their toxic effects by damaging the DNA, have increased potency in combination treatment with PARP inhibitors due to a decrease in DNA repair and an accumulation of collapsed replication forks (Thomas et al., 2007). This increase in DNA damage results in cell death, which is a favorable outcome in the treatment of cancer. Researchers have used this knowledge to initiate the development of PARP inhibitors and over the past four decades numerous PARP inhibitors have entered clinical trials, some of which have been successfully incorporated into chemotherapeutic regimens.

Initial PARP inhibitors were analogues of nicotinamide whose activities were useful for *in vitro* studies but were found to be of little clinical value. A second generation of inhibitors, which included benzimidazole-carboxamides, quinazolin-4-[3H]-ones and isoquinoline derivatives (NU1085, NU1025, PD128763) were shown to increase radio- and chemosensitivity *in vitro* (Delaney et al., 2000). NU1025 sensitized tumor cell lines to temozolomide and camptothecin, yet the specificity of NU1025 was not developed enough for use in preclinical trials. AG14361, a PARP inhibitor identified by a PARP1 activity assay, exploits the PARP1-catalyzed NAD-dependent incorporation of poly(ADP)ribose to histones (Thomas et al., 2007). Briefly, the incorporation of ³²P-ADP-ribose into an insoluble material is measured following the transfer of radiolabeled phosphate from ³²P-NAD. Data from this assay demonstrate a decrease in ADP transfer, suggestive of the inhibition of PARP1 activity. Additionally, AG14361 treatment increased a cells' susceptibility to three chemotherapeutics irinotecan, γ -irradiation and temozolomide with an IC₅₀ value below 5 nM (Calabrese et al., 2004). Although a number of PARP inhibitors have entered preclinical and clinical trials, most of these are competitive inhibitors with NAD⁺ and there is no data demonstrating disruption of the PARP-DNA interaction by SMIs. This is consistent with the separation of activities between the two zinc finger DNA binding domains and the catalytic domain though recent evidence proposes a model for the DNA dependent catalytic activity involving a DNA bound Zn domain 1 interaction with another critical domain required for catalytic activity (Langelier et al., 2011). How this is regulated by SMI binding again is yet to be determined.

Currently there are over 40 ongoing clinical trials focusing on PARP inhibitors and patient response. Olaparib, a novel PARP1 inhibitor has entered into clinical trials and in conjunction with BRCA1/2 mutations has proven to increase efficacy of treatments and is in Phase II clinical trials. ABT-888 has been shown to not only inhibit PARP1 activity but to also inhibit PARP2 activity based on a PARP enzyme assay using purified protein (Donawho et al., 2007; Penning et al., 2009). More recently crystal structure data provided a potential mechanism for this specificity (Karlberg et al., 2010) and highlights the deep binding pocket that allow low nM inhibitors to be developed (Figure 1). While ABT-888 has entered into early phase clinical trials the true target remains to be determined (Kummar et al., 2010).

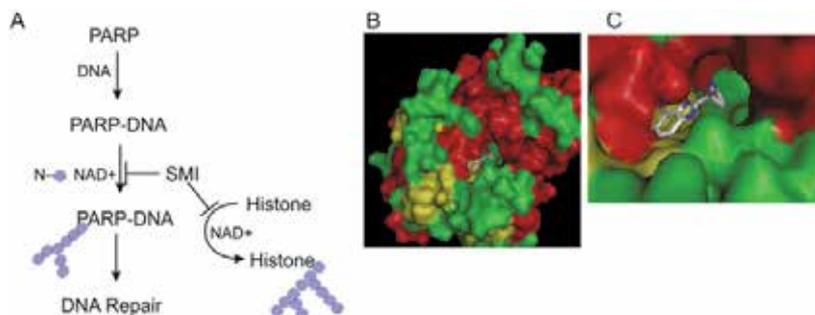


Fig. 1. Small Molecule Inhibitor of PARPs DNA Repair Activity. A) Depicted is the inhibitory effect of a general PARP SMI which disrupts the PARP catalyzed addition of poly(ADP)ribose onto various substrates or PARP itself. Disruption of this activity results in a decrease in DNA repair. B, C) Structural analysis of PARP- ABT-888 complex . PARP is colored according to secondary structure with red indicating α -helix, yellow β -sheet and green unstructured. ABT-888 is colored by element.

2.2 RPA inhibitors – targeting nucleotide excision repair, homologous recombination repair and DNA replication

Replication protein A (RPA) is involved in nearly all DNA metabolic pathways. It's involvement in the nucleotide excision repair (NER) pathway and the HR repair pathway is particularly important in the context of genome stability. RPA is the major human single strand DNA binding protein and participates in DNA replication at the fork, stabilizing single-stranded DNA (Wold, 1997). In NER RPA interacts with undamaged single strand DNA following the recognition of damaged nucleotides by Xeroderma Pigmentosum Group C (XPC), Rad23B and Centrin 2 in the initial steps of the NER pathway. RPA, together with the Xeroderma Pigmentosum Group A (XPA) protein and Transcription Factor II H (TFIIH), form the preincision complex of the NER pathway which is necessary for the proper functioning of the NER machinery (Araujo et al., 2000; Shuck et al., 2008). This preincision complex further guides the placement of downstream proteins such as Xeroderma Pigmentosum Group F (XPF) and Xeroderma Pigmentosum Group G (XPG) the proteins responsible for excising the damaged nucleotides. RPA also plays an indirect role in the HR repair pathway by interacting with Rad51 which initiates the HR pathway and promotes DNA strand exchange (Stauffer and Chazin, 2004; Sigurdsson et al., 2001). Disruption of RPA's DNA binding activity via a SMI would disrupt the formation of a functional NER preincision complex and decrease the efficiency of the NER machinery. In relation to cancer therapy, a decrease in NER efficiency is beneficial since this is the main pathway utilized for the removal of bulky DNA adducts, such as those resulting from cisplatin treatment.

Cisplatin is a common chemotherapeutic used as front line treatment for a number of cancers including those associated with the head and neck, non-small cell lung cancer (NSCLC), and testicular cancer. Cisplatin has a >90% cure rate for the treatment of testicular cancer (Einhorn, 2002); however in the case of NSCLC the response to cisplatin treatment varies significantly (Simon, 2008). Recurrence and resistance to cisplatin treatment are two significant clinical issues. In the cell, cisplatin elicits its toxic effect by forming covalent adducts with purine residues within the DNA. The NER preincision complex recognizes these bulky adducts and initiates the removal of the damaged nucleotides. Therefore,

inhibiting the NER machinery would result in an accumulation of cisplatin lesions on genomic DNA resulting in increased efficacy following treatment.

TDRL-505 - Recently a number of putative SMIs of RPA's DNA binding activity were identified and a subset of these putative inhibitors were analyzed *in vitro*. One inhibitor, TRDL-505, demonstrated an inhibitory effect on RPA's DNA binding activity as shown by electrophoretic mobility shift assays (EMSA) and by fluorescence polarization assays using both single- and double-strand DNA substrates with an IC_{50} value of 12.9 μ M (Shuck and Turchi, 2010). This reversible RPA inhibitor disrupted not only the interaction of full-length RPA but also of the p70 domain of RPA with DNA (Figure 2). The p70 domain of RPA contains two of the main OB-folds which are involved in the RPA-DNA interaction (Bochkarev and Bochkareva, 2004). After demonstrating that TRDL-505 reduced the formation of an RPA-DNA complex, *in vivo* studies were initiated to study the cellular effect of TDRL-505. Although single agent treatment of lung cancer cell lines H460 and A549 with TDRL-505 resulted in a G1 arrest and increased non-apoptotic cellular death, combination treatment with cisplatin resulted in a synergistic level of efficacy in both cell lines. Other *in vivo* analyses, such as immunofluorescence and foci formation demonstrated both a decrease in cellular concentration of RPA and foci formation following treatment with TDRL-505, suggesting that TDRL-505 may be affecting the NER activity and the replication activity of RPA (Shuck and Turchi, 2010). Although a number of *in vitro* and *in vivo* studies have demonstrated not only an interaction between TRDL-505 and RPA but also a synergistic effect increasing cisplatin efficacy, the molecule interactions between RPA and TDRL-505 have not been determined. Further, mouse model studies, in addition to clinical testing, are necessary to further characterize the inhibitory effect of TDRL-505. The initial data, however, provide substantial support for studies involving SMIs, which interact directly with proteins in the NER pathway, specifically those necessary for the preincision complex formation.

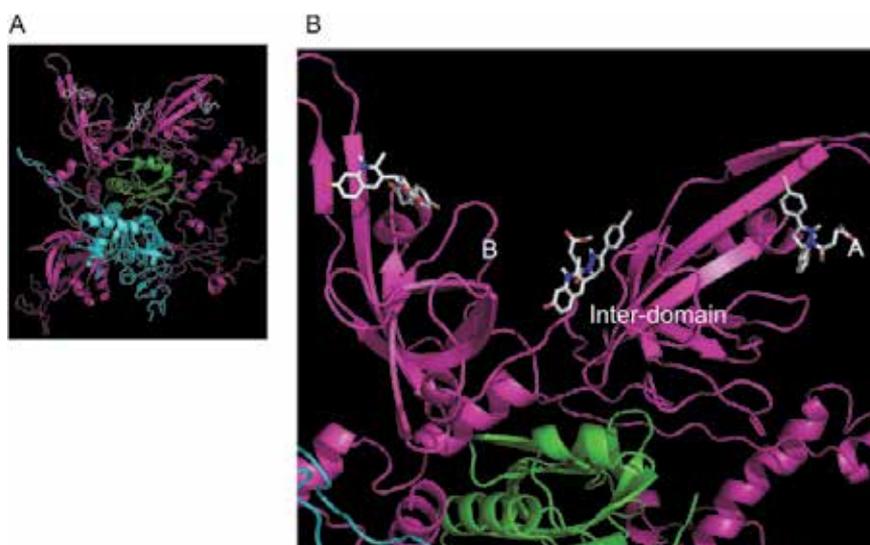


Fig. 2. Interaction of TDRL-505 with Replication Protein A. TDRL-505 is an *in vitro* characterized inhibitor of RPA's DNA binding activity. The modeled image demonstrates the potential interaction between TDRL-505 and RPA.

MCI - A second class of RPA inhibitors, containing a central isoborneol compound, irreversibly inhibit RPA's DNA binding activity *in vitro* (Anciano Granadillo et al., 2010).

This class of SMIs demonstrated an IC₅₀ value below 5 µM and in some cases as low as 1 µM, depending on the cell line analyzed. Following single agent treatment, this class of compounds induced a classical apoptotic response and lengthened either G1 or S phases of the cell cycle. Furthermore, this class of compounds synergized with cisplatin following combination treatment and a sequential dosing schedule in which cisplatin was the first drug employed. Taken together, this data suggests that this class of isoborneol compounds may be employed as effective single agent chemotherapeutics or in combination with current chemotherapy drugs such as cisplatin. However, the MCI SMIs have potential off-target cellular effects, which render the compounds more effective, further analyses studying the direct *in vivo* targets of the MCI SMIs is necessary to form solid conclusions as to the exact mechanisms of the inhibitors.

More recently, novel SMIs of the N-terminal domain of RPA70, which is responsible for numerous protein-protein interactions important for DNA damage signaling and damage response, were identified using *in silico* and *in vitro* methodologies (Glanzer et al., 2011). One specific SMI, NSC15520, disrupted the RPA-p53 and RPA-Rad9 interactions but did not affect RPA's ability to bind to ssDNA. Although this SMI needs to be further characterized, this data demonstrates the feasibility of targeting protein-DNA interactions for therapeutic benefit.

2.3 XPA inhibitors – targeting nucleotide excision repair

XPA's only known role is in the NER pathway and this protein is essential for the formation of the preincision complex following damage recognition. Previous studies have demonstrated that a decrease in XPA expression in testicular cancer cell lines leads to a decrease in NER capacity and an increase in cisplatin efficacy (Koberle et al., 2006; Welsh et al., 2004; Koberle et al., 1999). Small molecules docked against XPA's minimal DNA binding domain (MBD), via *in silico* screening, led to the identification of a number of compounds with the potential to disrupt XPA's DNA binding activity (Neher et al., 2010). Primary screening, consisting of fluorescence polarization using a single stranded oligonucleotide containing a fluorescein label, identified 1 compound TDRL-X80, which disrupted the formation of the XPA-DNA complex (Neher et al., 2010).

TDRL-X80 - Fluorescence polarization and ELISA based assays were used to study the disruption of the XPA-DNA interaction in the presence of TDRL-X80. The assays were performed using single-, double-strand, and double-strand cisplatin-damaged DNA; and with all three substrates the XPA-DNA interaction was reduced with an IC₅₀ value near 30 µM. Modeling of TDRL-X80 with XPA's MBD demonstrated that TDRL-X80 was in proximity to interact with Lysine 137 possibly disrupting XPA's DNA binding ability (Figure 3). These data provide support for the accuracy of the novel *in silico* screen in addition to providing a SMI of XPA's DNA binding activity. In addition, the data suggest the importance of Lys 137 in XPA's DNA binding activity rendering this amino acid potentially important for XPA's DNA binding function; however further analyses are necessary before this conclusion can be made. The possibility remains that *in vivo* TDRL-X80 may reduce the formation of a stable preincision complex, involving XPA and therefore reduce the NER capacity. If this occurs, as seen with the testicular data provided above, combination treatment with TDRL-X80 and cisplatin should increase the efficacy of drug treatment reducing cell division and increasing apoptosis.

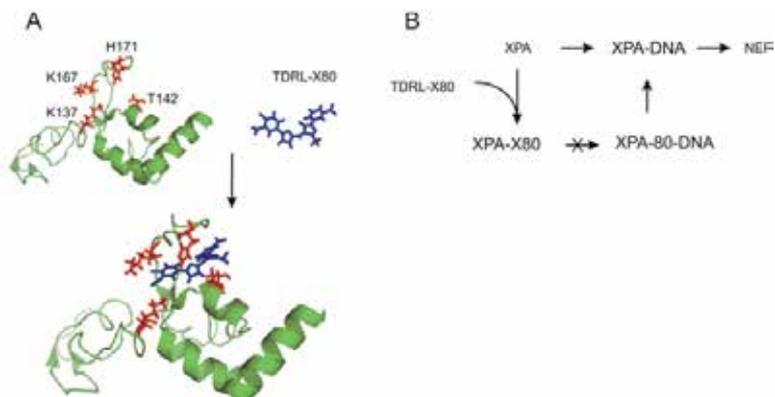


Fig. 3. Putative Interactions between TDRL-X80 and Amino Acids Localized in XPA's Minimal DNA Binding Domain. TDRL-X80 was modeled *in silico* in XPA's minimal DNA binding domain and amino acids within proximity are highlighted and may be potentially important for the XPA-X80 interaction.

2.4 DNA-PK inhibitors – targeting non-homologous end joining double strand break repair via ATP competitive inhibitors

DNA-PK, a heterotrimeric protein complex consisting of Ku70/80 and DNA-PKcs, plays an important role in the NHEJ pathway and the repair of double strand breaks (DSBs) by interacting with the free ends of genomic DNA and recruiting downstream end processing proteins such as artemis and fill-in/ligation proteins such as the XRCC4/Ligase IV complex (Pawelczak et al., 2010). Inhibiting proteins involved in the NHEJ pathway would result in a decrease in the repair of DNA DSBs and an accumulation of DNA damage, resulting in cellular death or apoptosis. DNA DSBs can be caused by a number of agents, including ionizing radiation (IR), which is commonly used for cancer therapy. IR treatment not only directly causes DSBs but also induces free radicals and reactive oxygen species which in turn may damage the DNA, creating abasic sites. IR, therefore, results in DNA ends which are difficult to ligate and require significant processing, which is the function of the NHEJ pathway. Thus, by decreasing the efficiency of the NHEJ pathway an increase in the effectiveness of IR may be possible. A number of SMIs of DNA-PK have been identified and extensively characterized *in vitro* and *in vivo*, although none target the DNA-PK-DNA interaction. Most of the inhibitors identified to date are competitive with the ATP substrate and do not appear to impact the necessary interaction of DNA-PK with DNA.

Wortmannin, one of the first identified DNA-PK inhibitors is an irreversible inhibitor that displays non-competitive kinetics, while competing with ATP (Izzard et al., 1999). The lack of specificity while limiting the utility of this agent as a chemical genetic probe, did provide key structural information used for the synthesis of analogs. Similarly other early PI-3K inhibitors, OK1035 and LY294002 were useful structurally but had limited utility to probe DNA DSB repair (Take et al., 1995, Hollick et al., 2003). Second generation ATP competitive inhibitor NU-7026 ((2-(morpholin-4-yl)-benzo[h]chromen-4-one) was shown to be more selective and more potent (Hollick et al., 2003). 2-N-morpholino-8-dibenzothiophenyl-chromen-4-one (NU7441) also based on LY294002 (Hardcastle et al., 2005), was shown to be more potent and more specific for DNA-PK with an IC_{50} value of 14 nM as demonstrated in an ELISA-based assay (Leahy et al., 2004). With low nM activity and increased specificity

these agents were active as radiosensitizers and sensitized cells to etoposide, a common chemotherapeutic that indirectly induces DNA DSBs (Zhao et al., 2006). These data support the possibility that NU7441 is an effective SMI of DNA-PK and in combination treatment with current chemotherapeutic regimens, such as IR or etoposide treatment, a decrease in repair would result in an increase in treatment efficacy. Continued development of both classes of inhibitors including NU7613 continue to target the ATP binding pocket of DNA-PK and result in increased potency and selectivity while also increasing cellular activity (Clapham et al., 2011; Cano et al., 2010; sage-El et al., 2008; Hollick et al., 2007; Griffin et al., 2005), however, their true utility as therapeutic agents awaits clinical assessment.

2.5 ATM inhibitors – targeting the DSB DNA damage response

The role of Ataxia telangiectasia mutated (ATM) in DSB repair has been studied in detail. This protein kinase plays an important role in the signaling cascade initiated in response to DNA DSBs. As mutation in ATM results in hypersensitivity to IR, similar to DNA-PK, chemical inhibition is a means of radiosensitizing cells to radiation treatment. Initial work also focused on non-specific inhibitors including wortmannin and caffeine. More recently a small molecule, KU-55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one), was identified and from a screen of a combinatorial library and characterization revealed potent and specific inhibition of ATM (Hickson et al., 2004). Specificity for ATM was 100x greater than that for any other tested proteins in the PIKK family and demonstrated an IC₅₀ value of ~12.9 nM. Cells treated with KU-55933 after IR demonstrated a significant downregulation of the IR-induced phosphorylation of p53. Furthermore, UV-induced phosphorylation events were not altered by the addition of KU-55933 following UV damage, suggesting that KU-55933 is a specific inhibitor of ATM and not interacting indirectly with proteins in the NER pathway, which is used to repair UV-damaged DNA. Additional cell cycle analysis demonstrated that KU-55933 alone had no effect on the cell cycle; however, KU-55933 treatment following IR results in a shift to the G2/M phase in contrast to the G2/S or G2/M phase with IR treatment alone and KU-55933 did not alter the cell cycle for fibroblast cells even after IR treatment, again suggesting specificity and direct target effects by this ATM inhibitor. More recently, KU-55933 was observed to induce apoptosis as a single agent and ATM regulation of AKT was suggested to mediate this effect (Li and Yang, 2010). A similar effect was also observed with KU-60019, an improved more potent inhibitor (Golding et al., 2009). The mechanism of these inhibitors has not been described in detail however, these SMIs are based on the LY294002 compound (see section 2.4) and therefore one would expect both KU-55933 and KU-60019 to be competitive inhibitors with respect to ATP. Very similar results were also observed with another ATM inhibitor identified by screening a focused library (Rainey et al., 2008). Similar to DNA-PK inhibitors the utility of these as radiosensitizing agents for cancer therapy remains to be determined.

2.6 Indirect targeting of Rad51 via receptor tyrosine kinase inhibitors

Rad51 is a critical protein in homologous recombination (HR) and homology directed repair. RAD51 binds RPA coated single stranded DNA and mediates the search for homology. Rad51 is overexpressed in a variety of cancers and has been proposed to be a target for cancer therapy. Despite these intriguing data, no direct Rad51 inhibitors have been described to date. Rad51 however has been found to be down regulated in cells treated with an inhibitor of the c-Met Receptor tyrosine kinase (RTK). RTKs are involved in the

regulation of numerous cellular processes such as cell growth, differentiation and repair of damaged DNA. Additionally, overexpression and mutation of tyrosine kinases have been shown to be involved in the progression of many cancers. c-Met, one such RTK has been implicated in tumorigenesis and has recently become a target for SMIs (Eder et al., 2009; Welsh et al., 2009). The SMI, MP470 was designed as a general RTK inhibitor and was initially shown to inhibit c-Met in addition to other RTKs (Qi et al., 2009). More specifically, MP470 inhibits c-Met tyrosine kinase phosphorylation and has been shown to sensitize cells to IR and platinum-based therapies in addition to inducing apoptosis (Qi et al., 2009). Cells treated with MP470 were not only shown to have a reduction in c-Met but also a slight reduction in Akt- and IR-induced Rad51 expression, which is involved in the repair of DSB. Literature suggests that MP470 may impart its function by modulating Rad51 expression in a dose dependent manner; however, data supporting this statement remain sparse at best. Although MP470 has advanced to clinical trials, the plethora of indirect effects make determination of the contribution of HR repair to any observed activity nearly impossible to determine. Thus validation of Rad51 as a clinical target for cancer therapy will likely require an agent that directly targets this protein and its role in HR.

3. Inhibitors of transcription factors

Myriad of human diseases are associated with irregular transcription factor activity making this class of proteins and complexes highly desirable targets for therapy. Many factors however, have led some to consider these targets as “undruggable”. The complexity of the interactions involving multi-point contact over large surfaces and lack of small pockets and crevices in which to design SMI’s are only a few of the issues needed to be addressed. While not comprehensive, below we highlight a few of these efforts towards targeting the transcription factors and identify those with direct action blocking protein-DNA interactions versus indirect action.

3.1 Stat3 transcription factor

Signal transducer and activator of transcription 3 (Stat3) is a member of the Stat protein family that acts as a transcription activator. In response to cytokines and growth factors, Stat3 is activated by phosphorylation on tyrosine 705, and forms homo- or hetero-dimers via reciprocal interaction between its Src homology 2 (SH2) domain and the phosphorylated tyrosine residues. Stat3 dimers then translocate into the nucleus, where they bind to specific promoter sequences activating transcription. Stat3 target genes have been identified and include regulators of crucial steps in proliferation and survival, metastasis and angiogenesis, and immune evasion (reviewed in ref (Bowman et al., 2000; Darnell, Jr., 1997; Hsieh et al., 2005; Ihle, 1996). Constitutive activation of Stat3 has been observed in various human cancers such as breast, lung, head and neck, and prostate cancers and correlates with poor prognosis of these diseases (Bowman et al., 2000). Subcutaneously-injected cells expressing constitutively-activated Stat3 also formed tumors in xenograft animal models (Bromberg et al., 1999). Selective inhibition of aberrant Stat3 activity has been reported to inhibit cell proliferation and induce apoptosis in a variety of cancer cell lines (Yue and Turkson, 2009). Thus, Stat3 is one of the promising targets for antineoplastic drug discovery. Indeed, inhibitors directed against the Stat3 pathway have recently entered into clinical trials. For example, a phase-1 study of OPB-31121 in patients with advanced solid tumors is currently

recruiting participants. OPB-31121 appears to strongly inhibit Interleukin 6 (IL-6)-induced phosphorylation of Stat3, thereby inducing tumor cell apoptosis and regression. A variety of Stat3 inhibitors have been previously identified, although targeting the upstream kinases for Stat3 activation such as Janus kinase inhibitor AG490 (Burke et al., 2001), WP1066 (Iwamaru et al., 2007), TG101209 (Ramakrishnan et al., 2010) and AZD1480 (Hedvat et al., 2009) or upstream factors for its expression such as antisense oligonucleotides (Kunigal et al., 2009; Ling and Arlinghaus, 2005) have been considered and tested (Yue and Turkson, 2009). The following discussion focuses on the inhibitors specifically targeting protein-protein and protein-DNA interactions (Figure 4). For inhibitors directed against protein-protein interactions the first proof-of-concept approach was peptidic and peptidomimetic inhibitors mimicking the sequence that binds to the Stat3 SH2 domain disrupting Stat3 dimerization. These inhibitors include small peptides PpYLKTK (Turkson et al., 2001), pYLPQTV (Ren et al., 2003), certain peptide aptamers (Nagel-Wolfrum et al., 2004) and mimetics ISS610 (Turkson et al., 2004a). However, these inhibitors face challenges in membrane permeability and stability. Although the mimetics such as ISS610 have improved inhibition of Stat3 and selectivity over Stat1 and Stat5 in *in vitro* DNA-binding assays, their intracellular activity remains low as it cannot be efficiently taken up by cells (Turkson et al., 2004a). Nevertheless, these studies show that targeting the SH2 domain and dimerization of Stat3 is feasible. Recently, computational approaches and assay-based screening have been used to identify several potential SMIs targeting the SH2 domain. These SMIs include S3I-M2001 (Siddiquee et al., 2007b), STA-21 (Song et al., 2005; Chen et al., 2007), S3I-201 (Siddiquee et al., 2007a; Fletcher et al., 2009; Lin et al., 2009), Stattic (Schust et al., 2006), and catechol containing compounds (Hao et al., 2008). Additionally, these SMIs appear to inhibit Stat3 dimerization and have anti-proliferative effects on cancer cells. Although these inhibitors exhibited better cell permeability, stability and bioavailability than the peptidic and peptidomimetics, their moderate activities at medium to high micromolar levels will likely limit their clinical development.

Although the DNA-binding domain of transcription factors has been long considered undruggable, the inhibition of the DNA-binding activity of Stat3 for drug discovery has been tested. Galiellalactone (Weidler et al., 2000; Hellsten et al., 2008), flavopiridol (Lee et al., 2006) and a class of platinum (IV) compounds including IS3295 (Turkson et al., 2005), CPA-1, CPA-7 and platinum (V) tetrachloride (Turkson et al., 2004b), and decoy oligodeoxynucleotides (Xi et al., 2005; Zhang et al., 2007; Gu et al., 2008; Sun et al., 2008; Zhang et al., 2010; Barton et al., 2004) were found to interfere with Stat3 binding to DNA as well as induce cell growth inhibition and apoptosis of human breast, lung and prostate cancer cells (Hellsten et al., 2008; Barton et al., 2004; Zhang et al., 2010; Zhang et al., 2007; Xi et al., 2005; Sun et al., 2008; Gu et al., 2008; Turkson et al., 2004b; Turkson et al., 2005; Lee et al., 2006). Some of these inhibitors have been tested in animal models and they appear to cause regression of xenograft tumors. These findings suggest that the DNA-binding domain of Stat3 is likely druggable. Recently, using computation-based drug screening, targeting the DNA-binding site of Stat3, we identified Stat3-specific SMIs that were able to inhibit cell proliferation and induce cellular apoptosis of human breast and lung cancer cells at sub-micromolar concentrations (un-published data). Further investigation of these DNA-binding inhibitors of Stat3 will likely result in promising candidates for therapeutic development. However, more studies are clearly needed to assess further the therapeutic suitability of these agents and establish a pharmacologic and toxicity profile.

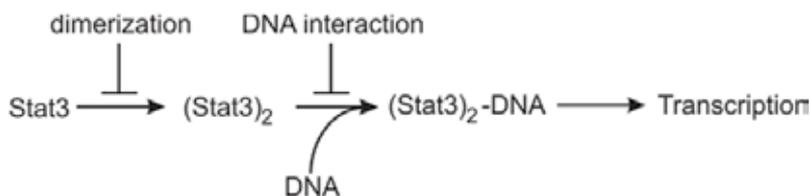


Fig. 4. Inhibitors of Transcription Factor Stat3. SMIs have been developed against Stat3's protein-protein and more recently protein-DNA interactions. Recently identified Stat3 inhibitors have demonstrated a decrease in the Stat3-DNA interaction and are being further characterized.

3.2 Direct inhibition of HOXA13-DNA interaction

HOXA13 is a member of the homeobox family of transcription factors involved in the regulation of development and has been found to play a role in the progression of a number of cancers (bate-Shen, 2002). These characteristics, like those of many transcription factors, make HOXA13 a viable target for inhibition towards therapeutic treatment of human cancer. As mentioned above, targeting the transcription factor-DNA interaction remains difficult but of high importance; therefore, a number of molecules with high complexity were designed to inhibit general transcription factors. An inhibitor of the HOXA13-DNA interaction was identified via a high-throughput screen of a library of lactam carboxamides (Ng et al., 2007). Researchers utilized a fluorescence polarization assay to screen the compounds and identified two lactam carboxamides which disrupted the HOXA13-DNA interaction. One identified inhibitor was further analyzed and demonstrated an IC_{50} value of $\sim 6.5 \mu\text{M}$. In addition, using a gene reporter assay, which places the HOXA13 transcription factor in control of the expression of luciferase, researchers demonstrated an increase in luciferase expression following the addition of the HOXA13 inhibitor (Ng et al., 2007). Although the data presented suggest the disruption of the HOXA13-DNA interaction, there is very little additional information regarding this inhibitor. Although further biochemical studies need to be performed to confirm the inhibitor activity of this lactam carboxamide on HOXA13, these data provide support for the utility of high-throughput screens and the importance of inhibition of transcription factor-DNA interactions.

3.3 Targeting Notch transcription complexes

Notch proteins are membrane bound receptors, which are expressed during organogenesis and throughout adult tissues. Cell communication occurs when a Notch receptor interacts with a Notch ligand. Once activated, Notch is cleaved and then able to translocate into the nucleus to activate target genes by forming a complex with the DNA-bound transcription factor CSL (Schwanbeck et al., 2010). The Notch-CSL-DNA complex then recruits co-activator proteins to ultimately stimulate transcription. Notch proteins play important roles in a number of cellular processes such as cellular differentiation and proliferation, for example, increased Notch expression correlates with a predisposition for cancers (Weng et al., 2004; O'Neil et al., 2007). Targeting Notch transcription was achieved with an α -helical peptide designed to bind the Notch-CSL-DNA complex termed SAHM1. SAHM1 was able to compete with the co-activator MAM11 *in vitro* and in cellular assays, capable of repressing Notch target gene expression (Moellering et al., 2009). Perhaps even more impressively, treatment of T-ALL cancer cells resulted in decreased proliferation in cell

culture models and also inhibited leukemic progression in an *in vivo* mouse model (Moellering et al., 2009). While this agent does not directly inhibit DNA binding of the transcription complex, the ability to target a DNA-bound factor on DNA represents a novel and exciting possibility of targeting other DNA bound proteins.

3.4 NF- κ B inhibitor

Nuclear transcription factor-kappa B (NF- κ B) has been shown to be activated in numerous tumors found in the breast, colon, prostate and skin (Amiri et al., 2004). This pleiotropic pathway has been the subject of intense study and inhibitors of NF- κ B signaling have been reported from numerous laboratories (Karin et al., 2004). The vast majority of these agents have been identified in cell based screening using reporter assays. While inhibition of NF- κ B DNA binding activity is reported for many of these, there is a scarcity of data supporting a direct interaction NF- κ B with any of these compounds. Again owing to the complexity of the system, there are numerous up-stream cellular events that could lead to reduced NF- κ B DNA binding activity as measured in cell extracts prepared from treated cells. A number of indirect NF- κ B inhibitors has been identified such as pristimerin, an inhibitor of NF- κ B-1 kinase (KINK-1)(Schon et al., 2008; Lu et al., 2010). While, the data demonstrate that cells treated with pristimerin had less NF- κ B activation, this is an indirect effect, which, while being therapeutically useful, is not mediated by a direct interaction with NF- κ B. Similarly, research has clearly demonstrated that DHMEQ, a derivative of the antibiotic epoxyquinomicin C, which was studied as an inhibitor of NF- κ B (Matsumoto et al., 2000), does not directly disrupt NF- κ B interactions, rather it inhibits NF- κ B activation by eliminating the nuclear translocation of NF- κ B via inhibition of the tumor necrosis factor-alpha (Yamamoto et al., 2008; Cardile et al., 2010). Recently, screening of a library of FDA approved compounds for their ability to inhibit NF- κ B signaling was reported and 19 drugs were identified that inhibited NF- κ B signaling with IC₅₀ values near 20 μ M (Miller et al., 2010). Although the 19 drugs identified as NF- κ B inhibitors are currently approved for clinical use they were not originally identified for direct interaction with NF- κ B. Therefore, further characterization of each of the drugs is necessary to gain insight into their exact biological and molecular mechanisms before any cellular or physiological effects can be attributed to inhibition of NF- κ B.

4. Conclusion

The molecular interactions between proteins and DNA have been investigated for decades in the context of nearly every nuclear DNA metabolic pathway. The results obtained have provided significant insight and advances to the scientific community and generated considerable biochemical, structural and physiological knowledge. In the last decade a researchers began the search for inhibitors of such protein-DNA interactions and have found a number of inhibitors, which are able to disrupt these critical interactions. While there is a wide range of implications, this review has focused on the potential clinical utility associated with the disruption of protein-DNA interactions as a means to treat cancer. Cancer associated deaths remain the number one cause of mortality in the US and resistance to current chemotherapeutics remains a major clinical hurdle. Sensitizing cancer cells to treatment with DNA damaging chemotherapeutics holds great value, and disruption of protein-DNA interactions via SMIs is one way to achieve this result. The development of small drug-like molecules targeting protein-DNA interactions represent a new paradigm for disrupting cellular processes toward clinical utility.

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6. References

- Alberts,B. (2009). Redefining cancer research. *Science* 325, 1319.
- Amiri,K.I. et al., (2004). Augmenting chemosensitivity of malignant melanoma tumors via proteasome inhibition: implication for bortezomib (VELCADE, PS-341) as a therapeutic agent for malignant melanoma. *Cancer Res.* 64, 4912-4918.
- Anciano Granadillo,V.J. et al., (2010). Targeting the OB-folds of Replication Protein A with Small Molecules. *Journal of Nucleic Acids* 304035.
- Araujo,S.J. et al., (2000). Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes & Development* 14, 349-359.
- Ballatore,C. et al., (2010). Modulation of Protein-Protein Interactions as a Therapeutic Strategy for the Treatment of Neurodegenerative Tauopathies. *Curr. Top. Med. Chem.*
- Barton,B.E. et al., (2004). Novel single-stranded oligonucleotides that inhibit signal transducer and activator of transcription 3 induce apoptosis in vitro and in vivo in prostate cancer cell lines. *Mol. Cancer Ther.* 3, 1183-1191.
- bate-Shen,C. (2002). Deregulated homeobox gene expression in cancer: cause or consequence? *Nat. Rev. Cancer* 2, 777-785.
- Bochkarev,A. and Bochkareva,E. (2004). From RPA to BRCA2: lessons from single-stranded DNA binding by the OB-fold. *Current Opinion in Structural Biology* 14, 36-42.
- Bowman,T. et al., . (2000). STATs in oncogenesis. *ONC* 19, 2474-2488.
- Bromberg,J.F. et al., (1999). Stat3 as an oncogene. *Cell* 98, 295-303.
- Burke,W.M. et al., (2001). Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. *ONC* 20, 7925-7934.
- Calabrese,C.R., et al., (2004). Anticancer chemosensitization and radiosensitization by the novel poly(ADP-ribose) polymerase-1 inhibitor AG14361. *J. Natl. Cancer Inst.* 96, 56-67.
- Cano,C. et al., (2010). DNA-dependent protein kinase (DNA-PK) inhibitors. Synthesis and biological activity of quinolin-4-one and pyridopyrimidin-4-one surrogates for the chromen-4-one chemotype. *J. Med. Chem.* 53, 8498-8507.
- Cardile,V. et al., (2010). Dehydroxymethylepoxyquinomicin, a novel nuclear factor-kappaB inhibitor, prevents inflammatory injury induced by interferon-gamma and histamine in NCTC 2544 keratinocytes. *Clin. Exp. Pharmacol. Physiol* 37, 679-683.
- Chen,C.L. et al., (2007). Signal transducer and activator of transcription 3 is involved in cell growth and survival of human rhabdomyosarcoma and osteosarcoma cells. *BMC. Cancer* 7, 111.
- Clapham,K.M. et al., (2011). DNA-dependent protein kinase (DNA-PK) inhibitors: structure-activity relationships for O-alkoxyphenylchromen-4-one probes of the ATP-binding domain. *Bioorg. Med. Chem. Lett.* 21, 966-970.
- Darnell,J.E., Jr. (1997). STATs and gene regulation. *Science* 277, 1630-1635.

- De,L.L. et al., (2009). Pharmacophore-based discovery of small-molecule inhibitors of protein-protein interactions between HIV-1 integrase and cellular cofactor LEDGF/p75. *ChemMedChem*. 4, 1311-1316.
- Delaney,C.A. et al., (2000). Potentiation of temozolomide and topotecan growth inhibition and cytotoxicity by novel poly(adenosine diphosphoribose) polymerase inhibitors in a panel of human tumor cell lines. *Clin. Cancer Res.* 6, 2860-2867.
- Donawho,C.K. et al., (2007). ABT-888, an orally active poly(ADP-ribose) polymerase inhibitor that potentiates DNA-damaging agents in preclinical tumor models. *Clin. Cancer Res.* 13, 2728-2737.
- Eder,J.P. et al., (2009). Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clin. Cancer Res.* 15, 2207-2214.
- Einhorn,L.H. (2002). Curing metastatic testicular cancer. *Proceedings of the National Academy of Sciences of the United States of America* 99, 4592-4595.
- Fletcher,S. et al., (2009). Disruption of transcriptionally active Stat3 dimers with non-phosphorylated, salicylic acid-based small molecules: potent in vitro and tumor cell activities. *Chembiochem*. 10, 1959-1964.
- Glanzer,J.G., Liu,S., and Oakley,G.G. (2011). Small molecule inhibitor of the RPA70 N-terminal protein interaction domain discovered using in silico and in vitro methods. *Bioorg. Med. Chem.* 19, 2589-2595.
- Golding,S.E. et al., (2009). Improved ATM kinase inhibitor KU-60019 radiosensitizes glioma cells, compromises insulin, AKT and ERK prosurvival signaling, and inhibits migration and invasion. *Mol. Cancer Ther.* 8, 2894-2902.
- Griffin,R.J. et al., (2005). Selective benzopyranone and pyrimido[2,1-a]isoquinolin-4-one inhibitors of DNA-dependent protein kinase: synthesis, structure-activity studies, and radiosensitization of a human tumor cell line in vitro. *J. Med. Chem.* 48, 569-585.
- Gu,J. et al., (2008). Blockage of the STAT3 signaling pathway with a decoy oligonucleotide suppresses growth of human malignant glioma cells. *J. Neurooncol.* 89, 9-17.
- Hao,W. et al., (2008). Discovery of the catechol structural moiety as a Stat3 SH2 domain inhibitor by virtual screening. *Bioorg. Med. Chem. Lett.* 18, 4988-4992.
- Hardcastle,I.R. et al., (2005). Discovery of potent chromen-4-one inhibitors of the DNA-dependent protein kinase (DNA-PK) using a small-molecule library approach. *J. Med. Chem.* 48, 7829-7846.
- Hedvat,M. et al., (2009). The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell* 16, 487-497.
- Helleday,T. (2010). Homologous recombination in cancer development, treatment and development of drug resistance. *Carcinogenesis*.
- Hellsten,R. et al., (2008). Galiellalactone is a novel therapeutic candidate against hormone-refractory prostate cancer expressing activated Stat3. *PROSTATE* 68, 269-280.
- Hickson,I. et al., (2004). Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res.* 64, 9152-9159.
- Hollick,J.J. et al., (2003). 2,6-disubstituted pyran-4-one and thiopyran-4-one inhibitors of DNA-Dependent protein kinase (DNA-PK). *Bioorg. Med. Chem. Lett.* 13, 3083-3086.
- Hollick,J.J. et al., (2007). Pyranone, thiopyranone, and pyridone inhibitors of phosphatidylinositol 3-kinase related kinases. Structure-activity relationships for

- DNA-dependent protein kinase inhibition, and identification of the first potent and selective inhibitor of the ataxia telangiectasia mutated kinase. *J. Med. Chem.* *50*, 1958-1972.
- Hsieh,F.C., Cheng,G., and Lin,J. (2005). Evaluation of potential Stat3-regulated genes in human breast cancer. *Biochem. Biophys. Res. Commun.* *335*, 292-299.
- Huang,J.W. et al., (2008). Fragment-based design of small molecule X-linked inhibitor of apoptosis protein inhibitors. *J. Med. Chem.* *51*, 7111-7118.
- Ihle,J.N. (1996). STATs: signal transducers and activators of transcription. *Cell* *84*, 331-334.
- Iwamaru,A. et al., (2007). A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both in vitro and in vivo. *ONC* *26*, 2435-2444.
- Izzard,R.A., Jackson,S.P., and Smith,G.C. (1999). Competitive and noncompetitive inhibition of the DNA-dependent protein kinase. *Cancer Res.* *59*, 2581-2586.
- Karin,M., Yamamoto,Y., and Wang,Q.M. (2004). The IKK NF-kappa B system: a treasure trove for drug development. *Nat. Rev. Drug Discov.* *3*, 17-26.
- Karlberg,T. et al., (2010). Crystal structure of the catalytic domain of human PARP2 in complex with PARP inhibitor ABT-888. *Biochemistry* *49*, 1056-1058.
- Koberle,B. et al., (1999). Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. *Current Biology* *9*, 273-276.
- Koberle,B., Roginskaya,V., and Wood,R.D. (2006). XPA protein as a limiting factor for nucleotide excision repair and UV sensitivity in human cells. *DNA Repair (Amst)* *5*, 641-648.
- Kumar,S. et al., (2010). Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. *Nat. Rev. Drug Discov.* *9*, 843-856.
- Kunigal,S. et al., (2009). Stat3-siRNA induces Fas-mediated apoptosis in vitro and in vivo in breast cancer. *Int. J. Oncol.* *34*, 1209-1220.
- Langelier,M.F. et al., (2011). Crystal Structures of Poly(ADP-ribose) Polymerase-1 (PARP-1) Zinc Fingers Bound to DNA: Structural and functional insights into DNA-dependent PARP-1 activity. *J. Biol. Chem.* *286*, 10690-10701.
- Leahy,J.J. et al., (2004). Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. *Bioorg. Med. Chem. Lett.* *14*, 6083-6087.
- Lee,Y.K. et al., (2006). Flavopiridol disrupts STAT3/DNA interactions, attenuates STAT3-directed transcription, and combines with the Jak kinase inhibitor AG490 to achieve cytotoxic synergy. *Mol. Cancer Ther.* *5*, 138-148.
- Li,Y. and Yang,D.Q. (2010). The ATM inhibitor KU-55933 suppresses cell proliferation and induces apoptosis by blocking Akt in cancer cells with overactivated Akt. *Mol. Cancer Ther.* *9*, 113-125.
- Lin,L. et al., (2009). The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. *ONC* *28*, 961-972.
- Ling,X. and Arlinghaus,R.B. (2005). Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. *Cancer Res.* *65*, 2532-2536.
- Lu,Z. et al., (2010). Pristimerin induces apoptosis in imatinib-resistant chronic myelogenous leukemia cells harboring T315I mutation by blocking NF-kappaB signaling and depleting Bcr-Abl. *Mol. Cancer* *9*, 112.

- Matsumoto,N. et al., (2000). Synthesis of NF-kappaB activation inhibitors derived from epoxyquinomicin C. *Bioorg. Med. Chem. Lett.* 10, 865-869.
- Miller,S.C. et al., (2010). Identification of known drugs that act as inhibitors of NF-kappaB signaling and their mechanism of action. *Biochem. Pharmacol.* 79, 1272-1280.
- Moellering,R.E. et al., (2009). Direct inhibition of the NOTCH transcription factor complex. *Nature* 462, 182-188.
- Nagel-Wolfrum,K. et al., (2004). The interaction of specific peptide aptamers with the DNA binding domain and the dimerization domain of the transcription factor Stat3 inhibits transactivation and induces apoptosis in tumor cells. *Mol. Cancer Res.* 2, 170-182.
- Neher,T.M. et al., (2010). Identification of novel small molecule inhibitors of the XPA protein using in silico based screening. *ACS Chem. Biol.*
- Ng,P.Y. et al., (2007). Synthesis of diverse lactam carboxamides leading to the discovery of a new transcription-factor inhibitor. *Angew. Chem. Int. Ed Engl.* 46, 5352-5355.
- O'Neil,J. et al., (2007). FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J. Exp. Med.* 204, 1813-1824.
- Pawelczak,K.S., Bennett,S.M., and Turchi,J.J. (2010). Coordination of DNA-PK Activation and Nuclease Processing of DNA Termini in NHEJ. *Antioxid. Redox. Signal.*
- Penning,T.D. et al., (2009). Discovery of the Poly(ADP-ribose) polymerase (PARP) inhibitor 2-[(R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide (ABT-888) for the treatment of cancer. *J. Med. Chem.* 52, 514-523.
- Qi,W. et al., (2009). MP470, a novel receptor tyrosine kinase inhibitor, in combination with Erlotinib inhibits the HER family/PI3K/Akt pathway and tumor growth in prostate cancer. *BMC. Cancer* 9, 142.
- Rainey,M.D. et al., (2008). Transient inhibition of ATM kinase is sufficient to enhance cellular sensitivity to ionizing radiation. *Cancer Res.* 68, 7466-7474.
- Ramakrishnan,V. et al., (2010). TG101209, a novel JAK2 inhibitor, has significant in vitro activity in multiple myeloma and displays preferential cytotoxicity for CD45+ myeloma cells. *Am. J. Hematol.* 85, 675-686.
- Ren,Z., Cabell,L.A., Schaefer,T.S., and McMurray,J.S. (2003). Identification of a high-affinity phosphopeptide inhibitor of Stat3. *Bioorg. Med. Chem. Lett.* 13, 633-636.
- Sage-El,M.M. et al., (2008). 8-Biarylchromen-4-one inhibitors of the DNA-dependent protein kinase (DNA-PK). *Bioorg. Med. Chem. Lett.* 18, 4885-4890.
- Saha,M.N. et al., (2010). MDM2 antagonist nutlin plus proteasome inhibitor velcade combination displays a synergistic anti-myeloma activity. *Cancer Biol. Ther.* 9.
- Schon,M. et al., (2008). KINK-1, a novel small-molecule inhibitor of IKKbeta, and the susceptibility of melanoma cells to antitumoral treatment. *J. Natl. Cancer Inst.* 100, 862-875.
- Schust,J. (2006). Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem. Biol.* 13, 1235-1242.
- Schwanbeck,R. et al., (2010). The Notch signaling pathway: Molecular basis of cell context dependency. *Eur. J. Cell Biol.*
- Shuck,S.C., Short,E.A., and Turchi,J.J. (2008). Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. *Cell Res.* 18, 64-72.

- Shuck,S.C. and Turchi,J.J. (2010). Targeted inhibition of Replication Protein A reveals cytotoxic activity, synergy with chemotherapeutic DNA-damaging agents, and insight into cellular function. *Cancer Res.* 70, 3189-3198.
- Siddiquee,K.A. et al., (2007a). Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc. Natl. Acad. Sci. U. S. A* 104, 7391-7396.
- Siddiquee,K.A. et al., (2007b). An oxazole-based small-molecule Stat3 inhibitor modulates Stat3 stability and processing and induces antitumor cell effects. *ACS Chem. Biol.* 2, 787-798.
- Sigurdsson,S. et al., (2001). Basis for avid homologous DNA strand exchange by human Rad51 and RPA. *J. Biol. Chem.* 276, 8798-8806.
- Simon,G.R. (2008). Individualizing chemotherapy for non-small cell lung cancer (NSCLC) in the adjuvant and advanced setting: current status and future directions. *Curr. Treat. Options. Oncol.* 9, 300-312.
- Song,H. et al., (2005). A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A* 102, 4700-4705.
- Stauffer,M.E. and Chazin,W.J. (2004). Physical interaction between replication protein A and Rad51 promotes exchange on single-stranded DNA. *J. Biol. Chem.* 279, 25638-25645.
- Sun,X. et al., (2008). Growth inhibition of human hepatocellular carcinoma cells by blocking STAT3 activation with decoy-ODN. *Cancer Lett.* 262, 201-213.
- Take,Y. et al., (1995). OK-1035, a selective inhibitor of DNA-dependent protein kinase. *Biochem. Biophys. Res. Commun.* 215, 41-47.
- Thomas,H.D. et al., (2007). Preclinical selection of a novel poly(ADP-ribose) polymerase inhibitor for clinical trial. *Mol. Cancer Ther.* 6, 945-956.
- Turkson,J., et al., (2001). Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. *J. Biol. Chem.* 276, 45443-45455.
- Turkson,J. et al., (2004a). Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. *Mol. Cancer Ther.* 3, 261-269.
- Turkson,J. et al., (2004b). Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent antitumor activity. *Mol. Cancer Ther.* 3, 1533-1542.
- Turkson,J. et al., (2005). A novel platinum compound inhibits constitutive Stat3 signaling and induces cell cycle arrest and apoptosis of malignant cells. *J. Biol. Chem.* 280, 32979-32988.
- Wang,M. et al., (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res.* 34, 6170-6182.
- Weber,L. (2010). Patented inhibitors of p53-Mdm2 interaction (2. *Expert. Opin. Ther. Pat* 20, 179-191.
- Weidler,M. et al., (2000). Inhibition of interleukin-6 signaling by galiellalactone. *FEBS Lett.* 484, 1-6.
- Welsh,C. et al., (2004). Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. *Int. J. Cancer* 110, 352-361.
- Welsh,J.W. et al., (2009). The c-Met receptor tyrosine kinase inhibitor MP470 radiosensitizes glioblastoma cells. *Radiat. Oncol.* 4, 69.

- Weng,A.P. et al., (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306, 269-271.
- Wold,M.S. (1997). Replication protein A: a heterotrimeric, single-stranded DNA- binding protein required for eukaryotic DNA metabolism. [Review] [190 refs]. *Annual Review of Biochemistry* 66, 61-92.
- Xi,S., Gooding,W.E., and Grandis,J.R. (2005). In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: implications for cancer therapy. *ONC* 24, 970-979.
- Yamamoto,M. et al., (2008). Inactivation of NF-kappaB components by covalent binding of (-)-dehydroxymethylepoxyquinomicin to specific cysteine residues. *J. Med. Chem.* 51, 5780-5788.
- Yang,R.Y. et al., (2010). Targeting the dimerization of epidermal growth factor receptors with small-molecule inhibitors. *Chem. Biol. Drug Des* 76, 1-9.
- Yue,P. and Turkson,J. (2009). Targeting STAT3 in cancer: how successful are we? *Expert. Opin. Investig. Drugs* 18, 45-56.
- Zhang,X. et al., (2010). Role of STAT3 decoy oligodeoxynucleotides on cell invasion and chemosensitivity in human epithelial ovarian cancer cells. *Cancer Genet. Cytogenet.* 197, 46-53.
- Zhang,X. et al., (2007). Therapeutic effects of STAT3 decoy oligodeoxynucleotide on human lung cancer in xenograft mice. *BMC. Cancer* 7, 149.
- Zhao,Y. et al., (2006). Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. *Cancer Res.* 66, 5354-5362.

Pharmacogenomic Approach of Telomerase in Cancer: Importance of End Zone Variability

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1. Introduction

Sixty-five years ago, the term *telomere* was introduced by Muller to describe the terminal elements of linear chromosomes (McClintock 1941). In 1985, Greider and Blackburn discovered the telomere synthesizing enzyme telomerase in Tetrahymena (Greider and Blackburn 1985). Three years after, the human telomeric DNA repeat sequence, TTAGGG, was identified (Moyzis, Buckingham et al. 1988). Since then, it has become apparent that several aspects of telomere biology are relevant to human cancer, offering potential opportunities for clinical intervention. In the work by Blackburn, Greider and Szostak, that was awarded the 2009 Nobel Prize in Physiology and Medicine (Zakian 2009), the structure of the chromosome ends was discovered to be specialized nucleoprotein structures that comprise the “end zone” of chromosomes, called telomeres.

Several recent studies have highlighted the remarkable importance of telomeres in clinical medicine. These advances provide an opportunity to revisit some of the concepts and data that provide a link between telomeres and the diverse pathology that is now linked to telomere dysfunction. Here, we review the telomere and telomerase biology and the pharmacogenomic implications in human cancer, considering the importance of telomerase in tumor development.

2. Telomeres and telomerase biology

Telomeres are terminal protein-DNA complexes forming capping structures that function to stabilize chromosomal ends and prevent them from being recognized by the cell as DNA double strand breaks. Functional telomeres require sufficient numbers of telomeric DNA repeats, as well as the correct assembly of telomere associated protein complexes (Blasco and Hahn 2003).

Telomeres consist of tandem repeats of a DNA sequence (TTAGGG in all vertebrates) bound by a six-protein complex, known as shelterin. Shelterin encompasses the Pot1-TPP1 heterodimer, the telomere-binding proteins TRF1 and TRF2, and the interacting factors Rap1 and Tin2 (de Lange 2005). Telomeric chromatin is also enriched in epigenetic marks that are characteristic of constitutive heterochromatin, such as histone tri-methylation and DNA

hypermethylation, which act as negative regulators of telomere length and telomere recombination (Blasco 2007).

Telomere shortening below a certain threshold length or alterations in the functionality of the telomere-binding proteins can result in loss of telomeric protection, leading to end-to-end chromosome fusions, cell cycle arrest and apoptosis. Telomeres also perform other functions, which include the transcriptional silencing of genes located close to the telomeres (subtelomeric silencing), as well as ensuring correct chromosome segregation during mitosis. Shortening of telomeres is associated with each round of cell division because of the inability of conventional DNA polymerases to replicate the ends of linear chromosomes, the so-called 'end replication problem'. Telomerase is a cellular enzyme capable of compensating this progressive telomere attrition through *de novo* addition of TTAGGG repeats to the chromosome ends (Greider and Blackburn 1985). Telomerase encompasses a catalytic subunit with reverse transcriptase activity (Tert) and an RNA component (Terc) that acts as a template for DNA synthesis (Blasco 2007).

Telomeres also solve the end protection problem. The DNA regions of the end zone are not blunt ended. Instead, they terminate in a long single-stranded run of the G-rich sequence on the 3' strand, called the 3' G-rich overhang. This long single-stranded tail of DNA is postulated to fold back onto itself to invade the duplex portion of telomeres, forming the t-loop. The t-loop effectively sequesters the free ends of chromosomes, protecting them from being sensed as double strand breaks (Griffith, Comeau et al. 1999).

High telomerase expression is a feature of pluripotent stem cells and early stages of embryonic development, although telomerase activity is also present in adult stem cell compartments (Blasco 2005). Telomerase activity in adult tissues, however, is not sufficient to prevent telomere shortening associated with ageing.

The specificity of shelterin for telomeric DNA is through direct recognition of the TTAGGG sequence by three of its components. In particular, TRF1 and TRF2 bind to the double stranded region of telomeric DNA, while Pot1 binds to the TTAGGG repeats of the G-overhang. TRF1 and TRF2 recruit the other four components of shelterin: Tin2 (a TRF1 and TRF2 interacting factor), Rap1, TPP1 and Pot1. These last two proteins form a heterodimer. Shelterin can form a stable complex in the absence of telomeric DNA (de Lange 2005). Mutations in the different components of telomerase (Tert, Terc and Dkc1), as well as in some shelterins (Tin2), have been linked to rare human genetic diseases, such as dyskeratosis congenita, aplastic anaemia and idiopathic pulmonary fibrosis. These diseases are associated with the presence of short/dysfunctional telomeres and they all exhibit a characteristic failure in the regenerative capacity of tissues and severe skin hyperpigmentation (Mitchell, Wood et al. 1999; Armanios, Chen et al. 2007).

Adult stem cells reside at specific compartments within tissues, which are enriched in cells with the longest telomeres. In young or adult organisms with sufficient telomere reserve, adult stem cells efficiently repopulate tissues and repair lesions as needed. In old organisms, however, stem cell telomeres may be too short, and this could impair the mobilization of stem cells and the ability to repair tissues efficiently (Flores, Canela et al. 2008).

When telomeres have shortened down to a critical length, they are recognized as DNA damage, activating a p53-mediated DNA damage signaling response that prevents the mobilization of the stem cells out of their niches. Decreased stem cell mobilization reduces the probability of accumulating abnormal cells in tissues, thus providing a mechanism for cancer protection. However, the ultimate consequence of impaired mobilization of the stem cells will be organ failure owing to tissue degeneration. By using mouse models over-

expressing telomerase, some investigators showed that elevated TERT expression increases stem cell mobilization. Under these conditions of higher mobilization, the fitness of the tissues would be maintained for longer times, therefore increasing the lifespan. The probabilities of initiating a tumor, however, are also higher, especially if telomerase reactivation occurs in a context of mutations in tumor suppressor genes (Serrano and Blasco 2007).

3. Telomere shortening: Consequences

Normally, cells respond to shortened or uncapped telomeres either by entering an irreversible cell cycle arrest, termed replicative senescence, or by undergoing apoptosis. Such responses involve both the Rb and p53 tumor suppressor pathways and likely evolved to prevent replication of mutation-prone cells harboring unstable chromosomes. If these checkpoints fail, chromosomal instability may occur and with it the potential for developing oncogenic mutations. If left unchecked, however, such instability will likely become lethal to the cell, thereby presenting an additional barrier to cell growth. Although some level of genetic instability is thought to be required for tumor initiation and progression, the vast majority of human cancers stabilizes their telomeres either by activating the enzyme telomerase or, in a minority of cases, by an alternative pathway, termed alternative lengthening of telomeres, or ALT, that seems to involve recombination (Reddel 2003).

It would be advantageous in long-lived organisms for cells to be limited in the maximal number of cell divisions permitted, in order to ensure appropriate growth and DNA repair early in life, but not so many divisions that could lead to the early onset of diseases, such as cancer (Wright and Shay 2002). Therefore, selection for enhanced DNA repair and maintenance would be highly desirable during reproductive years, but this could be lost in later life (Kirkwood and Austad 2000). In our modern 'protected environment', humans are living on average twice as long and are dying of heart disease, cancer and neurodegenerative disorders. Thus, normal cells in the context of genotoxic injuries have innate and probably highly conserved defense mechanisms that initiate programs leading to growth arrest or apoptosis, perhaps in part as a mechanism to prevent cancer.

3.1 Short telomeres activate DNA damage signals leading to cell cycle arrest in normal human cells

Normal human somatic cells have a limited lifespan *in vitro*. This was first demonstrated in human fibroblasts by Hayflick and Moorhead in 1961 (Hayflick and Moorhead 1961). Since then, it has been demonstrated that cultured normal human fibroblasts go through finite numbers of population doublings. Toward the end of a cell's lifespan, cell proliferation slows down and finally stops and the cell enters a state of irreversible growth arrest. The timing of growth arrest is determined by the number of population doublings the cells have undergone, not by the calendar time they have stayed in culture. In addition to fibroblasts, other somatic cells show replicative senescence, including epithelial cells, endothelial cells, lymphocytes, smooth muscle cells, and astrocytes, which serves as an intrinsic mechanism to prevent normal somatic cells from replicating indefinitely (Bierman 1978; Evans, Wyllie et al. 2003). Senescent human cells have increased p53 activity with the involvement of several DNA double strand break repair and checkpoint factors, such as ATM (Atadja, Wong et al. 1995). These multiple DNA damage response factors are assembled at the short telomeres in senescent cells, indicating that dysfunctional short telomeres trigger the response (d'Adda di

Fagagna, Reaper et al. 2003; Zou, Sfeir et al. 2004). The sustained DNA damage response, signaling through p53, can induce both G1 and G2 phase arrest. Numerous studies have also demonstrated that human fibroblasts or epithelial cells undergoing natural replicative senescence have elevated protein levels of hypophosphorylated Rb, p16INK4a, as well as p21CIP1, or decreased hyper-phosphorylated Rb, compared with early and proliferating cells (Atadja, Wong et al. 1995; Beausejour, Krtolica et al. 2003). Telomere-mediated senescence is therefore induced by activation of the multiple DNA damage responses, which then leads to cell cycle arrest in normal human cells.

Several lines of evidence have shown that replicative senescence also occurs *in vivo* and is thought to be associated with cellular aging. An inverse correlation between donor age and the number of population doublings at which human cells senesce has been demonstrated (Martin, Sprague et al. 1970). Moreover, comparisons between different species showed that cells from organisms with longer lifespan *in vivo* achieve more population doublings *in vitro* (Rohme 1981). Furthermore, cells from humans with premature aging syndromes have shorter lifespan in culture than those from age-matched controls (Smith and Pereira-Smith 1996). These data suggest that there is a genetic basis that controls the cells' replicative lifespan. However, although telomere-associated senescence is apparently an intrinsic barrier to cellular immortalization, which represents an early first step in the multi-stage process of cancer development, accumulation of senescent cells during aging *in vivo* seems to create a microenvironment that predisposes to cancer (Campisi 2005). Telomere shortening or dysfunction, therefore, presents a close association with cancer.

3.2 Telomere dysfunction leads to genomic instability in checkpoint defective cells

Broken chromosome ends tend to fuse with their sister chromatids or other broken chromosomes, leading to formation of anaphase bridges, cycles of chromosome breaks and further fusions during subsequent cell divisions (McClintock 1941). Telomere dysfunction initiates chromosomal instability through such breakage-fusion bridge (BFB) cycles. The p53 and Rb/p16INK4a pathways are crucial for DNA damage responses, cell cycle regulation, as well as apoptosis, which are indispensable for maintaining genomic stability. Most cancers have alterations in either or both of these pathways. Inactivation of the p53 and p16INK4a/Rb pathways can be accomplished *in vitro* by expression of viral oncogenes, such as HPV (human papillomavirus) E6 and E7 or adenovirus E1B and E1A (Moran 1993; Moran 1993) to stimulate the sustained inactivation of p53 and Rb genes, observed in cancers. Such alterations extend the lifespan and allow the cells to override cell cycle checkpoints and continue to proliferate. However, as telomeres continue to shorten with further cell divisions, the chromosome ends are no longer protected and become unstable. At this stage, the cells enter a mortality barrier, termed crisis (Wright and Shay 1992), which is characterized by wide-spread cell death and extensive chromosomal instability (Ducray, Pommier et al. 1999). However, a low percentage of cells may survive the crisis period by a mutation or an epigenetic event that activates a telomere maintenance mechanism. This may involve activation of telomerase, which elongates telomeres by synthesizing telomeric DNA and maintaining pre-existing telomeres, or an alternative telomere lengthening (ALT) mechanism, which involves copying of DNA sequences from telomere to telomere by means of homologous recombination. The resulting telomere preservation allows the cells to continue their long-term proliferation to become immortalized (Figure 1) (Dunham, Neumann et al. 2000).

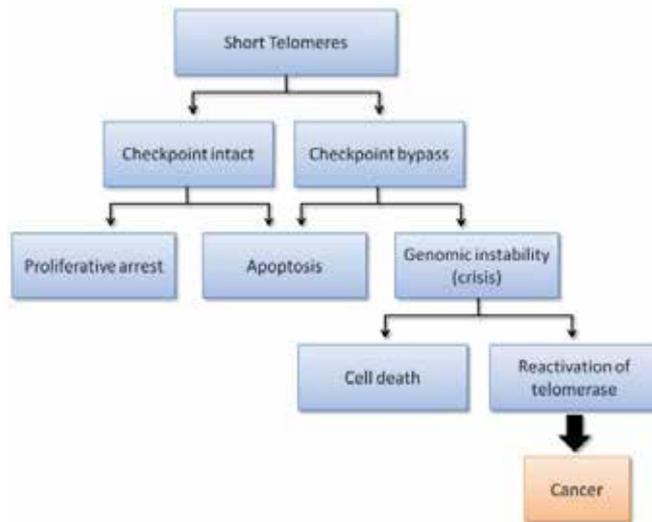


Fig. 1. Telomeres shorten with cell proliferation when not balanced by telomere synthesis. In healthy somatic cells, critically short telomeres activate a checkpoint that induces either apoptosis or the proliferative arrest of replicative senescence. In the absence of checkpoint function, telomeres may become substrates for aberrant DNA repair. Occasionally, spontaneous activation of telomerase during the crisis phase of genomic instability stabilizes and allows maintenance of the rearranged genome, conferring indefinite renewal capacity.

Critically short telomeres are hotspots for erroneous recombination. Whereas very few chromosome end-to-end fusions are observed in senescent normal human cells (Benn 1976; Zou, Sfeir et al. 2004), the frequencies of chromosome end-to-end fusions, reach a peak during crisis and decrease after this period due to telomere preservation (Ducray, Pommier et al. 1999). This is because the two centromeres are pulled in opposite directions during mitotic anaphase, forming a bridge between the daughter cells. The bridge can cause cell death independently of p53 and Rb genes, partly because the bridge compromises the integrity of cellular and nuclear membranes. The broken ends trigger DNA recombination by rejoining to other broken or unprotected chromosome ends. This can produce additional aberrations, such as structurally stable translocations and unstable dicentrics, which can undergo another round of BFB. Repeated cycles of sister-chromatid fusion-bridge breakage may, therefore, generate multiple copies of the same gene on the same chromosome in the same cell, while causing the loss of the genes in its sister cells after several rounds of cell divisions (Gisselsson, Jonson et al. 2001). In summary, regardless of how telomeres become disrupted, when telomeres become dysfunctional and can no longer protect the chromosome ends, they elicit a DNA damage response to activate the p53 and p16INK4a/Rb pathways, which prompt the cell to undergo irreversible cell cycle arrest (if both p53 and pRb checkpoints are intact) or p53-mediated apoptosis (if only the p53 checkpoint is intact) (d'Adda di Fagagna, Reaper et al. 2003). In cells defective in both p53 and p16INK4a/Rb pathways, excessive shortening of telomeres, coupled with the freedom to continue proliferation, promote aberrant fusions of unprotected chromosome ends and trigger massive chromosomal instability, increasing the risk of malignant transformation (Feldser, Hackett et al. 2003). Thus, telomere shortening can be both a barrier and a facilitator for cancer development, depending on the integrity of checkpoint response.

4. Role of telomere-mediated genomic instability in carcinogenesis

Carcinogenesis is a multistep process, characterized by a stepwise accumulation of genetic and molecular abnormalities. These events generally follow exposure to carcinogens and result in the selection of clonal cells with uncontrolled growth capacities (Hanahan D 2000). Thus, cancer develops through a series of stepwise events, from preinvasive histological changes to invasive disease. The earliest events are mutations, deletions, or polysomy at the genomic level, but these do not systematically lead to changes in cell morphology or tissue structure. Current knowledge indicates that cancer progression from premalignancy to malignancy is slow and multiple mutations are required to reach a full metastatic potential (Schedin and Elias 2004).

Tumor cells acquire the so-called hallmarks of cancer during this carcinogenic selection process (Hanahan D 2000). Cell immortality is one of the most prevalent of these major acquired features. Immortality involves the stabilization of telomere lengths, which is achieved by telomerase activation in about 80% of cases of human tumors. However, the specific changes in telomere length, telomeric proteins and telomerase expression that occur during the multistep carcinogenic process remain undetermined and thus represent a field of active research.

One of the core concepts in cancer research is that genomic instability helps drive development of human cancer (Maser and DePinho 2002). Rapid evolution of genomic alterations in genetically unstable cells makes them advantageous in natural selection by acquiring new features. Of the two categories of genomic instability, microsatellite instability exists only in a small subset of solid tumors, whereas chromosomal instability is present in most cancers. Chromosomal instability includes numerical instability reflected by alterations in chromosome numbers, and structural instability, which is characterized by continuous generation of new structural chromosome aberrations. Regarding the role of chromosome aberrations in cancer development, it is well recognized that chromosome aberrations have oncogenic potential. First, chromosome translocations can result in the formation of fusion genes or deregulation of gene transcription at or near the translocation points, as demonstrated in most leukemia and many soft-tissue tumors. Second, gains or losses of chromosome elements or whole chromosomes can lead to large scale genomic imbalances or alterations in gene dosage in human cancers (Albertson, Collins et al. 2003). These abnormalities persist along with continued acquisition of additional abnormalities with tumor progression towards late stage malignancies. It is known that chromosomal instability is an important mechanism leading to genomic rearrangement and imbalances that provide a platform for continuous selection of aberrant cells for cellular immortalization and cancer development. The mouse model with the depletion of telomerase RNA component (mTerc) shows increased incidence of spontaneous malignancies in late generation animals (Rudolph, Chang et al. 1999; Maser and DePinho 2002).

Extensive studies have shown that telomeres in normal human somatic cells shorten with cell divisions *in vitro* as well as *in vivo*. In cultured human fibroblasts, this progresses at a rate of 50-200 bp per population doubling (Harley, Futcher et al. 1990; Allsopp, Vaziri et al. 1992). *In vivo* studies have also shown that the average telomere lengths in normal somatic cells shorten at an estimated rate of 15-40 bp per year and that telomere erosion declines with age (Hastie, Dempster et al. 1990). One major mechanism leading to telomere shortening is associated with the end-replication problem intrinsic to linear chromosomes. To date, numerous experiments have demonstrated that telomeric DNA is indeed progressively lost with cell divisions in most human somatic cells that lack telomerase

activity. In addition, some studies also suggest that oxidative stress contributes to telomere shortening. Under conditions of additional oxidative stress induced by hydrogen peroxide treatment, human cells show preferential accumulation of single-strand breaks within the telomeres. The effects of oxidative stress on telomere erosion has important significance in the study of telomere dynamics in aging and cancer, since numerous oxidants are produced *in vivo* due to normal metabolism and extracellular stresses and oxidative stress increases with the aging process *in vivo* (Saretzki and Von Zglinicki 2002; von Zglinicki 2002).

Proof that telomeres shortening and cellular aging are causally related was demonstrated (Bodnar, Ouellette et al. 1998). The introduction of telomerase into normal telomerase silent cells was sufficient to bypass senescence, activate telomerase activity and lead to cell immortalization. It was further shown that ectopic expression of telomerase (TERT) in pre-senescent cells or in cells between senescence and crisis could be immortalized with ectopic introduction of TERT, demonstrating that telomeres are mechanistically important in both senescence and crisis. In the absence of intact critical checkpoint pathways, genomic instability occurs when telomeres are short, leading to end-to-end fusions, anaphase bridges, the development of aneuploidy and eventually to telomerase reactivation. One possibility is that the re-expression or upregulation of telomerase in cancer reduces the ongoing chromosomal instability that occurs in cells in crisis to a level compatible with both viability and sufficient instability to generate mutational evolution of the malignancy. In summary, telomere shortening may be a common underlying cause of chromosomal rearrangements in cancer (Bodnar, Ouellette et al. 1998). In a recent study, DePinho and colleagues determined whether entrenched multi-system degeneration in adult mice with severe telomere dysfunction can be halted or possibly reversed by reactivation of endogenous telomerase activity. These authors engineered a knock-in allele encoding a 4-hydroxytamoxifen (4-OHT)-inducible telomerase reverse transcriptase-oestrogen receptor (TERT-ER) under transcriptional control of the endogenous TERT promoter. Homozygous TERT-ER mice presented short dysfunctional telomeres and sustained increased DNA damage signalling and classical degenerative phenotypes upon successive generational matings and advancing age. Telomerase reactivation in such late generation TERT-ER mice extended telomeres, reduced DNA damage signalling and associated cellular checkpoint responses, allowing resumption of proliferation in quiescent cultures and eliminating degenerative phenotypes across multiple organs including testes, spleens and intestines. The authors demonstrated that telomere damage acts as a driver of age-associated organ decline and disease risk and that the marked reversal of systemic degenerative phenotypes in adult mice supports the development of regenerative strategies designed to restore telomere integrity (Jaskelioff, Muller et al. 2011).

Since cancer cells have to bypass senescence and crisis, it is consistent to assume that the cancer cell that first became immortal by upregulating telomerase would have had short telomeres (Figure 2). For example, in most cases of preneoplasia, it has been shown that cells have very short telomeres. In prostate cancer for example, telomere shortening is detected in low grade prostatic intraepithelial neoplasia (PIN) lesions (Koenenman, Pan et al. 1998; Meeker, Hicks et al. 2002). This indicates that the tumor initiating cells are likely to originate from a subset of transient amplifying cells which may have critically shortened telomeres.

Telomere length abnormalities are nearly universal in preinvasive stages of human epithelial carcinogenesis. Indeed, telomere shortening occurs in most cases of early stage bladder, cervix, colon, oesophageal and oral cavity cancer. Similar results were found for prostate cancer; the majority of high-grade PIN lesions (Prostatic intraepithelial neoplasia)

examined had much shorter telomeres than adjacent, apparently normal epithelial cells. This was also observed in fully invasive prostate carcinomas (Meeker, Hicks et al. 2002; Meeker, Hicks et al. 2004). Markedly or moderately shortened telomeres have also been found in lesions of ductal carcinoma *in situ* (Meeker and Argani 2004). Unlike normal breast epithelium, a moderate telomere shortening was observed in benign secretory cells in about half of histologically normal terminal duct lobular units. These findings clearly support a role for telomere shortening in pre-invasive stages, as well as in invasive cancer. Overall, these observations suggest that telomere length abnormality is one of the earliest and most frequently acquired genetic alterations involved in the multistep process of malignant transformation.

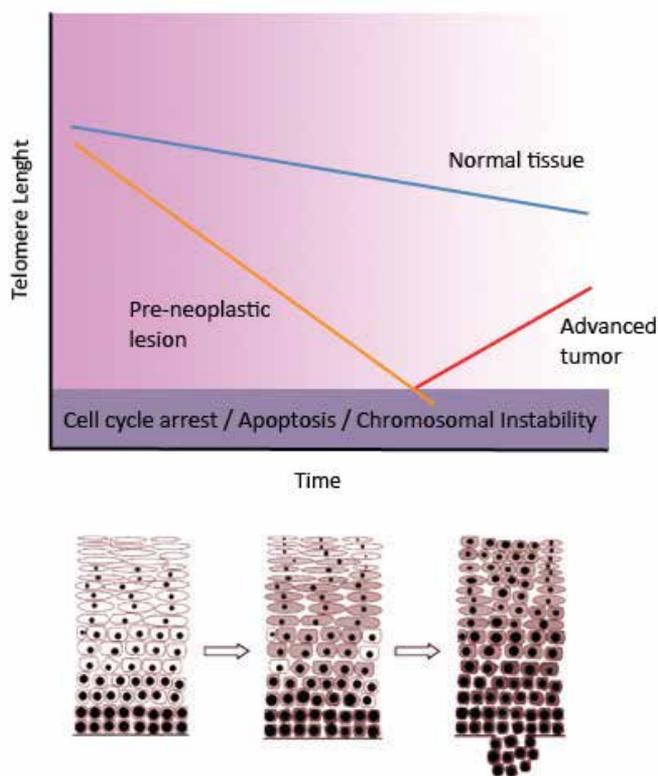


Fig. 2. Schematic representation of telomere length associated with multistep carcinogenesis.

As mentioned above, telomere shortening appears to be a signal for replication arrest in many cells. However, in some cells, rather than inducing cell cycle arrest, the substantial shortening of telomeres results in cycles of end-to-end fusions and BFB (Chin, de Solorzano et al. 2004; Lantuejoul, Soria et al. 2005). In most cells, damage-surveillance mechanisms detect genomic instability and induce cell death. Occasionally, however, one or a few cells escape this protective mechanism, leading to accumulation of multiple genomic and epigenomic aberrations and thus acquisition of additional cancer hallmark features (Chin, de Solorzano et al. 2004).

5. Heterogeneity in telomere lengths - impact on cancer development and prognosis

It is known that telomere length varies considerably among individuals. To date, the major conclusions on human telomere lengths include that there are large differences in telomere lengths between the arms of the same chromosomes, as well as between the same arms of homologous chromosomes in the cells of the same individual; different individuals have different profiles of telomere length heterogeneity, which seem to be largely inherited; and different tissues of the same individual have similar telomere length heterogeneity (Lansdorp, Verwoerd et al. 1996; Graakjaer, Bischoff et al. 2003; Nordfjall, Larefalk et al. 2005).

There is growing evidence that TL (Telomere Length) carries information that may be of clinical importance for cancer patients. It is well established that TL is important for senescence in normal cells. Malignant cells in general have shorter telomeres than their normal counterparts and there seems to be a connection between telomere shortening, genetic aberrations and risk of transformation (Hackett and Greider 2002). The interest of investigating TL as a potential clinical biomarker in cancer has grown considerably in recent years (Ohyashiki, Sashida et al. 2002; Bisoffi, Heaphy et al. 2006).

The vast majority of studies in the field have been performed on tumor samples, but there are also investigations on blood cell TL and its possible relation to cancer risk and prognosis. When tumor DNA is studied, TL reflects the cumulative result of a variety of tumor-associated factors with impact on telomere homeostasis. TL in peripheral blood is often used as a surrogate for TL in other healthy tissues. The fact that TL in peripheral blood may carry clinical information for cancer patients has been indicated by a growing number of studies. One question to be solved, however, is whether altered blood TL can contribute to disease development, or whether the TL alterations arise as a consequence of the disease. The latter case would suggest that an altered blood TL is the result of unknown mechanisms associated with the presence of a malignant tumor in the body. Previous data indicate that blood TL may serve both as a marker for cancer risk and as an independent prognostic marker for survival. Thus, both tumor TL and TL in peripheral blood may carry information with important clinical implications.

In hematological malignancies, there are convincing data suggesting that short telomeres in malignant hematopoietic cell populations indicate progressive disease and poor survival [reviewed in (Ohyashiki, Sashida et al. 2002; Bisoffi, Heaphy et al. 2006)].

A number of studies have reported that TL in solid tumors has potential to be used as a prognostic indicator. Several different tumor types have been investigated regarding tumor TL and clinical outcome. The majority of studies have found associations between altered tumor TL, i.e. attrition or elongation, and a poor outcome. Several studies have reported associations between reduced telomere content and poorer survival in both breast and prostate carcinomas. Similarly, in sarcoma, short TL was linked to genomic instability and poor survival (Bisoffi, Heaphy et al. 2006). A number of studies indicated that telomere alterations are associated with parameters of clinical outcome in patients with lung cancer (Shirovani, Hiyama et al. 1994; Hirashima, Komiya et al. 2000; Frias, Garcia-Aranda et al. 2008). A study (Frias, Garcia-Aranda et al. 2008) indicated a significant poor clinical outcome in lung patients presenting telomere shortening, a finding that emerged as an independent prognostic marker in multivariate analysis. In other tumor types, such as prostate cancer, it has been suggested that reduced telomere content is associated with poor

clinical outcome and markers of disease progression. Thus, reduced telomere content values conferred a relative hazard of 5.02 compared with tumors with greater telomere content (Fordyce, Heaphy et al. 2005). Also, studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, with low telomere content conferring an adjusted relative hazard of 4.43 (Bisoffi, Heaphy et al. 2006; Fordyce, Heaphy et al. 2006).

In contrast, long telomeres, or a high tumor to non-tumor TL ratio, have been coupled to tumors of more advanced stages and a worse prognosis in colorectal carcinoma, Barrett carcinoma and head and neck tumors (Patel, Parekh et al. 2002; Gertler, Rosenberg et al. 2004; Garcia-Aranda, de Juan et al. 2006; Gertler, Doll et al. 2008). Although not entirely consistent, most studies have indicated that tumor TL alterations are associated with a worse clinical outcome. The type of alteration linked to a poorer survival (short vs. long TL) might depend on the tumor type. At the present time, the underlying mechanisms remain unclear. As for short TL and its association to a poorer outcome, it is logical to consider the relation between short TL and genomic instability.

5.1 Blood telomere length as a potential predictor for cancer risk and prognosis

There is a need for reliable, easily measurable biological markers for risk and prognosis assessment of malignancies. In recent years, a growing number of studies have focused on analyzing telomeres in peripheral blood cells in relation to cancer risk. Since blood is an easily accessible biological sample, blood TL is an interesting candidate as a biological marker. However, previous reports from this research area are not unambiguous. In renal, lung, bladder, head and neck and oesophagus carcinomas, short TL in peripheral blood have been associated with increased cancer risk (Wu, Amos et al. 2003; McGrath, Wong et al. 2007; Risques, Vaughan et al. 2007; Jang, Choi et al. 2008). Similar data have been reported for malignant lymphoma (Widmann, Herrmann et al. 2007). Regarding breast cancer, a study in sister sets found a non-significant association between short TL and increased cancer risk (Shen, Terry et al. 2007). In contrast, in newly diagnosed women with spontaneous breast tumors, cancer risk increased with increasing TL (Svenson, Nordfjall et al. 2008). Hence, the majority of studies have found blood TL alterations in cancer patients when compared to healthy controls. It might be that the type of TL alteration differs depending on the tumor type. Differences in the methodology of measuring TL might also contribute to the discrepancy. In addition, the type of blood cells that are analyzed can vary between different studies.

The biological mechanisms behind the reported association between blood TL alterations and increased cancer risk are not clear. It is not evident whether the TL alterations observed in peripheral blood reflect a secondary event caused by the cancer disease, or if the alterations are in fact part of the cancer etiology, or both. Oxidative stress has been associated with tissue aging, as well as telomere shortening, and it has been proposed that the cumulative burden of oxidative stress through life is registered in the telomeres of leukocytes (von Zglinicki 2002; Balaban, Nemoto et al. 2005). Accordingly, it has been speculated that blood TL may act as a surrogate marker of tissue dysfunctions. In 2003, Wu et al. measured TL in peripheral lymphocytes and found an association between short TL and increased risk of bladder, head and neck, lung and renal cell cancers (Wu, Amos et al. 2003). The authors proposed telomere dysfunction as a potential predisposition factor for cancer development. It cannot be excluded that the disease itself, or responses to the disease, can originate a systemic effect which directly or indirectly affects the telomere length of

peripheral blood cells. The immune response to the tumor might need to be considered. Some inflammatory molecules, such as cytokines and reactive oxygen species, may have an impact on leukocyte TL. A number of cytokines have shown potential to activate telomerase, whereas oxidative stress, as mentioned above, might increase telomere attrition (Akiyama, Hideshima et al. 2002; von Zglinicki 2002). The proliferation rate of immune cells, and hence the telomere attrition rate, may also differ depending of the state of the immune system, which in turn might be altered due to the presence of a tumor.

It was previously reported that the inter-individual TL variation was reduced in the healthy aged individuals (≥ 85 years) (Halaschek-Wiener, Vulto et al. 2008). The authors had hypothesized that healthy old individuals without age-related diseases, such as cancer or cardiovascular disease, would have unusually long telomeres. The study, however, showed that telomere length in the "normal range" may be most protective against tumor development, since long telomeres may favor escape from senescence, whereas short TL may cause genomic instability.

Studies investigating blood telomere length as a prognostic indicator in malignancies are sparse (Svenson, Ljungberg et al. 2009; Svenson and Roos 2009; Willeit, Willeit et al. 2010).

A recent prospective, population-based study estimated the impact of peripheral blood leukocyte telomere length on overall cancer manifestation and mortality (Willeit, Willeit et al. 2010). This study demonstrated significant inverse correlations between baseline leukocyte telomere length and both cancer incidence and mortality, which emerged as independent of standard cancer risk factors. The authors found evidence of heterogeneous effects according to cancer type, whereby tumors with a high fatality rate tended to exhibit more prominent relationships with telomere length and tumors with a more favorable prognosis showed modest or no associations. These results corroborate with previous evaluations demonstrating a link between short telomere length and lung cancer, bladder cancer, renal cell carcinoma, non-Hodgkin lymphoma and head and neck tumors (Wu, Amos et al. 2003; Broberg, Bjork et al. 2005; McGrath, Wong et al. 2007; Shao, Wood et al. 2007; Jang, Choi et al. 2008; Lan, Cawthon et al. 2009), but failed to obtain significant correlations between short telomere length and colorectal and breast cancer (De Vivo, Prescott et al. 2009; Shen, Gammon et al. 2009; Zee, Castonguay et al. 2009). A variety of experimental and genetic studies support the hypothesis that telomere attrition contributes to the manifestation and dissemination of malignancies. While fully functional telomeres confer protection of the genome, shortened telomeres facilitate chromosomal instability (Calado and Young 2009).

In fibroblast cultures, accruing senescent cells were shown to produce and release high amounts of growth factors, causing an overwhelming proliferation of the surrounding tissue and to secrete metalloproteinase, diminishing intercellular adhesions and potentially favoring metastatic spread of tumors (Krtolica, Parrinello et al. 2001; Liu and Hornsby 2007). Moreover, a significantly higher production of vascular endothelial growth factor in senescent cells may stimulate tumor growth and dissemination by promoting neovascularization (Coppe, Kauser et al. 2006). The association between short leukocyte telomere length and cancer formation may be partially mediated by the aging of the immune system itself. Aging of leukocytes reflected by short telomere length may impair immune surveillance and reduce the clearance of tumor cells.

A study demonstrated that patients with multiple myeloma with high telomerase activity and short telomere length presented a poor prognosis (Wu, Orme et al. 2003). Accordingly, another study reports the same results in adult T-cell leukemia, suggesting that telomerase

activity and low telomere content may be novel markers for the prognosis of these diseases (Kubuki, Suzuki et al. 2005).

5.2 Telomerase genetic variants

In recent years, a growing number of studies have focused on genetic polymorphisms influencing cancer risk and prognosis (Araujo, Ribeiro et al. 2007; Nogal, Coelho et al. 2007; Teixeira, Ribeiro et al. 2009; Nogueira, Catarino et al. 2010).

Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age (Graakjaer, Pascoe et al. 2004; Londono-Vallejo 2004).

Rafnar et al (Rafnar, Sulem et al. 2009) demonstrated that polymorphisms in the gene encoding *TERT*, the catalytic subunit of telomerase, were associated with an increased risk for cancer of the lung, urinary bladder, prostate and cervix.

Matsubara and colleagues (Matsubara, Murata et al. 2006) screened the promoter region of *hTERT* for functional polymorphisms and a frequent T to C transition was found 1327 bp upstream the transcription starting site (-1327T/C). Individuals homozygous for the -1327C/C genotype showed lower telomerase activity and shorter telomere length in their peripheral leukocytes compared to the -1327T/T and -1327T/C genotypes.

Matsubara and co-workers (Matsubara, Murata et al. 2006; Matsubara, Murata et al. 2006) demonstrated that the -1327T/C polymorphism within the *hTERT* promoter region has functional roles: the -1327T sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the -1327T/C polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Transcriptional regulation of *hTERT* has a key role in telomerase activity and telomere shortening. Approximately 25% higher promoter activity in the -1327T-sequence was found compared the -1327C-sequence and the T allele was strongly associated with longer telomere length. Thus, *hTERT* T allele with higher *hTERT* transcriptional activity is associated with more effective extension of the telomeric end during cell division. Another study found an overrepresentation of the -1327C/C genotype in patients with coronary artery disease compared to controls, presenting shorter telomeres compared to other patients with alternative genotypes, indicating that a subgroup of patients is more prone to telomere shortening (Matsubara, Murata et al. 2006).

A previous study performed by our group indicates an influence of the telomerase genetic variants in overall survival of NSCLC patients. Multivariate Cox regression analysis indicated an increased overall survival for T carrier patients, when compared with CC genotype, after adjustment for tumor histological type, stage, smoking status, age and gender (hazard ratio, HR=0.52). The median estimated cumulative survival was significantly higher in T carrier patients, of 26.5 months, comparing with CC patients, of 19.3 months (Catarino, Araujo et al. 2010).

Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation and metastasis, i.e., an aggressive tumor phenotype. It has been hypothesized that altered telomere length could predispose cells to gain the necessary properties to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome (Bisoffi, Heaphy et al. 2006; Willeit, Willeit et al. 2010).

6. Concluding remarks

Cancer remains an important public health problem in developed countries. Therapeutic failure and side effects of anticancer therapy are essential issues requiring future research. One of the main aims of clinical or translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biologic activity and toxic effects. Therefore, the study of tumor and patient genetic profiles, relative to drug-related genes, may offer new opportunities for tailoring treatments.

The assessment of telomere length and telomerase genetic variants could supplement prognosis of survival in the course of cancer and may be a promising molecular marker of treatment response in cancer patients. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor could facilitate screening for cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely in order to reduce adverse effects, and establishing safety profiles based on individual genetic analyses.

Most cancer cells have strong telomerase activity to maintain telomere lengths for long-term cell proliferation. It has been proposed that cancer cells can be killed by inducing critical shortening of telomeres, and hence senescence or apoptosis, through inhibition of telomerase.

In most cancers (80%) unlimited replicative potential is achieved through telomerase reactivation, allowing telomere lengthening. This major feature of cancer cell biology is observed from the earliest stages in most tissues. Early telomerase activation gives a clear advantage to established tumor cells, but also contributes to cancer development. Moreover, the role of telomeres in carcinogenesis could partly underlie individual, tissue-specific predisposition. Indeed, telomere length varies between individuals in the population; some studies suggest that hereditary factors are involved in determining telomere length. Thus, individual telomere length heterogeneity and telomere-driven instability may be central events in early carcinogenesis.

Overall, these observations highlight the need for improved understanding of telomere-driven senescence and of the mechanisms involved in the failure of this process to protect against cancer progression in the early stages of disease onset. In particular, improved understanding of the role and mechanism of action of telomerase in preneoplasia and established tumors is essential for current and future developments of accurate and individually adapted anti-telomerase anti-cancer therapy. Thus, improvement of the methods used to measure telomerase expression and activity will be required. Finally, a combination of techniques – each one adapted for studying telomerase, telomere associated proteins, DNA damage proteins and telomere length and structure – will be necessary for use in future studies, to fully elucidate the molecular mechanisms underpinning telomere-related biology.

A possible prognostic impact of TL abnormalities seems to be tumor type dependent and more data on well characterized tumor materials are required. It is evident that the appearance of a malignancy affects the whole body, and it is possible that the alterations in blood TL seen at diagnosis reflect responses to the tumor. Of most interest is perhaps the fact that blood TL appears to constitute a significant prognostic indicator, but additional studies on different patient cohorts are needed in order to further substantiate this indication. Large longitudinal studies and improved standardized protocols are needed for the future, to fully be able to evaluate TL as a marker in malignancies.

New discoveries pertaining to telomere structure and telomere homeostasis that shed light on the complex relationship between telomeres, telomerase, chromosomal instability and cancer will undoubtedly have an important impact in cancer therapeutics.

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8. References

- Akiyama, M, T Hideshima, T Hayashi, YT Tai, CS Mitsiades, N Mitsiades, D Chauhan, P Richardson, NC Munshi and KC Anderson (2002). Cytokines modulate telomerase activity in a human multiple myeloma cell line. *Cancer Res* 62(13): 3876-3882.
- Albertson, DG, C Collins, F McCormick and JW Gray (2003). Chromosome aberrations in solid tumors. *Nat Genet* 34(4): 369-376.
- Allsopp, RC, H Vaziri, C Patterson, S Goldstein, EV Younglai, AB Futcher, CW Greider and CB Harley (1992). Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci U S A* 89(21): 10114-10118.
- Araujo, A, R Ribeiro, I Azevedo, A Coelho, M Soares, B Sousa, D Pinto, C Lopes, R Medeiros and GV Scagliotti (2007). Genetic polymorphisms of the epidermal growth factor and related receptor in non-small cell lung cancer--a review of the literature. *The oncologist* 12(2): 201-210.
- Armanios, MY, JJ Chen, JD Cogan, JK Alder, RG Ingersoll, C Markin, WE Lawson, M Xie, I Vulto, JA Phillips, 3rd, PM Lansdorp, CW Greider and JE Loyd (2007). Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med* 356(13): 1317-1326.
- Atadja, P, H Wong, I Garkavtsev, C Veillette and K Riabowol (1995). Increased activity of p53 in senescing fibroblasts. *Proc Natl Acad Sci U S A* 92(18): 8348-8352.
- Balaban, RS, S Nemoto and T Finkel (2005). Mitochondria, oxidants, and aging. *Cell* 120(4): 483-495.
- Beausejour, CM, A Krtolica, F Galimi, M Narita, SW Lowe, P Yaswen and J Campisi (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J* 22(16): 4212-4222.
- Benn, PA (1976). Specific chromosome aberrations in senescent fibroblast cell lines derived from human embryos. *Am J Hum Genet* 28(5): 465-473.
- Bierman, EL (1978). The effect of donor age on the in vitro life span of cultured human arterial smooth-muscle cells. *In Vitro* 14(11): 951-955.
- Bisoffi, M, CM Heaphy and JK Griffith (2006). Telomeres: prognostic markers for solid tumors. *Int J Cancer* 119(10): 2255-2260.
- Blasco, MA (2005). Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* 6(8): 611-622.

- Blasco, MA (2007). The epigenetic regulation of mammalian telomeres. *Nat Rev Genet* 8(4): 299-309.
- Blasco, MA and WC Hahn (2003). Evolving views of telomerase and cancer. *Trends Cell Biol* 13(6): 289-294.
- Bodnar, AG, M Ouellette, M Frolkis, SE Holt, CP Chiu, GB Morin, CB Harley, JW Shay, S Lichtsteiner and WE Wright (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* 279(5349): 349-352.
- Broberg, K, J Bjork, K Paulsson, M Hoglund and M Albin (2005). Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer. *Carcinogenesis* 26(7): 1263-1271.
- Calado, RT and NS Young (2009). Telomere diseases. *N Engl J Med* 361(24): 2353-2365.
- Campisi, J (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 120(4): 513-522.
- Catarino, R, A Araujo, A Coelho, M Gomes, A Nogueira, C Lopes and RM Medeiros (2010). Prognostic significance of telomerase polymorphism in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16(14): 3706-3712.
- Chin, K, CO de Solorzano, D Knowles, A Jones, W Chou, EG Rodriguez, WL Kuo, BM Ljung, K Chew, K Myambo, M Miranda, S Krig, J Garbe, M Stampfer, P Yaswen, JW Gray and SJ Lockett (2004). In situ analyses of genome instability in breast cancer. *Nat Genet* 36(9): 984-988.
- Coppe, JP, K Kauser, J Campisi and CM Beausejour (2006). Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem* 281(40): 29568-29574.
- d'Adda di Fagagna, F, PM Reaper, L Clay-Farrace, H Fiegler, P Carr, T Von Zglinicki, G Saretzki, NP Carter and SP Jackson (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 426(6963): 194-198.
- de Lange, T (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 19(18): 2100-2110.
- De Vivo, I, J Prescott, JY Wong, P Kraft, SE Hankinson and DJ Hunter (2009). A prospective study of relative telomere length and postmenopausal breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 18(4): 1152-1156.
- Ducray, C, JP Pommier, L Martins, FD Boussin and L Sabatier (1999). Telomere dynamics, end-to-end fusions and telomerase activation during the human fibroblast immortalization process. *Oncogene* 18(29): 4211-4223.
- Dunham, MA, AA Neumann, CL Fasching and RR Reddel (2000). Telomere maintenance by recombination in human cells. *Nat Genet* 26(4): 447-450.
- Evans, RJ, FS Wyllie, D Wynford-Thomas, D Kipling and CJ Jones (2003). A P53-dependent, telomere-independent proliferative life span barrier in human astrocytes consistent with the molecular genetics of glioma development. *Cancer Res* 63(16): 4854-4861.
- Feldser, DM, JA Hackett and CW Greider (2003). Telomere dysfunction and the initiation of genome instability. *Nat Rev Cancer* 3(8): 623-627.
- Flores, I, A Canela, E Vera, A Tejera, G Cotsarelis and MA Blasco (2008). The longest telomeres: a general signature of adult stem cell compartments. *Genes Dev* 22(5): 654-667.

- Fordyce, CA, CM Heaphy, M Bisoffi, JL Wyaco, NE Joste, A Mangalik, KB Baumgartner, RN Baumgartner, WC Hunt and JK Griffith (2006). Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Res Treat* 99(2): 193-202.
- Fordyce, CA, CM Heaphy, NE Joste, AY Smith, WC Hunt and JK Griffith (2005). Association between cancer-free survival and telomere DNA content in prostate tumors. *J Urol* 173(2): 610-614.
- Frias, C, C Garcia-Aranda, C De Juan, A Moran, P Ortega, A Gomez, F Hernando, JA Lopez-Asenjo, AJ Torres, M Benito and P Iniesta (2008). Telomere shortening is associated with poor prognosis and telomerase activity correlates with DNA repair impairment in non-small cell lung cancer. *Lung Cancer* 60(3): 416-425.
- Garcia-Aranda, C, C de Juan, A Diaz-Lopez, A Sanchez-Pernaute, AJ Torres, E Diaz-Rubio, JL Balibrea, M Benito and P Iniesta (2006). Correlations of telomere length, telomerase activity, and telomeric-repeat binding factor 1 expression in colorectal carcinoma. *Cancer* 106(3): 541-551.
- Gertler, R, D Doll, M Maak, M Feith and R Rosenberg (2008). Telomere length and telomerase subunits as diagnostic and prognostic biomarkers in Barrett carcinoma. *Cancer* 112(10): 2173-2180.
- Gertler, R, R Rosenberg, D Stricker, J Friederichs, A Hoos, M Werner, K Ulm, B Holzmann, H Nekarda and JR Siewert (2004). Telomere length and human telomerase reverse transcriptase expression as markers for progression and prognosis of colorectal carcinoma. *J Clin Oncol* 22(10): 1807-1814.
- Gisselsson, D, T Jonson, A Petersen, B Strombeck, P Dal Cin, M Hoglund, F Mitelman, F Mertens and N Mandahl (2001). Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. *Proc Natl Acad Sci U S A* 98(22): 12683-12688.
- Graakjaer, J, C Bischoff, L Korsholm, S Holstebro, W Vach, VA Bohr, K Christensen and S Kolvraa (2003). The pattern of chromosome-specific variations in telomere length in humans is determined by inherited, telomere-near factors and is maintained throughout life. *Mech Ageing Dev* 124(5): 629-640.
- Graakjaer, J, L Pascoe, H Der-Sarkissian, G Thomas, S Kolvraa, K Christensen and JA Londono-Vallejo (2004). The relative lengths of individual telomeres are defined in the zygote and strictly maintained during life. *Aging Cell* 3(3): 97-102.
- Greider, CW and EH Blackburn (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 43(2 Pt 1): 405-413.
- Griffith, JD, L Comeau, S Rosenfield, RM Stansel, A Bianchi, H Moss and T de Lange (1999). Mammalian telomeres end in a large duplex loop. *Cell* 97(4): 503-514.
- Hackett, JA and CW Greider (2002). Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis. *Oncogene* 21(4): 619-626.
- Halaschek-Wiener, J, I Vulto, D Fornika, J Collins, JM Connors, ND Le, PM Lansdorp and A Brooks-Wilson (2008). Reduced telomere length variation in healthy oldest old. *Mech Ageing Dev* 129(11): 638-641.
- Hanahan D, WR (2000). The Hallmarks of Cancer. *Cell* 100: 57-70.
- Harley, CB, AB Futcher and CW Greider (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* 345(6274): 458-460.

- Hastie, ND, M Dempster, MG Dunlop, AM Thompson, DK Green and RC Allshire (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 346(6287): 866-868.
- Hayflick, L and PS Moorhead (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res* 25: 585-621.
- Hirashima, T, T Komiya, T Nitta, Y Takada, M Kobayashi, N Masuda, K Matui, M Takada, M Kikui, T Yasumitu, A Ohno, K Nakagawa, M Fukuoka and I Kawase (2000). Prognostic significance of telomeric repeat length alterations in pathological stage I-III non-small cell lung cancer. *Anticancer Res* 20(3B): 2181-2187.
- Jang, JS, YY Choi, WK Lee, JE Choi, SI Cha, YJ Kim, CH Kim, S Kam, TH Jung and JY Park (2008). Telomere length and the risk of lung cancer. *Cancer Sci* 99(7): 1385-1389.
- Jaskelioff, M, FL Muller, JH Paik, E Thomas, S Jiang, AC Adams, E Sahin, M Kost-Alimova, A Protopopov, J Cadinanos, JW Horner, E Maratos-Flier and RA Depinho (2011). Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature* 469(7328): 102-106.
- Kirkwood, TB and SN Austad (2000). Why do we age? *Nature* 408(6809): 233-238.
- Koeneman, KS, CX Pan, JK Jin, JM Pyle, 3rd, RC Flanigan, TV Shankey and MO Diaz (1998). Telomerase activity, telomere length, and DNA ploidy in prostatic intraepithelial neoplasia (PIN). *J Urol* 160(4): 1533-1539.
- Krtolica, A, S Parrinello, S Lockett, PY Desprez and J Campisi (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* 98(21): 12072-12077.
- Kubuki, Y, M Suzuki, H Sasaki, T Toyama, K Yamashita, K Maeda, A Ido, H Matsuoka, A Okayama, T Nakanishi and H Tsubouchi (2005). Telomerase activity and telomere length as prognostic factors of adult T-cell leukemia. *Leuk Lymphoma* 46(3): 393-399.
- Lan, Q, R Cawthon, M Shen, SJ Weinstein, J Virtamo, U Lim, HD Hosgood, 3rd, D Albanes and N Rothman (2009). A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of non-Hodgkin lymphoma. *Clin Cancer Res* 15(23): 7429-7433.
- Lansdorp, PM, NP Verwoerd, FM van de Rijke, V Dragowska, MT Little, RW Dirks, AK Raap and HJ Tanke (1996). Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet* 5(5): 685-691.
- Lantuejoul, S, JC Soria, L Morat, P Lorimier, D Moro-Sibilot, L Sabatier, C Brambilla and E Brambilla (2005). Telomere shortening and telomerase reverse transcriptase expression in preinvasive bronchial lesions. *Clin Cancer Res* 11(5): 2074-2082.
- Liu, D and PJ Hornsby (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res* 67(7): 3117-3126.
- Londono-Vallejo, JA (2004). Telomere length heterogeneity and chromosome instability. *Cancer Lett* 212(2): 135-144.
- Martin, GM, CA Sprague and CJ Epstein (1970). Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype. *Lab Invest* 23(1): 86-92.
- Maser, RS and RA DePinho (2002). Connecting chromosomes, crisis, and cancer. *Science* 297(5581): 565-569.

- Matsubara, Y, M Murata, K Watanabe, I Saito, K Miyaki, K Omae, M Ishikawa, K Matsushita, S Iwanaga, S Ogawa and Y Ikeda (2006). Coronary artery disease and a functional polymorphism of hTERT. *Biochem Biophys Res Commun* 348(2): 669-672.
- Matsubara, Y, M Murata, T Yoshida, K Watanabe, I Saito, K Miyaki, K Omae and Y Ikeda (2006). Telomere length of normal leukocytes is affected by a functional polymorphism of hTERT. *Biochem Biophys Res Commun* 341(1): 128-131.
- McClintock, B (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. *Genetics* 26(2): 234-282.
- McGrath, M, JY Wong, D Michaud, DJ Hunter and I De Vivo (2007). Telomere length, cigarette smoking, and bladder cancer risk in men and women. *Cancer Epidemiol Biomarkers Prev* 16(4): 815-819.
- Meeker, AK and P Argani (2004). Telomere shortening occurs early during breast tumorigenesis: a cause of chromosome destabilization underlying malignant transformation? *J Mammary Gland Biol Neoplasia* 9(3): 285-296.
- Meeker, AK, JL Hicks, CA Iacobuzio-Donahue, EA Montgomery, WH Westra, TY Chan, BM Ronnett and AM De Marzo (2004). Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. *Clin Cancer Res* 10(10): 3317-3326.
- Meeker, AK, JL Hicks, EA Platz, GE March, CJ Bennett, MJ Delannoy and AM De Marzo (2002). Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res* 62(22): 6405-6409.
- Mitchell, JR, E Wood and K Collins (1999). A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 402(6761): 551-555.
- Moran, E (1993). DNA tumor virus transforming proteins and the cell cycle. *Curr Opin Genet Dev* 3(1): 63-70.
- Moran, E (1993). Interaction of adenoviral proteins with pRB and p53. *FASEB J* 7(10): 880-885.
- Moyzis, RK, JM Buckingham, LS Cram, M Dani, LL Deaven, MD Jones, J Meyne, RL Ratliff and JR Wu (1988). A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* 85(18): 6622-6626.
- Nogal, A, A Coelho, R Catarino, A Morais, F Lobo and R Medeiros (2007). The CYP3A4 *1B polymorphism and prostate cancer susceptibility in a Portuguese population. *Cancer genetics and cytogenetics* 177(2): 149-152.
- Nogueira, A, R Catarino, A Coelho, A Araujo, M Gomes and R Medeiros (2010). Influence of DNA repair RAD51 gene variants in overall survival of non-small cell lung cancer patients treated with first line chemotherapy. *Cancer chemotherapy and pharmacology* 66(3): 501-506.
- Nordfjall, K, A Larefalk, P Lindgren, D Holmberg and G Roos (2005). Telomere length and heredity: Indications of paternal inheritance. *Proc Natl Acad Sci U S A* 102(45): 16374-16378.
- Ohyashiki, JH, G Sashida, T Tauchi and K Ohyashiki (2002). Telomeres and telomerase in hematologic neoplasia. *Oncogene* 21(4): 680-687.
- Patel, MM, LJ Parekh, FP Jha, RN Sainger, JB Patel, DD Patel, PM Shah and PS Patel (2002). Clinical usefulness of telomerase activation and telomere length in head and neck cancer. *Head Neck* 24(12): 1060-1067.

- Rafnar, T, P Sulem, SN Stacey, F Geller, J Gudmundsson, A Sigurdsson, M Jakobsdottir, H Helgadottir, S Thorlacius, KK Aben, T Blondal, TE Thorgeirsson, G Thorleifsson, K Kristjansson, K Thorisdottir, R Ragnarsson, B Sigurgeirsson, H Skuladottir, T Gudbjartsson, HJ Isaksson, GV Einarsson, KR Benediktsdottir, BA Agnarsson, K Olafsson, A Salvarsdottir, H Bjarnason, M Asgeirsdottir, KT Kristinsson, S Matthiasdottir, SG Sveinsdottir, S Polidoro, V Hoiom, R Botella-Estrada, K Hemminki, P Rudnai, DT Bishop, M Campagna, E Kellen, MP Zeegers, P de Verdier, A Ferrer, D Isla, MJ Vidal, R Andres, B Saez, P Juberias, J Banzo, S Navarrete, A Tres, D Kan, A Lindblom, E Gurzau, K Koppova, F de Vegt, JA Schalken, HF van der Heijden, HJ Smit, RA Termeer, E Oosterwijk, O van Hooij, E Nagore, S Porru, G Steineck, J Hansson, F Buntinx, WJ Catalona, G Matullo, P Vineis, AE Kiltie, JI Mayordomo, R Kumar, LA Kiemeny, ML Frigge, T Jonsson, H Saemundsson, RB Barkardottir, E Jonsson, S Jonsson, JH Olafsson, JR Gulcher, G Masson, DF Gudbjartsson, A Kong, U Thorsteinsdottir and K Stefansson (2009). Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet* 41(2): 221-227.
- Reddel, RR (2003). Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett* 194(2): 155-162.
- Risques, RA, TL Vaughan, X Li, RD Odze, PL Blount, K Ayub, JL Gallaher, BJ Reid and PS Rabinovitch (2007). Leukocyte telomere length predicts cancer risk in Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 16(12): 2649-2655.
- Rohme, D (1981). Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. *Proc Natl Acad Sci U S A* 78(8): 5009-5013.
- Rudolph, KL, S Chang, HW Lee, M Blasco, GJ Gottlieb, C Greider and RA DePinho (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* 96(5): 701-712.
- Saretzki, G and T Von Zglinicki (2002). Replicative aging, telomeres, and oxidative stress. *Ann N Y Acad Sci* 959: 24-29.
- Schedin, P and A Elias (2004). Multistep tumorigenesis and the microenvironment. *Breast Cancer Res* 6(2): 93-101.
- Serrano, M and MA Blasco (2007). Cancer and ageing: convergent and divergent mechanisms. *Nat Rev Mol Cell Biol* 8(9): 715-722.
- Shao, L, CG Wood, D Zhang, NM Tannir, S Matin, CP Dinney and X Wu (2007). Telomere dysfunction in peripheral lymphocytes as a potential predisposition factor for renal cancer. *J Urol* 178(4 Pt 1): 1492-1496.
- Shen, J, MD Gammon, MB Terry, Q Wang, P Bradshaw, SL Teitelbaum, AI Neugut and RM Santella (2009). Telomere length, oxidative damage, antioxidants and breast cancer risk. *Int J Cancer* 124(7): 1637-1643.
- Shen, J, MB Terry, I Gurvich, Y Liao, RT Senie and RM Santella (2007). Short telomere length and breast cancer risk: a study in sister sets. *Cancer Res* 67(11): 5538-5544.
- Shirotani, Y, K Hiyama, S Ishioka, K Inyaku, Y Awaya, S Yonehara, Y Yoshida, K Inai, E Hiyama, K Hasegawa and et al. (1994). Alteration in length of telomeric repeats in lung cancer. *Lung Cancer* 11(1-2): 29-41.
- Smith, JR and OM Pereira-Smith (1996). Replicative senescence: implications for in vivo aging and tumor suppression. *Science* 273(5271): 63-67.

- Svenson, U, B Ljungberg and G Roos (2009). Telomere length in peripheral blood predicts survival in clear cell renal cell carcinoma. *Cancer Res* 69(7): 2896-2901.
- Svenson, U, K Nordfjall, B Stegmayr, J Manjer, P Nilsson, B Tavelin, R Henriksson, P Lenner and G Roos (2008). Breast cancer survival is associated with telomere length in peripheral blood cells. *Cancer Res* 68(10): 3618-3623.
- Svenson, U and G Roos (2009). Telomere length as a biological marker in malignancy. *Biochim Biophys Acta* 1792(4): 317-323.
- Teixeira, AL, R Ribeiro, A Morais, F Lobo, A Fraga, F Pina, FM Calais-da-Silva, FE Calais-da-Silva and R Medeiros (2009). Combined analysis of EGF+61G>A and TGFB1+869T>C functional polymorphisms in the time to androgen independence and prostate cancer susceptibility. *The pharmacogenomics journal* 9(5): 341-346.
- von Zglinicki, T (2002). Oxidative stress shortens telomeres. *Trends Biochem Sci* 27(7): 339-344.
- Widmann, TA, M Herrmann, N Taha, J Konig and M Pfreundschuh (2007). Short telomeres in aggressive non-Hodgkin's lymphoma as a risk factor in lymphomagenesis. *Exp Hematol* 35(6): 939-946.
- Willeit, P, J Willeit, A Mayr, S Weger, F Oberhollenzer, A Brandstatter, F Kronenberg and S Kiechl (2010). Telomere length and risk of incident cancer and cancer mortality. *JAMA* 304(1): 69-75.
- Wright, WE and JW Shay (1992). The two-stage mechanism controlling cellular senescence and immortalization. *Exp Gerontol* 27(4): 383-389.
- Wright, WE and JW Shay (2002). Historical claims and current interpretations of replicative aging. *Nat Biotechnol* 20(7): 682-688.
- Wu, KD, LM Orme, J Shaughnessy, Jr., J Jacobson, B Barlogie and MA Moore (2003). Telomerase and telomere length in multiple myeloma: correlations with disease heterogeneity, cytogenetic status, and overall survival. *Blood* 101(12): 4982-4989.
- Wu, X, CI Amos, Y Zhu, H Zhao, BH Grossman, JW Shay, S Luo, WK Hong and MR Spitz (2003). Telomere dysfunction: a potential cancer predisposition factor. *J Natl Cancer Inst* 95(16): 1211-1218.
- Zakian, VA (2009). The ends have arrived. *Cell* 139(6): 1038-1040.
- Zee, RY, AJ Castonguay, NS Barton and JE Buring (2009). Mean telomere length and risk of incident colorectal carcinoma: a prospective, nested case-control approach. *Cancer Epidemiol Biomarkers Prev* 18(8): 2280-2282.
- Zou, Y, A Sfeir, SM Gryaznov, JW Shay and WE Wright (2004). Does a sentinel or a subset of short telomeres determine replicative senescence? *Mol Biol Cell* 15(8): 3709-3718.

Differential Effects of the G-Quadruplex Ligand 360A in Human Normal and Cancer Cells

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1. Introduction

Telomeres are essential for chromosome replication and genome integrity. The 3' single-stranded overhang of human telomere may adopt particular conformations such as T-loops and G-quadruplexes. Reactivated in most tumors, telomerase, a specific reverse transcriptase that elongates the telomeres, is thought to enable cancer cells to proliferate in an unlimited manner, thereby correcting the normal telomere erosion that occurs during cell division. The level of interest in G-quadruplex has increased due to their ability to inhibit telomerase activity. We have investigated chromosomal binding and the cellular effects induced by pyridine derivative G-quadruplex ligand in human normal and tumour cells. We show from our analysis that this G-quadruplex ligand preferentially binds the terminal regions of chromosomes in both normal and tumour cells. This compound also induces DNA damage signals in a strictly ATM-dependent manner, inhibits cell proliferation and induces apoptosis. We further observed by telo-FISH and Chromosome Orientation-FISH that this compound induces specific telomere aberrations either during or after replication and mainly consisting of sister telomere fusions and recombination events principally involving the lagging strand telomeres. We have also observed that ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia Related) reduce telomere instability independently of apoptosis suggesting its direct role in preventing inappropriate DNA repair at the telomeres. We have further demonstrated that, even at elevated concentrations, G-quadruplex ligand has limited effects on proliferation of normal cells, and does not induce apoptosis or telomere aberrations. Interestingly, we observed induction of reversible premature senescence in primary fibroblasts. Taken together, our results suggest that the protein composition and/or organization of the telomeres differ markedly between normal and cancer cells, and provide higher telomere stability to normal cells.

1.1 Structure of telomeres

Telomeres are nucleoprotein structures located at the ends of chromosomes (Figure 1A). Human telomeric DNA contains double-stranded repeats of the motif TTAGGG (5 - 20 Kb) followed by a G-rich 3'-overhang (Makarov et al., 1997) (Figure 1B). In human chromosomes, the long terminal protrusions of single-stranded G-rich sequence have been reported to vary from 50 to more than 500 nucleotides (Makarov et al., 1997; Stewart et al., 2003). Telomeres have also been considered to be transcriptionally silent, but mammalian

telomeres are transcribed into telomeric repeat-containing RNA (Azzalin et al., 2007; Schoeftner & Blasco, 2008; Ho et al., 2008) (Figure 1B).

Telomeric DNA is capped by shelterin, a telomere-specific multiprotein complex (de Lange, 2005). Three shelterin subunits, TRF1 (Telomeric Repeat Factor 1) (Zhong et al., 1992; Broccoli et al., 1997), TRF2 (Telomeric Repeat Factor 2) (Broccoli et al., 1997; Bilaud et al., 1997) and POT1 (Protection Of Telomere 1) (Baumann & Cech, 2001; Loyza et al., 2004; Hockmeyer et al., 2005) directly recognize TTAGGG repeats and are interconnected by three additional shelterin proteins, TIN2 (TRF1 Interacting Nuclear Protein 1) (Kim et al., 1999; O'Connor et al., 2006), TPP1 (TINT1-PIP1-PTOP1) (Hockmeyer et al., 2007; Ye et al., 2004; O'Connor et al., 2006; Chen et al., 2007; Xin et al., 2007) and Rap1 (Repressor activator protein 1) (Li et al., 2000; Li & de Lange, 2003; O'Connor et al., 2006), forming a complex that allows cells to distinguish telomeres from sites of DNA damage.

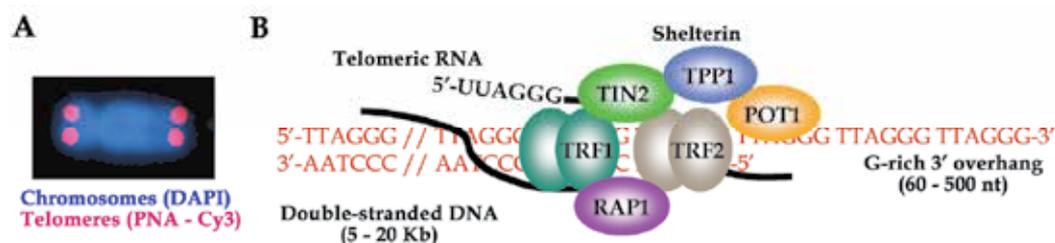


Fig. 1. Human telomeres evidenced by hybridization with a PNA-Cy3 (Peptide Nucleic Acid - Cyanine 3) probe on chromosome counterstained with DAPI (A) and schematic representation of shelterin on telomeric DNA in the presence of telomeric repeat-containing RNA (B).

Telomeres can adopt a protective conformation, the T-loop structure, in which the telomeric 3'-overhang is incorporated into the proximal double stranded telomeres (Griffith et al., 1999) (Figure 2A). Junction-specific binding would also allow TRF2 to stabilize a strand invasion structure that is thought to exist at the strand invasion site of the T-loop (Stansel et al., 2001; Fouche et al., 2006). The T-loop has been proposed to prevent telomeres from being recognized as a DNA double strand breaks (DSBs) and thus from activating cell cycle checkpoints, inappropriate DNA repair and cell death (Smogorzewska & de Lange, 2004). Nevertheless, the DNA damage machinery recognizes functional telomeres during replication (Verdun et al., 2005). At the telomeres, a localized DNA damage response (DDR) seems to be required for processing and the formation of protective structures such as the T-loop after replication.

The guanine-rich sequences of telomeric DNA are susceptible to form *in vitro* G-quadruplex as a consequence of the propensity of guanine to associate with each other in a stable hydrogen-bonded arrangement, the G-quartet (Neidle & Parkinson, 2003) (Figure 2B and 2C). G-quartet is stabilized by a monovalent cation (Na^+ or K^+) localized in the centre of the structure (Figure 2B) (Williamson et al., 1989; Sen & Gilbert, 1990). A three-dimensional arrangement of three G-quartets (Figure 2D) can result in a variety of G-quadruplex structures. The four-stranded quadruplex structural types depend on the number and the orientation of the DNA strands. Indeed, intramolecular G-quadruplexes comprise one DNA strand whereas dimeric and tetrameric intermolecular quadruplex involve 2 and 4 DNA

strands respectively. G-quadruplex heterogeneity also depends on the orientation of the DNA strands (parallel or anti-parallel) and the guanine conformation (syn or anti).

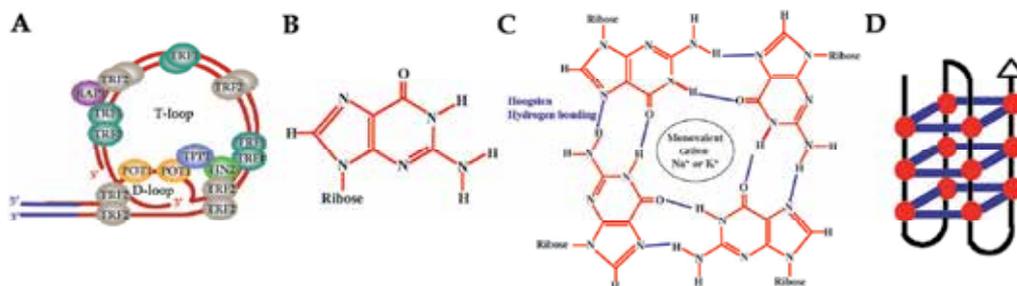


Fig. 2. Structure of the telomere. Schematic representation of T-loop structure (A). Chemical structure of guanine (B), of a G-quartet (C) and depiction of a G-quadruplex (D).

1.2 Telomerase

The maintenance of telomeric repeats in most eukaryotic organisms requires telomerase, which consists of a reverse transcriptase and an RNA template that dictates the synthesis of the G-rich strand of telomere terminal repeats (Greider & Blackburn, 1987; Autexier & Lue, 2006). The catalytic function of this enzyme depends minimally on two components: TERT (Telomerase Reverse Transcriptase) protein (Kilian et al., 1997) and telomerase RNA (TR: Telomerase RNA template) (Feng et al., 1995; Blasco et al., 1995). However, other proteins (dyskerin, Ku70, Ku80, nucleolin, hsp23, hsp90) have also been proposed to associate with human telomerase (Mitchell et al., 1999; Chai et al., 2002; Ting et al., 2005; Khurts et al., 2004; Forsyth et al., 2001; Cohen et al., 2007). By copying a short template sequences within its intrinsic RNA moiety, telomerase synthesizes the telomeric DNA strand running 5' to 3' toward the distal end of the chromosome, thereby extending it. Regulated extension of the chromosomal DNA termini occurs to compensate for the shortening that results from nuclease action and incomplete terminal DNA replication (Makarov et al., 1997; Blackburn, 2005; de Lange, 2005). Telomerase gene expression is active only in germ cells and stem cells and is repressed in most somatic cells, although limited expression is found in normal cycling cells (Masutomi et al., 2003). The expression of telomerase is reactivated in most tumours and is thought to enable cancer cells to proliferate in an unlimited manner by maintaining and protecting telomeres. Telomerase has therefore become a target for the development of new cancer drugs. In addition to telomerase however, other mechanisms to maintain telomere length have been identified in human tumours. Indeed, telomere lengthening is achieved in some cancer cells by recombination events between telomeres, known as alternative lengthening of telomeres (ALT) (Muntoni & Reddel, 2005).

1.3 Functions of telomeres

1.3.1 Telomeres protect chromosome ends

Telomeres are nucleoprotein structures essential for chromosome replication and genome integrity because they protect against instability-promoting events (degradation of the terminal regions of chromosomes, fusion of a telomere either with another telomere or with a broken DNA end, or inappropriate recombination) (Zakian, 1995). Telomeres allow cells to distinguish natural chromosome ends from damaged DNA by inhibiting the canonical DNA

damage response (NHEJ and HR). Telomerase, the T-loop and the shelterin complex serve to prevent telomeres from being recognized as DNA damage. Indeed, TRF2 can inhibit the ATM-dependent DNA damage response (Celli & de Lange, 2006; Karlseder et al., 2004; Denchi & de Lange, 2007, Bae & Baumann, 2007) whereas POT1 can inhibit inappropriate recombination at the telomeres (He 2006) and NHEJ (Denchi & de Lange, 2007). However, functional human telomeres are recognized by proteins involved in the DNA damage response, highlighting that a localized DNA damage response at the telomeres after replication is essential for recruiting the processing machinery that promotes formation of a chromosome end protection complex (Verdun et al., 2005). Without the protective activity of shelterin or when they shorten, telomeres are no longer hidden from the DNA damage surveillance and DNA repair pathways that may inappropriately process chromosome ends (Palm & de Lange, 2008).

1.3.2 Telomere shortening and replicative senescence

The replication of telomeres by conventional mechanisms is inevitably incomplete, leading to telomere shortening at each round of cell division (the end-replication problem) (Olovnikov, 1973). On the other hand, the processing reactions required to re-create a 3' end also lead to telomere shortening after replication (Dionne & Wellinger, 1998). Telomerase, the unique enzyme in the cell that can add telomeric repeats *de novo* to the 3' end, counteracts these losses. In humans, however the expression of the telomerase is highly regulated and most somatic cells do not possess any telomerase activity, and as a result telomere shortening is prevalent in proliferating cells. Because end-replication results in telomere shortening with each round of replication, the telomeres of human somatic cells act as a mitotic clock, shortening with age both *in vitro* and *in vivo* in a replication dependent manner (Olovnikov, 1973; Makarov et al., 1997). When telomeres reach a critical length (Hayflick & Moorhead, 1961), dysfunctional telomeres trigger a damage response leading to growth arrest (cellular senescence) or cell death (apoptosis) (Figure 3).

Indeed, uncapping of one telomere, because of damage or loss of telomere sequences or because of destabilization of the protein complex, triggers a DNA damage response and an attempt by the cell to repair the unprotected extremity (Takai et al., 2003; d'Adda di Fagagna et al., 2003). Dysfunctional telomeres are sensed as double strand DNA breaks, activating the DNA damage response checkpoints, including ATM and ATR (Karlseder et al., 1999; d'Adda di Fagagna et al., 2003). Hence, dysfunctional telomeres became associated with DNA damage response factors such as γ -H2AX, 53BP1, Rad17 and Mre11 leading to the formation of TIFs (Telomere dysfunction-Induced Foci) in an ATM- and ATR-dependent manner (Takai et al., 2003; Konishi & de Lange, 2008) and to the induction of replicative senescence. Cellular senescence is a cell-cycle arrest event in which cells show characteristic morphological changes and stain positively for senescence-associated β -galactosidase (SA- β -gal) activity (Hayflick & Moorhead, 1961; Dimri et al., 1995; Cosme-Blanco et al., 2007), and proceed through central signalling pathways leading to the activation of the p53 and retinoblastoma tumour suppressor proteins (pRb). Therefore, telomere shortening is considered to be the main causal mechanism underlying replicative senescence, but telomere deprotection, DNA damage, numerous stresses and/or signalling imbalance can also induce senescence (d'Adda di Fagagna et al., 2003; Cosme-Blanco et al., 2007). Other sources of telomere damage (exogenous events, such as UV irradiation, or endogenous factors, such as reactive oxygen species) may lead to sudden telomere shortening and

uncapping presumably with very similar consequences (von Zglinicki, 2000; Oikawa et al., 2001). Owing to its anti-proliferative effects, cellular senescence triggered by telomere dysfunction and/or erosion is considered to be a strong protective mechanism against unlimited proliferation (Harley et al., 1991) and an efficient tumour suppressor mechanism (Sedivy, 2007; Feldser & Greider, 2007).

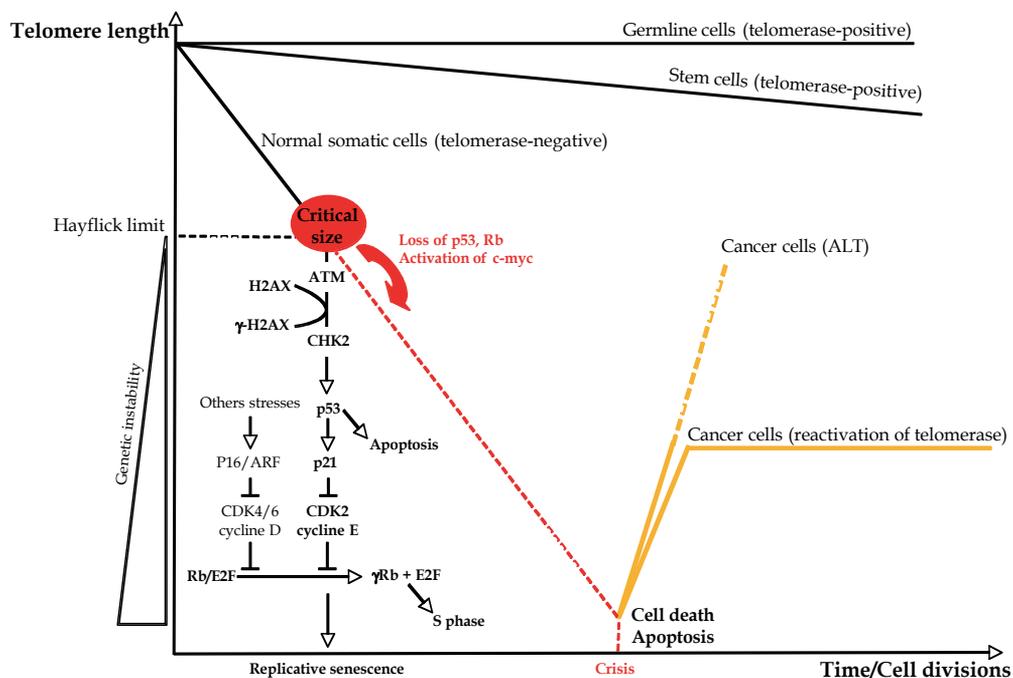


Fig. 3. Induction of replicative senescence and cellular immortalization.

1.4 Replication of telomeres

Bidirectional replication initiates at defined origins but telomeric chromatin that has the ability to form unusual structures might be a source of difficulties for the passage of the replication fork (Fouche et al., 2006). Leading strand replication occurs continuously whereas lagging strand replication is discontinuous and the DNA is synthesized *via* Okazaki fragments. Telomere replication occurs in two phases, S-phase and G₂/M phase (Verdun & Karlseder, 2006 and 2007) which implicate specific mechanisms to initiate, control and coordinate the synthesis of leading and lagging strands at the telomeres and to allow G-tail formation and recapping of the telomeres after their replication.

The telomere structure, T-loop, G-quadruplex and D-loop, cause fork progression problems (Fouche et al., 2006; Verdun & Karlseder, 2006). During the G₁ phase, the T-loop structure prevents telomeres from being recognized as DSBs. During S-phase however, T-loop opening and/or difficulties during the passage of the replication fork induce a localized DNA damage response (Verdun et al., 2005) but do not engage the downstream elements of the cascade that would lead to cell cycle checkpoint enforcement. Naturally, stalling of the replication fork at the telomeres induces an ATM/ATR-dependent DNA damage response. Proteins involved in the DNA damage response such as MRE11/RAD50/NBS, RPA,

trimeric complex of Rad9/Rad1/Hus1 and β DNA polymerase are detected at telomeres at the end of S phase (Verdun et al., 2005; Verdun & Karlseder, 2006 and 2007; Jazayeri et al., 2006; Gilson & Geli, 2007). RPA, POT1 and helicases from the RecQ family (BLM and WRN) regulated by TRF2 and POT1 could be implicated in the resolution of unusual DNA structure and G-quadruplexes on the G-rich strand (Crabbe et al., 2004; Shen & Loeb, 2001; Opresko et al., 2004 and 2005) to facilitate progression of the replication fork. ATM and MRE11 located at telomeres during G2/M phase suggest that ATM-dependent damage is required for generation of the 3' overhang at the leading telomere (Verdun & Karlseder, 2006) implicating Apollo through its 5' resection activity (Lenain et al., 2006; van Overbeek & de Lange, 2006; Dimitrova & de Lange, 2009). Apollo, a member of the SNM1/PSO2 family of nucleases, contributes to the repair of interstrand crosslink and the cleavage of hairpins during V(D)J recombination and is recruited to chromosome ends *via* interaction with TRF2 (Ye et al., 2010; Wu et al., 2010). Sfeir et al. have reported that 80% of the C-rich strands terminate in CCCAATC-5' (Sfeir et al., 2005), suggesting the actions of a specific nuclease that is POT1-dependent. Indeed POT1 is implicated in the generation of the correct sequence at chromosome ends (Hockmeyer et al., 2005). ATM-dependent signalling at telomeres in G2 is required for T-loop re-formation (Verdun et al., 2005; Verdun & Karlseder, 2006). Proteins implicated in homologous recombination (RAD51, RAD52 and XRCC3) and telomeric proteins (TRF2, TRF1 and TIN2) are recruited at telomeres to facilitate T-loop formation after replication (Stansel et al., 2001; de Lange, 2005; Verdun et al., 2005; Verdun & Karlseder, 2006; Amiard et al., 2007; Fouche et al., 2006).

1.5 Telomeres, telomerase and cancer

1.5.1 Telomere stabilization

When human cells bypass replicative senescence *via* the inactivation of p53 or Rb, they continue to divide in spite of very short telomeres leading to the deprotection of chromosome ends (Counter et al., 1994; Stewart & Weinberg, 2000; Kim et al., 2002; Londono-Vallejo, 2008). As the substrates of repair mechanisms, critically shortened telomeres are highly recombinogenic leading to the formation of dicentric chromosomes by NHEJ, anaphase bridges, engagement of the breakage-fusion-breakage (BFB) cycle (Murnane, 2006; Bailey & Murnane, 2006), and the generation of novel chromosomal variants that could lead to the emergence of a pro-cancer genome. Indeed, genomic instability driven by dysfunctional telomeres is associated with the transition from benign lesions to malignant cancer. Eventually, the number of unstable chromosomes being too high, cells cannot divide further without losing vital genetic material and thus they initiate mitotic catastrophe and die (crisis) (Figure 3). Consequently, crisis represents another powerful barrier to uncontrolled cell proliferation. To escape from death, cells that enter crisis must acquire a mechanism of telomere maintenance that is most often achieved by the re-expression of telomerase (Counter et al., 1992 and 1994) (Figure 3). In stabilizing telomere length, telomerase reactivation facilitates an indefinite replication potential, which is a hallmark of tumour cells. The reactivation of telomerase thus allows cells to stabilize their genome and divide indefinitely. Amplification of the hTERT locus and duplication/translocation of this locus has been linked to the reactivation of telomerase. In addition, the promoter of the hTERT gene is a target for numerous oncogenes (such as c-myc) or tumour suppressors (e.g. p53). The telomerase ribonucleoprotein recognizes telomeric DNA during S phase (Jady et al., 2006; Tomlinson et al., 2006) and sequentially

adds telomeric repeats to the 3' end. Telomerase then undergoes a translocation reaction to enable another round of nucleotide addition (Morin, 1989; Autexier & Lue, 2006; Hug & Lingner, 2006).

Transformed cells may also acquire alternative mechanisms of telomere maintenance (ALT) based on homologous recombination between telomeres (Figure 3) (Bryan et al., 1997; Muntoni & Reddel, 2005; Stewart, 2005). ALT cells are characterized by high level of telomeric exchange (Londono-Vallejo et al., 2004; Bailey et al., 2004; Dunham et al., 2000), highly heterogeneous telomeres and ALT-associated PML bodies colocalized with telomeres (Muntoni & Reddel, 2005; Dunham et al., 2000).

1.5.2 Telomere recombination and chromosomal instability

Dysfunctional telomeres are sensed as double strand DNA breaks which activates the DNA damage response checkpoints, including the ATM and ATR pathways (Karlseder et al., 1999; d'Adda di Fagagna et al., 2003). In human cells, the activation of ATM and ATR, members of the phosphatidylinositol 3-kinase-like kinase family, leads to the phosphorylation and activation of the central cell cycle regulator p53, CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2), which in turn facilitates G1 and G2 arrest (Shiloh, 2003; Khanna et al., 2001). ATM responds to DSBs (Shiloh, 2003) whereas ATR responds to lesions after they have been processed to single-stranded DNA intermediates (Zhou et al., 2003). Two main pathways, HR (homologous recombination) or NHEJ (non-homologous end joining), can repair DNA damage. The HR pathway is a very accurate repair mechanism because sister chromatid serves as a template to guide repair of the broken strand during the S and G2 phases of the cell cycle. NHEJ, which is potentially less accurate because two termini of broken DNA are ligated, is the prevailing repair pathway during the G1 and M phases.

The loss of telomere function can result in telomere fusion events in an NHEJ-dependent manner (Smogorzewska et al., 2002; Celli & de Lange, 2005) containing telomeric repeat DNA. Prior to replication, telomere fusion results from the linkage of the G-strand of one chromosome end to the C-strand of another chromosome (van Steensel et al., 1998; Bailey et al., 2001). These telomere fusions induce the formation of dicentric chromosome with telomere sequences at the fusion point and anaphase bridge. In contrast, after replication, telomere fusion can result from the linkage of two chromatids of different chromosomes resulting from C-strand synthesis with blunt ends or two sister chromatids leading to the propagation of the breakage/fusion/breakage (B/F/B) cycle (Fouladi et al., 2000). The B/F/B cycle is a well-established mechanism that causes genome instability leading to complex chromosomal rearrangements and cancer genome amplification. Indeed, deficiency of several proteins such as ATM, TRF2, POT1 and DNA-PKcs result in telomere fusion events that are dependent on factors involved in NHEJ (van Steensel et al., 1998; Veldman et al., 2004; Yang et al., 2005; Bailey et al., 2001; Bailey & Murnane, 2006; Gilley et al., 2001; Metcalfe et al., 1996). In addition, CO-FISH (Chromosome Orientation-Fluorescence In Situ Hybridization) analysis has shown the existence of telomere-DSB fusion (Crabbe et al., 2004; Bailey & Murnane, 2006).

Homologous recombination has been observed at dysfunctional telomeres. Indeed, recombination can occur within the T-loop structure (T-loop HR or telomere rapid deletion) (Wang et al., 2004; de Lange & Petrini, 2000), between sister telomeres (Telomere Sister Chromatid Exchange or T-SCE) (Rudd et al., 2007; Baird, 2008; Bailey et al., 2004) and

between telomere and chromosome-internal telomeric sequences leading to the formation of TDM (Telomeric DNA-containing Double Minute Chromosomes) (Zhu et al., 2003, Palm & de Lange, 2008).

Another source of telomere-driven instability is the modification of the telomere nucleoprotein complex. The best characterized of these modifications is the inactivation of TRF2 in cells, which leads to rampant uncapping and chromosome fusions in the presence of telomere repeats (van Steensel et al., 1998).

1.6 Telomeric G-quadruplexes are new targets for cancer therapy

Because telomerase is reactivated in most human tumours but not in normal human cells, it is regarded as a potential drug target. Several classes of telomerase inhibitors have now been developed and inhibit this enzyme through the targeting of its RNA (Asia et al., 2003; Herbert et al., 2005; Jackson et al., 2007; Gomez-Millan et al., 2007; Gryanov et al., 2007) or catalytic components (Ward & Autexier, 2005; El-Daly et al., 2005). However compounds that stabilize the telomeric G-quadruplex have been shown to inhibit the activity of telomerase and disrupt telomere capping and maintenance, making the human telomeric DNA G-quadruplex also an attractive target for cancer therapeutic intervention (Mergny & Helene, 1998; Riou et al., 2002; Gowan et al., 2001; de Cian et al., 2008).

Indeed, the intramolecular telomeric G-quadruplex (Neidle & Parkinson, 2003; Dai et al., 2008) has been considered to be an attractive target for anticancer drug design since quadruplex ligands were found to inhibit telomerase (Sun et al., 1997; Zahler et al., 1991; Zaugg et al., 2005). However genomic analyses using several algorithms have revealed that more than 370 000 sequences have the potential to form G-quadruplex structure in the human genome (Huppert & Balasubramanian, 2005; Huppert, 2008). Indeed, many G-rich sequences in the human genome are susceptible to the formation of a G-quadruplex (e.g. ribosomal RNA, repetitive G-rich microsatellites and the promoters of several proto-oncogenes including *c-MYC* and *c-KIT*) (Maizels, 2006; Eddy & Maizels, 2008; Huppert & Balasubramanian, 2005 and 2007; Todd et al., 2005 and 2007; Qin & Hurley, 2008; Siddiqui-Jain et al., 2002; Phan et al., 2004; Yang & Hurley, 2006; Rankin et al., 2005; Fernando et al., 2006; Shirude et al., 2006). Several proteins have been reported to interact and/or resolve such unusual DNA conformations (Hurley, 2002; Bates et al., 2007; Oganessian & Bryan, 2007; Fry, 2007) supporting the existence of a G-quadruplex. Thus, G-quadruplex ligands have to selectively bind to and stabilize telomeric G-quadruplexes to inhibit telomerase activity. Many quadruplex ligands have now been identified such as telomestatin, BRACO19, TMPyP4 and RHSP4 (Monchaud et al., 2008; Gowan et al., 2002; Han et al., 2001; Gavathiotis et al., 2003; Shin Ya et al., 2001; de Cian et al., 2008), all selected *in vitro* for their ability to interact with telomeric G-quadruplex. However, many new compounds are likely to be discovered in the future (Bates et al., 2007). In this context, we have investigated chromosomal binding and the cellular effects induced by new pyridine-derived G-quadruplex ligands of the 2,6 pyridine-dicarboxamide series in human normal and tumour cells, and also in ATM- and ATR- (Ataxia Telangiectasia-mutated and Rad3-related) deficient cells. Pyridine derivatives that function as G-quadruplex ligands were selected *in vitro* on the basis of their ability to interact with a telomeric G-quadruplex (ΔT_m values of 21 - 26 °C) and to inhibit telomerase (IC₅₀ values of 0,22 - 0,45 μ M) (Pennarun et al., 2005). Among these new compounds, 307A and 360A (Figure 4) displayed the best compromise between activity and selectivity *in vitro*. Finally, we chose 360A as it is the most toxic G-quadruplex ligand in human normal cells (Pennarun et al., 2005).

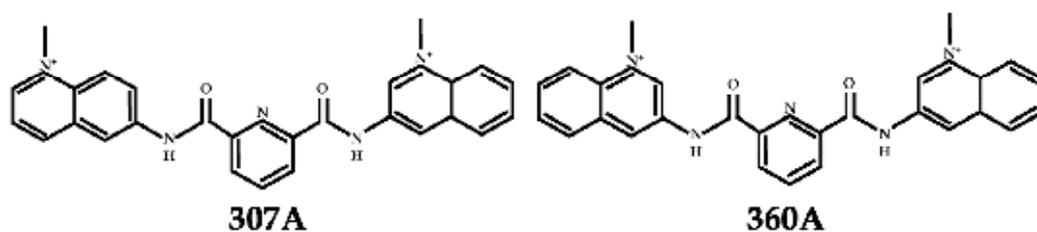


Fig. 4. Chemical formula of the G-quadruplex ligands, 307A and 360A.

2. *In vivo* binding of G-quadruplex ligand 360A

The existence of G-quadruplexes was shown *in vitro* many years ago (Wang & Patel, 1993; Parkinson et al., 2002; Neidle & Parkinson, 2003), but it has not yet been definitively demonstrated that G-quadruplexes exist *in vivo*, apart from *Escherichia coli* (Duquette et al., 2004) by electron microscopy and ciliates (Schaffitzel et al., 2001 and 2010) using antibodies specific for telomeric guanine-quadruplex DNA. The replication band, which is the region where replication and telomere elongation take place, is not stained suggesting that G-quadruplexes are resolved during replication. In human cells, G-quadruplex-interacting proteins (Oganesian & Bryan, 2007), the physiological relevance of G-quadruplexes at the telomere (Phatak & Burger, 2007; Qin & Hurley, 2008), and *in vitro* studies (Su et al., 2010; Yang et al., 2009) strongly support the *in vivo* existence of such structures at the telomere. BMVC (3,6-bis(4-methyl-4-vinylpyridinium)carbazole diiodide) has a high sensitivity and binding preference for quadruplex d(TTAGGG)₄ over duplex DNA (Chang et al., 2004). By analyzing BMVC fluorescence at the ends of metaphase chromosomes and other regions of chromosomes, Chang et al. have shown the presence of G-quadruplexes in the human genome and in telomere-proximal regions (Chang et al., 2004). G-quadruplex ligands were selected *in vitro* on the basis of their highly selective interactions with telomeric G-quadruplexes and for their potent inhibitory effects on telomerase.

Because the *in vivo* existence of G-quadruplex is still unresolved, we investigated the targeting in human normal and cancer cells by the G-quadruplex ligand using tritiated-360A (³H-360A) (Granotier et al., 2005). The selectivity of ³H-360A for G-quadruplex was first checked *in vitro* using competitive equilibrium dialysis (Granotier et al., 2005). The binding of ³H-360A to purified genomic DNA was then determined by competition experiments with various oligonucleotides and the results highlighted that ³H-360A has a preference for G-quadruplex. Interestingly, the addition of oligonucleotide that hybridized to the telomeric G-overhang decreased the binding of ³H-360A, suggesting that at least 35% of ³H-360A bound to genomic DNA at the telomeric G-overhang (Granotier et al., 2005). G-quadruplex formation would be more likely on the 3' telomeric overhang because the Watson-Crick double helix is the predominant DNA form under physiological conditions. Interaction of G-quadruplex ligand and the telomeric single-strand overhang have been reported for telomestatin (Gomez et al., 2004). In agreement with this, DMS footprinting and exonuclease hydrolysis has revealed that G-quadruplex preferentially forms at the very 3' end of the telomeric DNA (Tang et al., 2008). Using this method, it will be interesting in the future to analyze the position of the G-quadruplex stabilized by 360A. Indeed, G-quadruplex formation at the very 3' end of the chromosome may be a regulatory mechanism at the

telomeric overhang that mediates T-loop formation, DNA repair and telomere maintenance by telomerase and/or ALT.

Autoradiography has been used previously to investigate the distribution of tritium using a liquid emulsion, NTB2. Charged particles from tritium (β -rays) interact with a silver halide crystal of the autoradiography emulsion. This interaction induces the formation of a latent image within the crystal, initiating the formation of silver grains during subsequent photographic development of the emulsion (Boren et al., 1975). Thus, by autoradiography of cancer cells cultured with ^3H -360A, we were able to show previously in our laboratory that the G-quadruplex ligand enters the cells within six hours of the start of treatment and progressively accumulate in the nuclei of living cells (Figure 5A and Granotier et al., 2005). Autoradiography of metaphase spreads from cancer cells treated with ^3H -360A (T98G cells with short telomeres and CEM1301 cells with long telomeres) demonstrated that the silver grain density was significantly higher at the ends of the chromosomes than at the interstitial regions, thus highlighting that the G-quadruplex ligand preferentially bound to the terminal regions of the chromosomes (Figure 5B and 5C and Granotier et al., 2005). Interestingly, autoradiographs of the CEM1301 cells that have very long telomeres did not show a greater silver grain density at the ends of chromosomes compared with T98G cells (Figure 5C) revealing that the frequency of binding of ^3H -360A to chromosome ends does not appear to depend on telomere length and supporting the idea that ^3H -360A might be bound to telomeric overhangs (Granotier et al., 2005). The G-quadruplex ligand specifically interacts with the terminal regions of chromosomes but we cannot rule out the possibility of specific binding sites located in interstitial sequences. Indeed G-quadruplexes have been shown to exist in other physiologically important G-rich regions (Siddiquin-Jain et al., 2002; Xu et al., 2006; Sun et al., 2005; de Armond et al., 2005; Todd et al., 2007; Huppert, 2008).

RNase treatment of metaphase spreads decreased the number of silver grains (data not shown) suggesting that 360A also binds RNA, in accordance with the existence of an RNA G-quadruplex (Phan & Patel, 2003; Kumari et al., 2007; Huppert, 2008). Nevertheless, autoradiography analysis of metaphase spreads treated with RNase provided evidence that ^3H -360A binds to telomeric DNA quadruplex at the ends of chromosomes, but not to telomeric repeat-containing RNA (TERRA) (Azzalin et al., 2007; Azzalin & Lingner, 2008; Ho et al., 2008). Indeed, Azzalin et al. have shown previously that UUAGGG-containing RNA exists in cells at higher levels than complementary CCCUAA-containing RNA. High levels of UUUAGGG-containing RNA might result from the strong RNA resistance induced by G-quadruplex formation (Xu et al., 2008). Using a light-switching pyrene probe, Xu et al. have now demonstrated that human TERRA RNA forms a G-quadruplex structure in living cells (Xu et al., 2010) and hence we cannot exclude the possibility that ^3H -360A binds to the RNA G-quadruplexes located at the telomeres.

Autoradiography has revealed G-quadruplexes in interphase nuclei and in metaphase spreads, suggesting that their existence is not restricted to only one phase of the cell cycle. Telomere replication and T-loop resolution also do not seem to interfere with the formation and/or the stabilization of G-quadruplex but it is presently unclear whether a T-loop and G-quadruplex can form on the same telomere.

Silver grain densities were found again to be significantly higher at the ends of chromosomes in PHA-activated PBL cells from a healthy donor (Figure 5C), indicating the preferential binding of ^3H -360A to terminal regions of chromosomes from normal as well as cancer cells (Granotier et al., 2005).

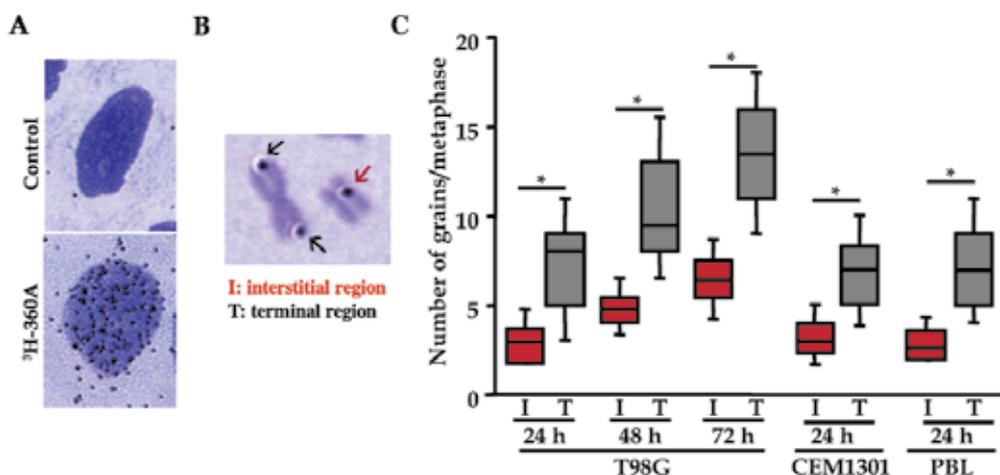


Fig. 5. Detection of ^3H -360A by autoradiography in human cancer and normal cells. A: Autoradiographs of T98G glioblastoma cells cultured without (Control) or with ^3H -360A for 24h and showing accumulation of the radioactive compound in the nuclei stained with Mayer's hemalun solution. B: Autoradiography of metaphase chromosomes from T98G cells cultured with ^3H -360A for 48 h. Black arrows indicate silver grains on the terminal regions and red arrows indicate silver grains on the interstitial regions. C: Densities of silver grains on terminal (T) and interstitial (I) regions of chromosomes from T98G (short telomeres) and CEM1301 (long telomeres) and normal cells (PBL, peripheral blood lymphocytes PHA-p stimulated). Silver grains were counted in 25 metaphases/group for the untreated control and in 50 metaphases/group for cultures incubated with ^3H -360A. I values were normalized to areas of terminal regions by dividing the total numbers of grains on the interstitial regions in each metaphase by the mean ratio of the interstitial and terminal areas estimated by Metamorph software (T98G: 2.93 ± 0.44 at 24 h, $n = 20$; 3.52 ± 0.48 at 48 h, $n = 20$ and 3.34 ± 0.29 at 72 h, $n = 20$; CEM1301: 3.03 ± 0.44 , $n = 12$; PBL: 3.64 ± 0.87 , $n = 20$). Boxes include 50% of the values centred on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile. T values were significantly greater than I values: *t-test $P < 0.0001$.

Overall, autoradiography analysis of metaphase spreads has revealed that ^3H -360A binds to telomeres, indicating G-quadruplex formation. However, this result might be an underestimation of this binding because not all the decays can be detected by this method. *In vitro* studies have indicated the polymorphic nature of the telomeric G-quadruplex (Dai et al., 2008), and the ^3H -360A ligand may therefore be unable to bind all the G-quadruplex structural conformations. In addition, ^3H -360A is naturally unstable due to progressive radiolysis, so we cannot totally exclude a partial degradation of this G-quadruplex ligand. We have shown elsewhere that tritium induces DNA damage and/or cell cycle alterations so that the metaphase spreads analyzed could be from the cells less affected by the G-quadruplex ligand. G-quadruplex elimination by the DNA repair machinery might also explain that not all telomeres are detected with silver grains.

Previous findings from our laboratory revealed that a specific G-quadruplex ligand interacts with the terminal ends of human chromosomes and support the hypothesis that G-quadruplex ligands induce and/or stabilize G-quadruplex structures at the telomeres in

both human normal and cancer cells. Fluorescence titration experiments with oligonucleotides have demonstrated that 360A might actively induce the formation of a tetramolecular quadruplex, acting as a chaperone for the association of the four strands (de Cian & Mergny, 2007), therefore highlighting that 360A triggers G-quadruplex formation and locks it into a preformed structure. We have also shown by autoradiography that the G-quadruplex ligand (360A) binds preferentially to the terminal regions of chromosomes in tumour and normal cells (Granotier et al., 2005). This led us to investigate the cellular effects induced by 360A in cancer cells.

3. Cellular effects induced by the G-quadruplex ligand 360A in human cancer cells

3.1 G-quadruplex ligands reduce viability and induce apoptosis in both telomerase-positive and ALT cancer cells

We have reported previously that pyridine derivatives displaying strong selectivity for G-quadruplex structures inhibit cell proliferation in telomerase-positive tumour cells and induce apoptosis after more than three population doublings (Pennarun et al., 2005 and 2008). This is similar to the effects of other G-quadruplex ligands (telomestatin, TMPyP4, RHSP4 and BRACO19) (Phatak & Burger, 2007; Tahara et al., 2006; Gomez et al., 2004; Incles et al., 2004; Gowan et al., 2002). In these earlier studies, cancer cells were treated with several doses of G-quadruplex ligands (ranging from 1 to 5 μ M for telomestatin, RHSP4 and BRACO19 and from 50 to 100 μ M for TMPyP4). Comparisons of the effects of these ligands is problematic however as they were evaluated in different cancer cell lines. It will be important therefore to compare the inhibition of cell proliferation by various G-quadruplex ligands in the same cancer cell types.

3.1.1 The action of the G-quadruplex ligands is independent of telomerase inhibition

We have shown that 360A blocks cell proliferation and induces apoptosis in ALT tumour cells, suggesting that this G-quadruplex ligand does not only inhibit telomerase activity (Pennarun et al., 2005). Consistently, other G-quadruplex ligands (TMPyP4, RHSP4, BRACO19) have also been shown to inhibit cell proliferation in ALT cells (Kim et al., 2003; Gowan et al., 2001; Incles et al., 2004; de Cian et al., 2008), although telomestatin does not do so (Kim et al., 2003). Moreover, the overexpression of hTERT or a dominant-negative of hTERT in telomerase-positive cell lines does not modify the anti-proliferative effects of the triazine derivative, 12459, (de Cian et al., 2008) indicating that the suppression of cell growth by G-quadruplex ligands is independent of its function in telomerase inhibition. Consistent with this, we have further shown that active pyridine derivatives require several rounds of replication to induce cell growth arrest.

Flow cytometry analysis have shown that the treatment of cancer cells with a G-quadruplex ligand induces a marked decrease in the percentage of cells in G0/G1 phase, dramatically increases the percentage of cells in S phase and also in sub-G1, indicating that 360A induces cell death in cancer cells. The detection of TUNEL-positive cells (Pennarun et al. 2005) in 360A-treated telomerase positive- and ALT cells confirmed that this G-quadruplex ligand induces apoptosis in human cancer cells regardless of the status of their telomere maintenance mechanism.

The effectiveness of the G-quadruplex ligand depends more on its specificity for the G-quadruplex structure. *In vitro* studies have found that 360A more actively induces the G-

quadruplex structure compared with other G-quadruplex ligands tested (telomestatin, TMPyP4 and BRACO19) (de Cian & Mergny, 2007). G-quadruplex ligands lock into the G-quadruplex structure but this situation is complicated by the existence of a number of different G-quadruplex structures (dimeric, tetrameric, intra- or inter-molecular). TMPyP4, that facilitates the formation of intermolecular G-quadruplex structures, suppresses the proliferation of ALT cells and telomerase-positive cells whereas telomestatin, which promotes the formation of intramolecular G-quadruplex, only suppresses the proliferation of telomerase-positive cells (Kim et al., 2003). TMPyP4 induces anaphase bridges in sea urchin embryos whereas telomestatin does not have this effect (Kim 2003) suggesting that the selectivity of G-quadruplex ligands for intra- or inter-molecular structures is important for their biological effects. It will be interesting in the future to analyze the selectivity of 360A by electrophoretic mobility shift assay (Kim et al., 2003) or by fluorescent screening method (Paramasivan & Bolton, 2008) using multiple reporter molecules that bind to different features of quadruplex DNA.

3.1.2 360A does not induce telomere shortening but provokes telomeric overhang degradation

360A was found not to induce telomere shortening in our previous analysis (Pennarun et al., 2005). Indeed, Southern blot analysis has revealed no significant change in the mean TRF (Telomere Restriction Fragment) lengths in cells treated with this G-quadruplex ligand, although the possibility that this treatment may have caused slight telomere erosion could not be excluded. In contrast, BRACO19 and TMPyP4 treatments shorten telomeres whereas telomestatin and RHPS4 do not do so. In highlighting that G-quadruplex ligands do not act as telomerase inhibitors these data suggest that they induce telomere deprotection. The inhibition of cell proliferation induced by G-quadruplex ligands in ALT cells and in CEM1301 cells with long telomeres (data not shown) confirms that G-quadruplex ligand targets telomeres independently of any effects upon telomerase activity.

In any case, the inhibition of cancer cell proliferation is not due to telomere shortening induced by G-quadruplex stabilization but could be triggered by telomeric overhang degradation. Indeed, non-denaturing hybridization assays have revealed that a pyridine derivative induces an alteration of the length of telomeric G-overhang in cancer cells (Pennarun et al., 2005) in a manner similar to G-quadruplex ligands. Taken together, these data suggest that G-quadruplex ligands destabilize telomere structures in human cancer cells.

3.2 G-quadruplex ligands induce telomere deprotection and telomere aberrations in cancer cells involving lagging strand telomeres

Giemsa staining of metaphase spreads from 360A-treated cancer cells has shown that G-quadruplex ligands induce the formation of dicentric or ring chromosomes and anaphase bridges in telomerase-positive cancer cell lines (Pennarun et al., 2005). This confirms that pyridine derivatives acts at the telomeres and induce telomeric instability that is comparable to deprotected telomeres. Detection of chromosome dicentrics in 360A-treated CEM1301 cells with long telomeres has further indicated that genetic instability is not provoked by critically short telomeres providing new evidence that G-quadruplex ligands modify telomere structures.

Indeed, we have shown by Telomere-Fluorescence *in situ* hybridization (Telo-FISH) and Chromosome Orientation-FISH (Bailey et al., 2004; Crabbe et al., 2004), that the G-quadruplex ligands induce specific telomere aberrations (sister telomere fusions, sister

telomere losses and telomere doublets) either during or after replication (Figure 6) (Pennarun et al., 2008 and 2010). Telomere aberrations induced by a G-quadruplex ligand mainly consist of sister telomere fusions and also recombination preferentially involving the lagging strand telomeres (Pennarun et al., 2008).

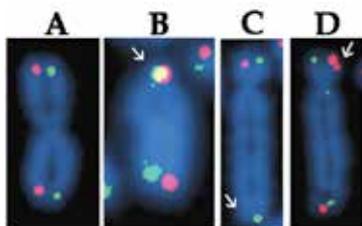


Fig. 6. Telomere aberrations revealed by CO-FISH in human cancer cells. Lagging strand telomeres are labelled in red and leading strand telomeres in green. Representative examples of a control chromosome (A), a sister telomere fusion (B), a sister telomere loss involving the lagging strand (C) and a telomere doublet involving the lagging strand (D) are shown.

Delocalization of telomeric proteins (for example POT1 and TRF2) from the telomere induced by 360A (Figure 7A and 7B) and other G-quadruplex ligands (Tahara et al., 2006; Gomez et al., 2006; Salvati et al., 2007; Rizzo et al., 2009) and telomere aberrations induced by 360A in cancer cells confirmed that those compounds provoke telomere deprotection events and not just the inhibition of telomerase. Rizzo et al. have provided evidence that RHSP4 damages telomeric chromatin during replication (Rizzo et al., 2009), confirming the results from our laboratory.

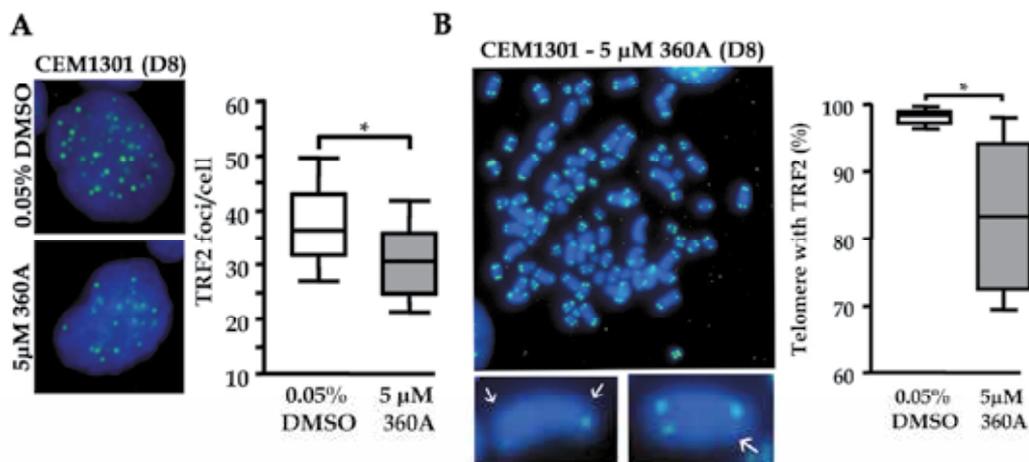


Fig. 7. TRF2 delocalization from the telomere revealed by immunostaining with anti-TRF2 antibody (green) in CEM1301 cancer cells treated for 8 days with 5 μ M 360A or 0.05% DMSO. Nuclei and metaphase chromosomes were counterstained with DAPI (blue). Histograms show the mean number of TRF2 foci per nucleus (A) and the mean number of telomeres on metaphase chromosomes with TRF2 foci \pm SE (B).

360A has been shown to stabilize the G4 structure within the TRF2 mRNA thereby repressing the expression of this telomeric protein (Gomez et al., 2010). By interacting with supercoiled DNA and participating to the formation and to the dissolution of the T-loop, TRF2 is essential for telomere replication. Therefore the decrease of TRF2 may reduce the presence of this telomeric protein at telomeres leading to dysfunctional telomeres.

Taken together, these data reveal that G-quadruplex ligands inhibit cell proliferation and stimulate apoptosis by inducing a broad spectrum of telomere aberrations and telomere deprotection in human cancer cells.

3.3 Increase in telomere aberrations in 360A-treated ATM- or ATR-deficient cells

Deprotection of the telomere is known to induce DNA damage response pathways leading to cell cycle arrest, genomic instability, apoptosis or senescence. In addition, several DNA damage response factors (e.g. ATM) are involved in telomere replication and in the formation of a T-loop in the S and G2 phases. ATR, another key protein involved in the DNA damage response, is essential for maintenance of genomic stability. ATR belongs to a family of phosphatidylinositol 3-kinase-like kinases, which includes ATM (Cimprich & Cortez, 2008). ATM is primarily activated by DNA double-stranded breaks, whereas ATR responds to a broad spectrum of DNA damage events, in particular those interfering with DNA replication. In that context, we investigated the cellular effects of 360A in ATM- and ATR-deficient cells. In cancer cells, immunofluorescence staining showed that 360A induces DNA damage signals (γ -H2AX foci) in a strictly ATM-dependent manner (Pennarun et al., 2008) similar to telomestatin (Tauchi et al., 2003), thus showing that G-quadruplex stabilization induces telomere deprotection. However, in cells treated with 360A, nuclear foci of 53BP1 only partly co-localize with PNA telomeric signals, indicating that G-quadruplex ligands, such as telomestatin (Bates et al., 2007), induce other DNA damage events at interstitial sites.

We have further characterized the biological effects of 360A in stable cells expressing siRNA molecules that target ATM or ATR. We have found that ATM, which is involved in the cellular response to DNA damage, strongly suppresses 360A-induced telomere instability (Pennarun et al., 2008). The increase in telomere aberrations in 360A-treated ATM deficient cells were found not to be related to defects in cell cycle checkpoints or to apoptosis induction, suggesting a direct role of ATM in preventing inappropriate DNA repair at the telomeres.

We have further reported that an ATR deficiency causes telomere instability both in primary human fibroblasts from Seckel syndrome patients and in HeLa cells (Pennarun et al., 2010). Telomere aberrations resulting from an ATR deficiency are mainly generated during and/or after telomere replication and involve both leading and lagging strand telomeres. Moreover, we have demonstrated that an ATR deficiency strongly sensitizes cells to the effects of the G-quadruplex ligand 360A, leading to enhanced sister fusions and chromatid-type telomere aberrations involving specifically the lagging strand telomere. RHSP4 has also been found to induce an ATR dependent ATM signalling (Rizzo et al., 2009) consistent with our results. These data emphasize that ATR also plays a critical role in telomere maintenance during and/or after telomere replication in human cells.

The increase in telomere instability that we observed in ATM- and in ATR-deficient cells treated with G-quadruplex ligand indicates the importance of ATM and ATR and suggests that key proteins implicated in the DNA damage response also contribute to telomere stability during and/or after replication, thus playing an important role in human telomere maintenance.

4. Effects of the G-quadruplex ligand 360A in human normal cells

G-quadruplex ligands inhibit cellular proliferation, and provoke cell cycle arrest and apoptosis by inducing telomere aberrations and telomere deprotection, i.e. they do not only function as a telomerase inhibitor. We previously observed that after seven days of treatment, no effects of G-quadruplex ligands could be found in primary astrocyte cultures (Pennarun et al., 2005), suggesting a differential effect of these factors in cancer and normal cells. In most somatic cells, telomerase activity is not detectable whereas telomerase is expressed in highly proliferative cells such as germ cells and stem cells. In this context, we investigated the cellular effects induced by 360A in human telomerase-positive (PBL, peripheral blood lymphocytes PHA-p stimulated) and telomerase-negative normal cells (NHF27 fibroblasts). It is noteworthy however that in normal fibroblasts, a transient expression of hTERT and very low telomerase activity can be detected during S phase (Masutomi et al., 2003). The telomerase activity detected in primary fibroblasts during S phase is considerably lower than that in cancer cells and other normal cells (such as activated lymphocytes). In normal cells, telomere maintenance by telomerase activity impedes replicative senescence but telomerase activity decreases with age, thereby inducing a telomere shortening.

4.1 The G-quadruplex ligand 360A marginally represses the proliferation of normal cells and can induce premature senescence

We previously observed that after seven days of treatment, no effect of G-quadruplex ligands was evident in primary astrocyte cultures (Pennarun et al., 2005). This result was confirmed by assessing the effects of this treatment on the population doublings in long-term cultures. Peripheral blood lymphocytes isolated from 15 healthy volunteers, and stimulated with PHA-p and IL2 (Interleukine 2) were treated with 360A. For long-term exposure studies, these cells were grown in flasks and exposed to 360A at various concentrations (1, 5 and 10 μM). Control cells were treated with the corresponding concentrations of DMSO (0.01, 0.05 and 0.1%). Every 3-4 days, the cells were counted using trypan blue, and then reseeded with fresh medium containing a new dose of compound. Indeed, the 1 μM dose of 360A was found not to decrease the rate of PBL proliferation (Figure 8), whereas this concentration dramatically reduced proliferation and induced cell death in cancer cells (Pennarun et al., 2005; 2008 and 2010). Higher concentrations of 360A (5 and 10 μM) slightly decreased PBL proliferation in a donor-dependent manner (Figure 8).

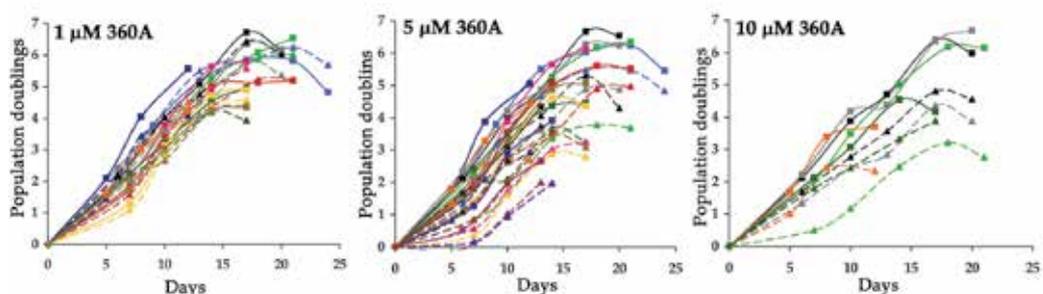


Fig. 8. Cell growth curves for PBLs isolated from the fresh blood samples of normal volunteers (one colour per donor) and cultured with 1, 5 and 10 μM 360A (▲) or the corresponding concentrations of DMSO (■).

In telomerase-positive cancer cells, 360A induces apoptosis after 12-14 days, (2-5 population doublings) (Pennarun et al., 2005; 2008 and 2010). After 14 days of treatment, DMSO had no effect on the mean population doubling time of PBLs (4.94 ± 0.55 for 0.01% - 4.96 ± 0.46 for 0.05% and 4.77 ± 0.33 for 0.1%), whereas 360A slightly decreased this rate (4.52 ± 0.36 for 1 μM , 3.31 ± 0.31 for 5 μM and 2.90 ± 0.59 for 10 μM). This result emphasizes that the lack of apoptosis in 360A-treated normal cells is not linked to their reduced cell proliferation compared with cancer cells. Autoradiography analysis have previously demonstrated the preferential binding of the G-quadruplex ligand to the chromosome ends of PBLs (Granotier et al., 2005), thus providing evidence that the differential effects of the G-quadruplex ligand in human normal cells is not linked to the lack of binding of 360A.

We found that 360A slightly decreases the rate of PBL proliferation without inducing cell cycle arrest (analyzed by propidium iodide staining and FACS, Figure 9). Determination of the cellular DNA content by flow cytometry shows that the G-quadruplex ligand induces a gradual increase in the percentage of cells in G1 phase over time in treated populations of PBLs (Figure 4). By TUNEL staining and sub-G1 quantification by FACS we have further found that 360A does not induce cell death in PBLs. Indeed, our TUNEL assay results reveal less than 5% of PBLs treated with 1, 5 and 10 μM 360A or the corresponding concentrations of DMSO for 13 days are apoptotic (data not shown).

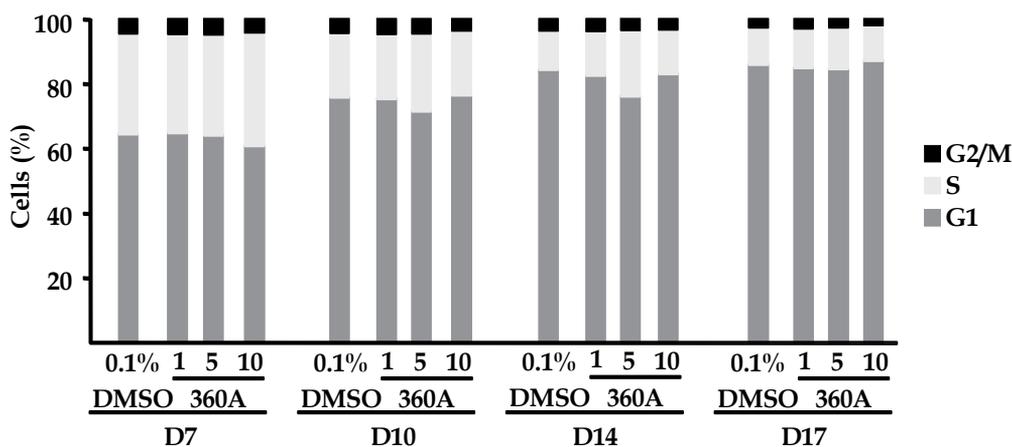


Fig. 9. Cell cycle analysis of PBLs cultured with 1, 5 and 10 μM 360A or 0.1% DMSO for 7, 10, 14 and 17 days. The percentages of cells in different phases of the cell cycle are expressed with respect to the total number of viable cells (corresponding to an analysis of 10^5 cells). Histograms are representative of two independent experiments.

In telomerase-negative normal cells, a WST assay revealed a reduced effect of 360A in primary NHF27 fibroblasts ($\text{IC}_{50} > 16 \mu\text{M}$) so we further characterized the biological effects of 360A by assessing population doublings in long-term cultures. Primary fibroblasts were treated with 1 and 5 μM 360A or the corresponding concentrations of DMSO (Figure 10A). A 20 day treatment with 1 and 5 μM of 360A slightly reduced the proliferation of primary fibroblasts in a dose-dependent manner without inducing cell death. After 50 days of treatment, 360A reduced the mean doubling population of primary fibroblasts (6 and 18 population doublings for 360A- and DMSO-treated NHF27 respectively). Moreover, telomerase-negative primary fibroblasts treated with 1 μM of 360A can be maintained in culture for 50 days without cell

death, highlighting the fact that 360A decreases cell viability and/or cell proliferation in telomerase-positive cancer cell lines, whereas telomerase-positive and telomerase-negative human normal cells are more resistant to G-quadruplex ligand treatment.

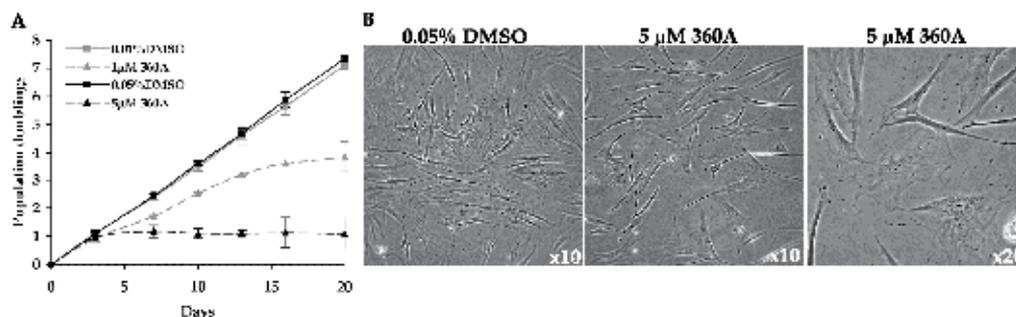


Fig. 10. Proliferation and morphology of primary fibroblasts treated with G-quadruplex ligand 360A or DMSO. A: Representative cell growth curves for primary fibroblasts cultured with various concentrations of 360A (1 and 5 μ M) or the corresponding concentrations of DMSO (0.01% and 0.05%). Mean population doublings from duplicate cultures \pm standard deviation, are representative of four independent experiments. B: Phase-contrast micrographs showing the cellular morphology of primary fibroblasts treated with 0.05% DMSO (left, *10 objective) or 5 μ M 360A (middle, *10 objective and right, *20 objective).

Light microscopy revealed a decrease in the cell density and morphological changes that became detectable after about 20 days of treatment. Indeed, treated primary fibroblasts exhibited a typical senescent phenotype with a flattened and enlarged cell shape (Figure 10B). By propidium iodide staining and FACS analysis, we analysed the progression of primary fibroblasts treated with 1 and 5 μ M of 360A or the corresponding concentrations of DMSO for 10, 14, 22 and 29 days. The G-quadruplex ligand was found not to increase the percentage of cells in S-phase as in cancer cells populations, or the percentage of cells in G1-phase such as in PBLs but did increase the percentage of cells in G2/M phase (Figure 11) without increasing the percentage of cells in sub-G1 phase. This confirmed the lack of a cell death in 360A-treated primary fibroblasts.

The induction of senescence was confirmed by the detection of senescent associated β -galactosidase-positive cells at pH6.0, an established senescence marker (Dimri et al., 1995) (Figure 12A).

At day 19, we detected 35.4 ± 15.2 and $61.7 \pm 2.3\%$ of senescent cells in primary fibroblast cultures treated with 1 and 5 μ M 360A, respectively, and a $1.8 \pm 0.6\%$ of senescent cells in DMSO-treated primary fibroblasts (Figure 12B), thus showing that G-quadruplex ligand does not induce apoptosis but premature senescence in normal cells in a dose-dependent manner. Some G-quadruplex ligands (e.g. telomestatin, BRACO19, RHPS4) but nor all (TMPyP4) show selective toxicity towards cancer cells (Gomez et al., 2006; Cheng et al., 2007; Salvati et al., 2007; Rha et al., 2000). Indeed TMPyP4 inhibits the cell proliferation of normal cells (fibroblasts, adult keratinocytes and epithelial cells) and cancer cells (neuroblastoma, breast cancer, cervical cancer, pancreatic cancer, colon cancer and a prostate cancer cell line) (Rha et al., 2000). The slow growth of normal cells versus the rapid proliferation of tumour cells may be involved in the selective toxicity of G-quadruplex ligand. But we have found that 360A does not induce cell death in normal cells and Salvati

et al, have further reported that highly proliferating human peripheral blood lymphocytes are resistant to RHPS4 suggesting that growth rate is not the main cause of G-quadruplex ligand tumour selectivity (Salvati et al., 2007).

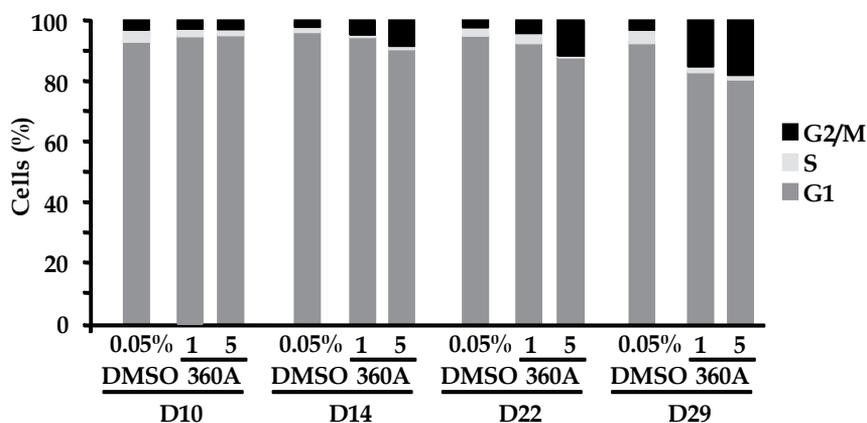


Fig. 11. Cell cycle analysis of primary fibroblasts cultured with 1 and 5 μM 360A or 0.05% DMSO for 10, 14, 22 and 29 days. The percentages of cells in different phases of the cell cycle are expressed with the respect to the total number of viable cells (corresponding to an analysis of 10^5 cells). Histograms show the mean of duplicate cultures and are representative of three independent experiments.

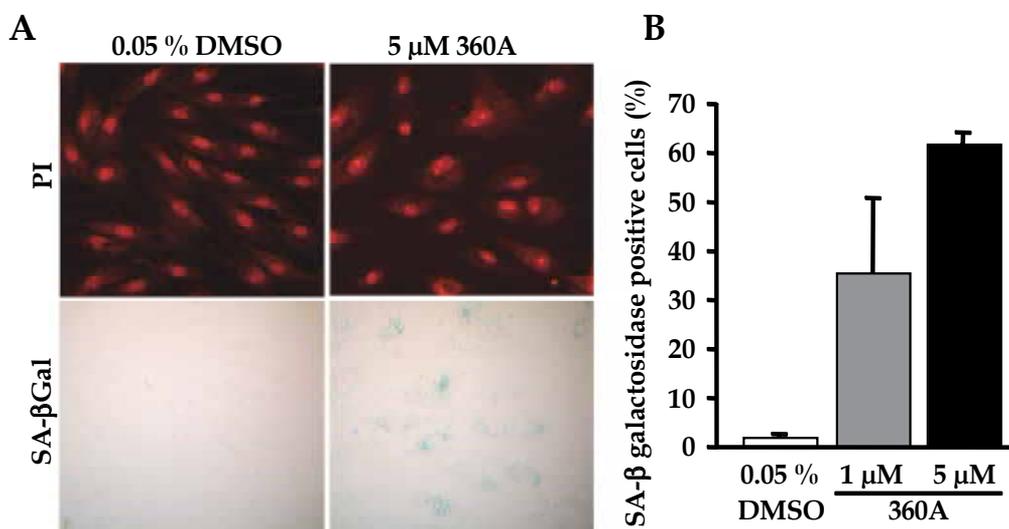


Fig. 12. Premature senescence induced by G-quadruplex ligand in primary fibroblasts cultured with 1 or 5 μM 360A or the corresponding concentration of DMSO for 19 days. A: Senescent associated- β galactosidase staining (blue) of senescent cells. Nuclei were stained with propidium iodide (red). B: Histograms showing the percentage of senescent cells (150-550 nuclei analyzed per condition).

G-quadruplex ligands induced premature senescence in primary fibroblasts but no cell death. Masutomi et al. have detected low levels of telomerase activity in primary fibroblasts during S phase. The disruption of telomerase activity in human primary fibroblasts slows down proliferation, alters the maintenance of the 3' single-stranded telomeric overhangs and induces senescence without cell death (Masutomi et al., 2003) suggesting that the periodic expression of hTERT benefits cell proliferation and delays the onset of replicative senescence. Because 360A induces a premature senescence, this G-quadruplex ligand may inhibit transient telomerase activity in normal cells. It was therefore of interest to evaluate the transient telomerase activity levels in 360A-treated primary fibroblasts during S phase. To this end, we analyzed BJ-TERT cells, primary human fibroblasts with long telomeres immortalized through the stable expression of the catalytic component of human telomerase (Jiang et al., 1999). Our results showed that 360A-treated BJ-TERT cells are more resistant to this ligand than primary fibroblasts. Indeed, BJ-TERT cells treated with 5 μM of 360A can be maintained in culture for 34 days without any evidence of premature senescence (<5% of senescent cells) confirming the link between telomerase activity and G-quadruplex resistance in normal cells.

We have previously shown by autoradiography that 360A binds preferentially to the terminal regions of chromosomes in human cancer and normal cells (Granotier et al., 2005), supporting the hypothesis for the *in vivo* formation of G-quadruplexes at the telomeres of these cells. The differential effects of 360A in normal and cancer cells are not linked to the lack of a G-quadruplex and/or the lack of 360A binding in normal cells. Nevertheless, we cannot at this stage exclude G-quadruplex structure variation and/or the differential regulation of G-quadruplexes during cell cycle progression between normal and cancer cells. *In vitro* studies using oligonucleotides have given some insights into the structural polymorphisms of the G-quadruplex. (Hurley, 2002; Bates et al., 2007; Oganessian & Bryan, 2007; Dai et al., 2008). These polymorphisms and the dynamic equilibrium of telomeric G-quadruplexes, between human normal and cancer cells, may be important for considerations for the design of future therapeutic interventions.

Finally, we analyzed the proliferation of cancer cells (human glioblastoma, T98G) and normal cells (primary fibroblasts, NH27) at the end of a 360A treatment. T98G cells and primary fibroblasts were cultured with 1 and 5 μM 360A or the corresponding concentration of DMSO for several days (4 and 7 days for T98G, 15 and 22 days for NHF27). After the treatment period had ended, T98G and NHF27 cells were cultured in medium without G-quadruplex ligand until day 20 for T98G and day 50 for NHF27 (Figure 13).

After treatment with 1 μM 360A, we observed a resumption of proliferation in T98G cells treated for 4 and 7 days (Figure 13A) and in NH27 cells treated for 15 and 22 days (Figure 13B). After a treatment with 5 μM 360A, elimination of the G-quadruplex ligand at day 4 provoked a resumption of proliferation in cancer cells, but elimination of 360A at day 7 did not do so (Figure 13C). However, elimination of this G-quadruplex ligand at the 5 μM dose did induce the resumption of proliferation, even after 15 days of treatment (Figure 13D). In primary fibroblasts, the percentage of senescent cells decreased after the end of treatment, when we detected the resumption of proliferation (data not shown). We found that 360A also induced reversible premature senescence in primary fibroblasts but provoked apoptosis in cancer cells. Replicative senescence is thought to be an essentially irreversible growth arrest phenomenon but a p53-dependent reversible senescence pathway has been identified in normal cells as a response to dysfunctional telomeres (Beausejour et al., 2003; Dirac & Bernards, 2003). It will be of interest to study the implications of p53 function in normal cells treated with G-quadruplex ligand.

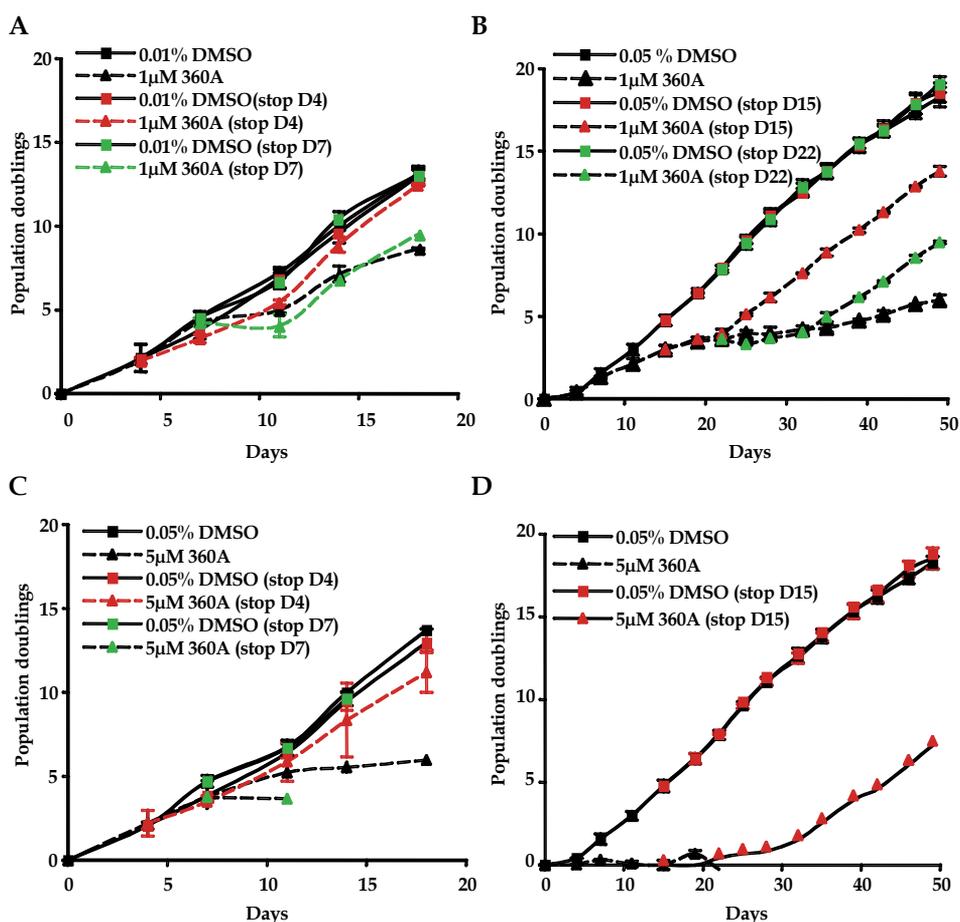


Fig. 13. Cell growth curves for T98G glioblastoma cells (A and C) and NHF27 primary fibroblasts (B and D) cultured with $1\mu\text{M}$ 360A (top), $5\mu\text{M}$ 360A (bottom) or the corresponding concentrations of DMSO. Cells were analyzed after the end of treatment at day 4 (stop D4) or day 7 (stop D7) for T98G cells and day 15 (stop D15) or day 22 (stop D22) for NHF27 cells. Mean population doublings from duplicate populations \pm standard deviation are shown.

4.2 The G-quadruplex ligand 360A does not induce telomere instability in normal human cells

To gain further insight into the cellular effects of 360A in primary human normal cells, we analyzed genetic stability as an endpoint. We have previously shown that G-quadruplex ligands induce dicentric and ring chromosomes in telomerase-positive cancer cell lines (Pennarun et al., 2005). However, Giemsa staining of PBL chromosomes did not reveal chromatid breaks or dicentric chromosomes, suggesting that 360A does not induce chromosome instability in PBLs at a 1, 5 or $10\mu\text{M}$ dose for 7, 10 and 15 days. Giemsa staining of primary fibroblasts chromosomes treated with $5\mu\text{M}$ 360A for 7 days confirmed that this G-quadruplex ligand does not induce chromosome instability in normal cells.

We further characterized telomere stability in 360A-treated PBL and primary fibroblasts by telo-FISH after a 7 day treatment. In PBLs, we detected telomere aberrations (sister telomere fusions, sister telomere losses and telomere doublets) but their frequency remained stable in G-quadruplex ligand-treated normal cells (Figure 14A). We detected $9.1\% \pm 1.3$ chromosomes with telomere aberrations in 360A-treated PBLs ($n=1387$ chromosomes) and $9.9\% \pm 1.1$ chromosomes with abnormal telomeres in DMSO-treated PBL ($n=1385$ chromosomes), indicating that 360A does not induce telomere instability in normal cells but does so in cancer cells (Pennarun et al., 2008 and 2010). It is noteworthy that the frequency of telomere aberrations in normal cells was found substantially lower than that in cancer cells. Indeed, Telo-FISH analysis revealed 2.98 ± 0.36 damaged telomere in 360-treated PBLs and 2.98 ± 0.34 in DMSO-treated PBLs (Figure 14B), highlighting that this G-quadruplex ligand does not induce telomere aberrations in human primary peripheral cells. Autoradiography analysis of metaphase spreads of PBLs treated with ^3H -360A further demonstrated the preferential binding of this G-quadruplex ligand to the terminal regions of chromosomes (Granotier et al., 2005) suggesting that the selective toxicity of 360A is not due to its lack of binding to telomeres in normal cells.

Telo-FISH of primary fibroblast cultures treated with 360A for seven days (data not shown) further confirmed that this G-quadruplex ligand does not induce telomere instability in human normal cells, in contrast with the cancer cells. RHPS4, is another G-quadruplex ligand that does not induce telomere aberration in normal fibroblasts (Salvati et al., 2007) further suggesting that the telomere organization and the cellular response to damaged telomeres is substantially different between normal and cancer cells.

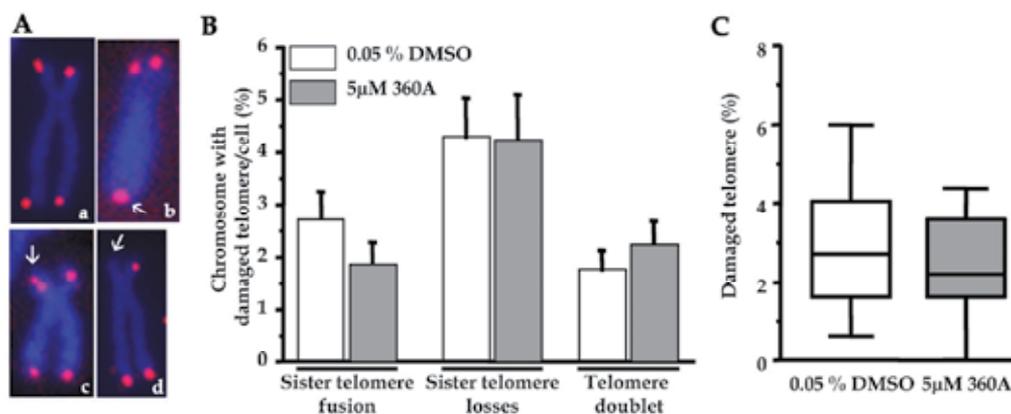


Fig. 14. The G-quadruplex ligand 360A does not induce telomere aberrations in primary blood lymphocytes at a $5 \mu\text{M}$ 360A dose in a 7 day culture. A: Example of telomere evidenced by Telo-FISH in normal cells (a-d): control chromosome (a), sister telomere fusion (b), telomere doublet (c) and sister telomere loss (d). B: Histograms showing the percentage of chromosomes with telomere aberrations (sister telomere fusions, sister telomere losses, and telomere doublets) \pm standard error ($n=32-34$ metaphases analyzed per condition). C: Box graphs showing the percentage distribution of damaged telomeres in DMSO- and in 360A-treated PBLs for seven days. Boxes include 50% of the values centred on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile.

5. Discussion

Taken together, our results demonstrate that even at elevated concentrations, the G-quadruplex ligand 360A has limited effects on the proliferation of normal cells, in which they do not induce apoptosis. Interestingly, we found that 360A induces reversible premature senescence in primary human fibroblasts. We mainly observed that this G-quadruplex ligand does not induce telomere aberrations in normal human telomerase-positive and telomerase-negative cells, highlighting the differential effects of this ligand in human normal and cancer cells (Table 1).

The cellular effects induced by this G-quadruplex ligand suggest that the organization and/or cellular responses activated by damaged telomeres differ significantly between human normal and cancer cells.

5.1 Telomere organization in the nucleus

Telomere organization is cell cycle dependent, with the assembly of telomeres into a telomeric disk in the centre of the nucleus occurring in G2 phase in normal cells (primary human lymphocytes, primary human fibroblasts and normal human epithelial tissue) (Chuang et al., 2004). Telomeres are widely distributed throughout the nucleus in the G0/G1 and S phases. Moreover, the three dimensional telomere organization is distorted in tumour cells and telomeric aggregates are thus formed. Transient telomeric aggregations that potentially cause irreversible chromosomal rearrangements are suggestive of genomic instability. The spatial organization and distribution of telomeres in the nucleus, and during the cell cycle, are important for genomic stability. On the other hand the telomere organization in the nucleus of cancer cells could promote the chromosome instability induced by G-quadruplex ligands. Hence, it will be very interesting in the future to investigate telomere organization in normal and cancer cells treated with G-quadruplex ligands to evaluate whether G-quadruplex stabilization disrupts the three dimensional arrangement of the telomere organization in the nucleus.

5.2 Telomeric 3' overhang length

At the very ends of the chromosomes, the telomeric 3' overhang is critical for telomere elongation by telomerase, for forming the T-loop structure, and therefore for normal telomere function and genome stability (Rahman et al., 2008). Most cancer cell lines possess shorter overhangs whereas most normal cells have longer 3' overhangs (Lee 2008). The size difference of the telomeric 3' overhang between normal and cancer cells could explain the differential effects of the G-quadruplex ligand. Stabilization of G-quadruplexes by specific ligands along a long 3' overhang in normal cells would not prevent the formation of the T-loop. We can also speculate that G-quadruplex resolution by endonucleases and/or helicases would leave a sufficiently long overhang for normal telomere function. In this regard we have previously shown that 360A reduces the telomeric overhang in cancer cells (Pennarun et al., 2005). It will be now interesting to measure the 3' overhang in human normal cells treated with G-quadruplex ligand using the previously described oligonucleotide ligation assay (Cimino-Reale et al., 2001). The telomerase inhibitor telomestatin, which is known to stabilize G-quadruplexes, preferentially inhibits the growth of cancer cells compared to normal cells (fibroblasts and epithelial cells). However, telomestatin does not inhibit cell growth, does not reduce the telomeric overhang and does not induce the dissociation of TRF2 from telomeres in normal and hTERT-positive

	Pyridine derivative G-quadruplex ligand (360A)		References
	Human cancer cells	Human normal cells	
Preferential binding to telomeres	Telomerase positive *	Telomerase positive (Peripheral blood lymphocytes)	
Cell proliferation	yes	yes	Granotier 2005
Cell cycle	significant decrease	slight decrease	Penmarun 2005 and 2008
Apoptosis	accumulation in S	no cell cycle arrest	Penmarun 2005 and 2008
Senescence	yes	no	Penmarun 2005
DNA damage signaling	no	n.d.	Penmarun 2005
Telomere shortening	yes	no	Penmarun 2008
Shortening of the 3' overhang	no	n.d.	Penmarun 2005
Delocalization of telomeric proteins	yes	n.d.	Penmarun 2005
Telomere aberrations	yes	n.d.	Penmarun 2005
*: human glioma (T98G, CBI93 and UJ18-MG), SV40-transformed human normal fibroblast (A53WT2), SV-40 transformed ataxia-telangiectasia fibroblast (GM09607), EBV-transformed lymphocytes derived from normal (GM03657) and ataxia-telangiectasia donors (GM03189), cervical carcinoma (HeLa)	no	no	Penmarun 2008 and 2010
n.d.: not determined			

Table 1. Comparison of the cellular effects of the G-quadruplex ligand 360A in human normal and cancer cells.

fibroblasts (Tahara et al., 2006). We can thus hypothesize that there is a link between the lack of reduction of the telomeric 3' overhang length, the TRF2 stability at telomeres and resistance to the effects of G-quadruplex ligands.

5.3 Telomeric proteins

Variations in the number of telomeric proteins fixed to double- or single-stranded telomeric DNA can modulate the sensitivity of cells to G-quadruplex ligands. In cancer cell lines, we have previously shown that 360A induces TRF2 delocalization from the telomeres but we don't yet know if 360A also induces TRF2 delocalisation in normal cells. In any event, the G-quadruplex ligands telomestatin and RHSP4 have been shown to induce TRF2 and POT1 delocalization from the telomeres only in cancer cells (Tahara et al., 2006; Zaug et al., 2005; Salvati et al., 2007; Bates et al., 2007) without inducing TRF1 delocalization. The differential effects of telomestatin on TRF1 and TRF2 could be explained by their DNA-binding activities. Indeed, TRF1 binds to the telomere more strongly than TRF2 (Hanaoka et al., 2005). The differential effects of telomestatin on TRF2 localization in cancer and normal cells could therefore be linked to different DNA-binding activities between these cell types.

The telomere-disrupting agent telomestatin also induces a DNA damage response and apoptosis in newly generated neurons which have low levels of TRF2, whereas neural progenitor cells which have high levels of telomerase and mature neurons with high levels of TRF2 are resistant to telomere damage (Cheng et al., 2007). Finally, TRF2 or POT1 over-expression correlates with the resistance to telomestatin and RHSP4 (Salvati et al., 2007; Cheng et al., 2007; Gomez et al., 2006). TRF2 might therefore counteract the effects of G-quadruplex ligands through the formation of a T-loop that would mask the 3' overhang and inhibit the recruitment of helicases which are expected to resolve G-quadruplexes and favour the binding of POT1 (Salvati et al., 2007; Opresko et al., 2002; O'Connor et al., 2006). Recently, Wang et al. have reported that the G-quadruplex formation at the 3' end of telomeric DNA inhibits extension by telomerase and that the BLM helicase requires few nucleotides beyond the G-quadruplex for efficient unwinding (Wang et al., 2011). These data suggest that G-quadruplexes at the extreme 3' end of a chromosome are resistant to the unwinding activity of helicases. The resistance of normal cells to 360A could therefore be due to the strong binding of TRF2 at the telomeres and/or the lack of TRF2 delocalization induced by G-quadruplex ligand.

5.4 Cellular responses to damaged telomeres

The cellular responses induced by damaged telomeres may significantly differ between normal and cancer cells. At the telomeres, the DNA damage response is activated by TRF2 delocalization (Karlseder et al., 1999; van Steensel et al., 1998; Takai et al., 2003; Celli & de Lange, 2005). In cancer cells, immunofluorescence staining results have shown that 360A-induced DNA damage signals occurs in a strictly ATM-dependent manner, but not in normal cells. Indeed, our own results indicate that 360A does not increase the number of γ -H2AX foci in human primary lymphocytes, suggesting that the cellular response activated by damaged telomeres is significantly different between human normal and cancer cells. However, we don't yet know if 360A induces less DNA damage in normal cells than in cancer cells and/or if the DNA damage response pathway is more efficient in normal cells than in cancer cells. In agreement with our preliminary results, RHSP4, which induces telomere damage in immortalized fibroblasts, does not induce H2AX phosphorylation in

normal fibroblasts (Salvati et al., 2007). This lack of H2AX phosphorylation is not due to impairment of DNA damage response pathways because bleomycin and the dominant-negative form of TRF2 phosphorylate H2AX in normal fibroblasts.

6. Conclusions

We showed herein that a specific G-quadruplex ligand, 360A, interacts with the terminal ends of human chromosomes in both tumour and normal cells. We find that 360A binds preferentially to human chromosome ends in normal and cancer cells but that the G-quadruplex ligand-induced cell death by apoptosis occurs only in cancer cells. We provide evidence that the G-quadruplex ligand provokes telomere deprotection during replication and induces telomere aberrations only in cancer cells. Taken together, our results indicate that 360A induces telomere instability in cancer cells but not in normal cells. Our results thus suggest that the cellular response activated by damaged telomeres and/or mechanisms implicated in telomere maintenance are significantly different between human normal and cancer cells. We cannot yet exclude the possibility that the protein composition at the telomeres and/or organization of the telomeres differ markedly between normal and cancer cells, and thereby provide normal cells with a higher degree of telomere stability.

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8. References

- Amiard, S., Doudeau, M., Pinte, S., Poulet, A., Lenain, C., Faivre-Moskalenko, C., Angelov, D., Hug, N., Vindigni, A., Bouvet, P., Paoletti, J., Gilson, E. & Giraud-Panis, M. J., (2007). A topological mechanism for TRF2-enhanced strand invasion. *Nat Struct Mol Biol*, Vol. 14, No.2, pp. 147-154, ISSN 1545-9993
- Asai, A., Oshima, Y., Yamamoto, Y., Uochi, T. A., Kusaka, H., Akinaga, S., Yamashita, Y., Pongracz, K., Pruzan, R., Wunder, E., Piatyszek, M., Li, S., Chin, A. C., Harley, C. B. & Gryaznov, S., (2003). A novel telomerase template antagonist (GRN163) as a potential anticancer agent. *Cancer Res*, Vol. 63, No.14, pp. 3931-3939, ISSN 0008-5472
- Autexier, C. & Lue, N. F., (2006). The structure and function of telomerase reverse transcriptase. *Annu Rev Biochem*, Vol. 75, pp. 493-517, ISSN 0066-4154
- Azzalin, C. M. & Lingner, J., (2008). Telomeres: the silence is broken. *Cell Cycle*, Vol. 7, No.9, pp. 1161-1165, ISSN 1551-4005
- Azzalin, C. M., Reichenbach, P., Khoriauli, L., Giulotto, E. & Lingner, J., (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science*, Vol. 318, No.5851, pp. 798-801, ISSN 1095-9203
- Bae, N. S. & Baumann, P., (2007). A RAP1/TRF2 complex inhibits nonhomologous end-joining at human telomeric DNA ends. *Mol Cell*, Vol. 26, No.3, pp. 323-334, ISSN 1097-2765

- Bailey, S. M., Cornforth, M. N., Kurimasa, A., Chen, D. J. & Goodwin, E. H., (2001). Strand-specific postreplicative processing of mammalian telomeres. *Science*, Vol. 293, No.5539, pp. 2462-2465, ISSN 0036-8075
- Bailey, S. M., Cornforth, M. N., Ullrich, R. L. & Goodwin, E. H., (2004). Dysfunctional mammalian telomeres join with DNA double-strand breaks. *DNA Repair (Amst)*, Vol. 3, No.4, pp. 349-357, ISSN 1568-7864
- Bailey, S. M. & Murnane, J. P., (2006). Telomeres, chromosome instability and cancer. *Nucleic Acids Res*, Vol. 34, No.8, pp. 2408-2417, ISSN 1362-4962
- Baird, D. M., (2008). Telomere dynamics in human cells. *Biochimie*, Vol. 90, No.1, pp. 116-121, ISSN 0300-9084
- Bates, P., Mergny, J. L. & Yang, D., (2007). Quartets in G-major. The First International Meeting on Quadruplex DNA *EMBO Rep*, Vol. 8, No.11, pp. 1003-1010, ISSN 1469-221X
- Baumann, P. & Cech, T. R., (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science*, Vol. 292, No.5519, pp. 1171-1175, ISSN 0036-8075
- Beausejour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P. & Campisi, J., (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J*, Vol. 22, No.16, pp. 4212-4222, ISSN 0261-4189
- Bilaud, T., Brun, C., Ancelin, K., Koering, C. E., Laroche, T. & Gilson, E., (1997). Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet*, Vol. 17, No.2, pp. 236-239, ISSN 1061-4036
- Blackburn, E. H., (2005). Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Lett*, Vol. 579, No.4, pp. 859-862, ISSN 0014-5793
- Blasco, M. A., Funk, W., Villeponteau, B. & Greider, C. W., (1995). Functional characterization and developmental regulation of mouse telomerase RNA. *Science*, Vol. 269, No.5228, pp. 1267-1270, ISSN 0036-8075
- Boren, H. G., Wright, E. C. & Harris, C. C., (1975). Quantitative light microscopic autoradiography. Emulsion sensitivity and latent image fading. *J Histochem Cytochem*, Vol. 23, No.12, pp. 901-909, ISSN 0022-1554
- Broccoli, D., Smogorzewska, A., Chong, L. & de Lange, T., (1997). Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet*, Vol. 17, No.2, pp. 231-235, ISSN 1061-4036
- Bryan, T. M., Englezou, A., Dalla-Pozza, L., Dunham, M. A. & Reddel, R. R., (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med*, Vol. 3, No.11, pp. 1271-1274, ISSN 1078-8956
- Celli, G. B. & de Lange, T., (2005). DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion. *Nat Cell Biol*, Vol. 7, No.7, pp. 712-718, ISSN 1465-7392
- Chai, W., Ford, L. P., Lenertz, L., Wright, W. E. & Shay, J. W., (2002). Human Ku70/80 associates physically with telomerase through interaction with hTERT. *J Biol Chem*, Vol. 277, No.49, pp. 47242-47247, ISSN 0021-9258
- Chang, C. C., Kuo, I. C., Ling, I. F., Chen, C. T., Chen, H. C., Lou, P. J., Lin, J. J. & Chang, T. C., (2004). Detection of quadruplex DNA structures in human telomeres by a fluorescent carbazole derivative. *Anal Chem*, Vol. 76, No.15, pp. 4490-4494, ISSN 0003-2700
- Chen, L. Y., Liu, D. & Songyang, Z., (2007). Telomere maintenance through spatial control of telomeric proteins. *Mol Cell Biol*, Vol. 27, No.16, pp. 5898-5909, ISSN 0270-7306
- Cheng, A., Shin-ya, K., Wan, R., Tang, S. C., Miura, T., Tang, H., Khatri, R., Gleichman, M., Ouyang, X., Liu, D., Park, H. R., Chiang, J. Y. & Mattson, M. P., (2007). Telomere

- protection mechanisms change during neurogenesis and neuronal maturation: newly generated neurons are hypersensitive to telomere and DNA damage. *J Neurosci*, Vol. 27, No.14, pp. 3722-3733, ISSN 1529-2401
- Chuang, T. C., Moshir, S., Garini, Y., Chuang, A. Y., Young, I. T., Vermolen, B., van den Doel, R., Mougey, V., Perrin, M., Braun, M., Kerr, P. D., Fest, T., Boukamp, P. & Mai, S., (2004). The three-dimensional organization of telomeres in the nucleus of mammalian cells. *BMC Biol*, Vol. 2, pp. 12, ISSN 1741-7007
- Cimino-Reale, G., Pascale, E., Battiloro, E., Starace, G., Verna, R. & D'Ambrosio, E., (2001). The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res*, Vol. 29, No.7, pp. E35, ISSN 1362-4962
- Cimprich, K. A. & Cortez, D., (2008). ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol*, Vol. 9, No.8, pp. 616-627, ISSN 1471-0080
- Cohen, S. B., Graham, M. E., Lovrecz, G. O., Bache, N., Robinson, P. J. & Reddel, R. R., (2007). Protein composition of catalytically active human telomerase from immortal cells. *Science*, Vol. 315, No.5820, pp. 1850-1853, ISSN 1095-9203
- Cosme-Blanco, W., Shen, M. F., Lazar, A. J., Pathak, S., Lozano, G., Multani, A. S. & Chang, S., (2007). Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence. *EMBO Rep*, Vol. 8, No.5, pp. 497-503, ISSN 1469-221X
- Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. & Bacchetti, S., (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J*, Vol. 11, No.5, pp. 1921-1929, ISSN 0261-4189
- Counter, C. M., Botelho, F. M., Wang, P., Harley, C. B. & Bacchetti, S., (1994). Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. *J Virol*, Vol. 68, No.5, pp. 3410-3414, ISSN 0022-538X
- Crabbe, L., Verdun, R. E., Haggblom, C. I. & Karlseder, J., (2004). Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. *Science*, Vol. 306, No.5703, pp. 1951-1953, ISSN 1095-9203
- d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P. & Jackson, S. P., (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, Vol. 426, No.6963, pp. 194-198, ISSN 1476-4687
- Dai, J., Carver, M. & Yang, D., (2008). Polymorphism of human telomeric quadruplex structures. *Biochimie*, Vol. 90, No.8, pp. 1172-1183, ISSN 0300-9084
- De Armond, R., Wood, S., Sun, D., Hurley, L. H. & Ebbinghaus, S. W., (2005). Evidence for the presence of a guanine quadruplex forming region within a polypurine tract of the hypoxia inducible factor 1alpha promoter. *Biochemistry*, Vol. 44, No.49, pp. 16341-16350, ISSN 0006-2960
- De Cian, A., Lacroix, L., Douarre, C., Temime-Smaali, N., Trentesaux, C., Riou, J. F. & Mergny, J. L., (2008). Targeting telomeres and telomerase. *Biochimie*, Vol. 90, No.1, pp. 131-155, ISSN 0300-9084
- De Cian, A. & Mergny, J. L., (2007). Quadruplex ligands may act as molecular chaperones for tetramolecular quadruplex formation. *Nucleic Acids Res*, Vol. 35, No.8, pp. 2483-2493, ISSN 1362-4962
- de Lange, T., (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev*, Vol. 19, No.18, pp. 2100-2110, ISSN 0890-9369

- de Lange, T. & Petrini, J. H., (2000). A new connection at human telomeres: association of the Mre11 complex with TRF2. *Cold Spring Harb Symp Quant Biol*, Vol. 65, pp. 265-273, ISSN 0091-7451
- Denchi, E. L. & de Lange, T., (2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature*, Vol. 448, No.7157, pp. 1068-1071, ISSN 1476-4687
- Dimitrova, N. & de Lange, T., (2009). Cell cycle-dependency role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G1 and resection-mediated inhibition of NEJ in G2. *Mol Cell Biol*, Vol. 29, pp. 5552-5563, ISSN 1098-5549
- Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. & et al., (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*, Vol. 92, No.20, pp. 9363-9367, ISSN 0027-8424
- Dionne, I. & Wellinger, R. J., (1996). Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. *Proc Natl Acad Sci U S A*, Vol. 93, No.24, pp. 13902-13907, ISSN 0027-8424
- Dirac, A. M. & Bernards, R., (2003). Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J Biol Chem*, Vol. 278, No.14, pp. 11731-11734, ISSN 0021-9258
- Dunham, M. A., Neumann, A. A., Fasching, C. L. & Reddel, R. R., (2000). Telomere maintenance by recombination in human cells. *Nat Genet*, Vol. 26, No.4, pp. 447-450, ISSN 1061-4036
- Duquette, M. L., Handa, P., Vincent, J. A., Taylor, A. F. & Maizels, N., (2004). Intracellular transcription of G-rich DNAs induces formation of G-loops, novel structures containing G4 DNA. *Genes Dev*, Vol. 18, No.13, pp. 1618-1629, ISSN 0890-9369
- Eddy, J. & Maizels, N., (2008). Conserved elements with potential to form polymorphic G-quadruplex structures in the first intron of human genes. *Nucleic Acids Res*, Vol. 36, No.4, pp. 1321-1333, ISSN 1362-4962
- El-Daly, H., Kull, M., Zimmermann, S., Pantic, M., Waller, C. F. & Martens, U. M., (2005). Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532. *Blood*, Vol. 105, No.4, pp. 1742-1749, ISSN 0006-4971
- Feldser, D. M. & Greider, C. W., (2007). Short telomeres limit tumor progression in vivo by inducing senescence. *Cancer Cell*, Vol. 11, No.5, pp. 461-469, ISSN 1535-6108
- Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J. & et al., (1995). The RNA component of human telomerase. *Science*, Vol. 269, No.5228, pp. 1236-1241, ISSN 0036-8075
- Fernando, H., Reszka, A. P., Huppert, J., Ladame, S., Rankin, S., Venkitaraman, A. R., Neidle, S. & Balasubramanian, S., (2006). A conserved quadruplex motif located in a transcription activation site of the human c-kit oncogene. *Biochemistry*, Vol. 45, No.25, pp. 7854-7860, ISSN 0006-2960
- Forsythe, H. L., Jarvis, J. L., Turner, J. W., Elmore, L. W. & Holt, S. E., (2001). Stable association of hsp90 and p23, but not hsp70, with active human telomerase. *J Biol Chem*, Vol. 276, No.19, pp. 15571-15574, ISSN 0021-9258
- Fouche, N., Cesare, A. J., Willcox, S., Ozgur, S., Compton, S. A. & Griffith, J. D., (2006). The basic domain of TRF2 directs binding to DNA junctions irrespective of the presence of TTAGGG repeats. *J Biol Chem*, Vol. 281, No.49, pp. 37486-37495, ISSN 0021-9258

- Fouladi, B., Sabatier, L., Miller, D., Pottier, G. & Murnane, J. P., (2000). The relationship between spontaneous telomere loss and chromosome instability in a human tumor cell line. *Neoplasia*, Vol. 2, No.6, pp. 540-554, ISSN 1522-8002
- Fry, M., (2007). Tetraplex DNA and its interacting proteins *Front Biosci*, Vol. 12, pp. 4336-4351, ISSN 1093-4715
- Gavathiotis, E., Heald, R. A., Stevens, M. F. & Searle, M. S., (2003). Drug recognition and stabilisation of the parallel-stranded DNA quadruplex d(TTAGGGT)₄ containing the human telomeric repeat. *J Mol Biol*, Vol. 334, No.1, pp. 25-36, ISSN 0022-2836
- Gilley, D., Tanaka, H., Hande, M. P., Kurimasa, A., Li, G. C., Oshimura, M. & Chen, D. J., (2001). DNA-PKcs is critical for telomere capping. *Proc Natl Acad Sci U S A*, Vol. 98, No.26, pp. 15084-15088, ISSN 0027-8424
- Gilson, E. & Geli, V., (2007). How telomeres are replicated. *Nat Rev Mol Cell Biol*, Vol. 8, No.10, pp. 825-838, ISSN 1471-0080
- Gomez, D., Guedin, A., Mergny, J. L., Salles, B., Riou, J. F., Teulade-Fichou, M. P. & Calsou, P., A G-quadruplex structure within the 5'-UTR of TRF2 mRNA represses translation in human cells. *Nucleic Acids Res*, Vol. 38, No.20, pp. 7187-7198, ISSN 1362-4962
- Gomez, D., Lemarteleur, T., Lacroix, L., Mailliet, P., Mergny, J. L. & Riou, J. F., (2004). Telomerase downregulation induced by the G-quadruplex ligand 12459 in A549 cells is mediated by hTERT RNA alternative splicing. *Nucleic Acids Res*, Vol. 32, No.1, pp. 371-379, ISSN 1362-4962
- Gomez, D., O'Donohue, M. F., Wenner, T., Douarre, C., Macadre, J., Koebel, P., Giraud-Panis, M. J., Kaplan, H., Kolkes, A., Shin-ya, K. & Riou, J. F., (2006). The G-quadruplex ligand telomestatin inhibits POT1 binding to telomeric sequences in vitro and induces GFP-POT1 dissociation from telomeres in human cells. *Cancer Res*, Vol. 66, No.14, pp. 6908-6912, ISSN 0008-5472
- Gomez-Millan, J., Goldblatt, E. M., Gryaznov, S. M., Mendonca, M. S. & Herbert, B. S., (2007). Specific telomere dysfunction induced by GRN163L increases radiation sensitivity in breast cancer cells. *Int J Radiat Oncol Biol Phys*, Vol. 67, No.3, pp. 897-905, ISSN 0360-3016
- Gowan, S. M., Harrison, J. R., Patterson, L., Valenti, M., Read, M. A., Neidle, S. & Kelland, L. R., (2002). A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. *Mol Pharmacol*, Vol. 61, No.5, pp. 1154-1162, ISSN 0026-895X
- Gowan, S. M., Heald, R., Stevens, M. F. & Kelland, L. R., (2001). Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes. *Mol Pharmacol*, Vol. 60, No.5, pp. 981-988, ISSN 0026-895X
- Granotier, C., Pennarun, G., Riou, L., Hoffschir, F., Gauthier, L. R., De Cian, A., Gomez, D., Mandine, E., Riou, J. F., Mergny, J. L., Mailliet, P., Dutrillaux, B. & Boussin, F. D., (2005). Preferential binding of a G-quadruplex ligand to human chromosome ends. *Nucleic Acids Res*, Vol. 33, No.13, pp. 4182-4190, ISSN 1362-4962
- Greider, C. W. & Blackburn, E. H., (1987). The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell*, Vol. 51, No.6, pp. 887-898, ISSN 0092-8674
- Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H. & de Lange, T., (1999). Mammalian telomeres end in a large duplex loop. *Cell*, Vol. 97, No.4, pp. 503-514, ISSN 0092-8674
- Gryaznov, S. M., Jackson, S., Dikmen, G., Harley, C., Herbert, B. S., Wright, W. E. & Shay, J. W., (2007). Oligonucleotide conjugate GRN163L targeting human telomerase as

- potential anticancer and antimetastatic agent. *Nucleosides Nucleotides Nucleic Acids*, Vol. 26, No.10-12, pp. 1577-1579, ISSN 1525-7770
- Han, H., Langley, D. R., Rangan, A. & Hurley, L. H., (2001). Selective interactions of cationic porphyrins with G-quadruplex structures. *J Am Chem Soc*, Vol. 123, No.37, pp. 8902-8913, ISSN 0002-7863
- Hanaoka, S., Nagadoi, A. & Nishimura, Y., (2005). Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities. *Protein Sci*, Vol. 14, No.1, pp. 119-130, ISSN 0961-8368
- Harley, C. B., Futcher, A. B. & Greider, C. W., (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, Vol. 345, No.6274, pp. 458-460, ISSN 0028-0836
- Hayflick, L. & Moorhead, P. S., (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res*, Vol. 25, pp. 585-621, ISSN 0014-4827
- Herbert, B. S., Gellert, G. C., Hochreiter, A., Pongracz, K., Wright, W. E., Zielinska, D., Chin, A. C., Harley, C. B., Shay, J. W. & Gryaznov, S. M., (2005). Lipid modification of GRN163, an N3'->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. *Oncogene*, Vol. 24, No.33, pp. 5262-5268, ISSN 0950-9232
- Ho, C. Y., Murnane, J. P., Yeung, A. K., Ng, H. K. & Lo, A. W., (2008). Telomeres acquire distinct heterochromatin characteristics during siRNA-induced RNA interference in mouse cells. *Curr Biol*, Vol. 18, No.3, pp. 183-187, ISSN 0960-9822
- Hockemeyer, D., Palm, W., Else, T., Daniels, J. P., Takai, K. K., Ye, J. Z., Keegan, C. E., de Lange, T. & Hammer, G. D., (2007). Telomere protection by mammalian Pot1 requires interaction with Tpp1. *Nat Struct Mol Biol*, Vol. 14, No.8, pp. 754-761, ISSN 1545-9993
- Hockemeyer, D., Sfeir, A. J., Shay, J. W., Wright, W. E. & de Lange, T., (2005). POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end. *EMBO J*, Vol. 24, No.14, pp. 2667-2678, ISSN 0261-4189
- Hug, N. & Lingner, J., (2006). Telomere length homeostasis. *Chromosoma*, Vol. 115, No.6, pp. 413-425, ISSN 0009-5915
- Huppert, J. L., (2008). Hunting G-quadruplexes. *Biochimie*, Vol. 90, No.8, pp. 1140-1148, ISSN 0300-9084
- Huppert, J. L. & Balasubramanian, S., (2005). Prevalence of quadruplexes in the human genome. *Nucleic Acids Res*, Vol. 33, No.9, pp. 2908-2916, ISSN 1362-4962
- Huppert, J. L. & Balasubramanian, S., (2007). G-quadruplexes in promoters throughout the human genome. *Nucleic Acids Res*, Vol. 35, No.2, pp. 406-413, ISSN 1362-4962
- Hurley, L. H., (2002). DNA and its associated processes as targets for cancer therapy. *Nat Rev Cancer*, Vol. 2, No.3, pp. 188-200, ISSN 1474-175X
- Incles, C. M., Schultes, C. M., Kempinski, H., Koehler, H., Kelland, L. R. & Neidle, S., (2004). A G-quadruplex telomere targeting agent produces p16-associated senescence and chromosomal fusions in human prostate cancer cells. *Mol Cancer Ther*, Vol. 3, No.10, pp. 1201-1206, ISSN 1535-7163
- Jackson, S. R., Zhu, C. H., Paulson, V., Watkins, L., Dikmen, Z. G., Gryaznov, S. M., Wright, W. E. & Shay, J. W., (2007). Antiadhesive effects of GRN163L--an oligonucleotide N3'->P5' thio-phosphoramidate targeting telomerase. *Cancer Res*, Vol. 67, No.3, pp. 1121-1129, ISSN 0008-5472
- Jady, B. E., Richard, P., Bertrand, E. & Kiss, T., (2006). Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. *Mol Biol Cell*, Vol. 17, No.2, pp. 944-954, ISSN 1059-1524
- Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J. & Jackson, S. P., (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol*, Vol. 8, No.1, pp. 37-45, ISSN 1465-7392

- Jiang, X. R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D. & Chiu, C. P., (1999). Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet*, Vol. 21, No.1, pp. 111-114, ISSN 1061-4036
- Karseder, J., Broccoli, D., Dai, Y., Hardy, S. & de Lange, T., (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science*, Vol. 283, No.5406, pp. 1321-1325, ISSN 0036-8075
- Karseder, J., Hoke, K., Mirzoeva, O. K., Bakkenist, C., Kastan, M. B., Petrini, J. H. & de Lange, T., (2004). The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol*, Vol. 2, No.8, pp. E240, ISSN 1545-7885
- Khanna, K. K., Lavin, M. F., Jackson, S. P. & Mulhern, T. D., (2001). ATM, a central controller of cellular responses to DNA damage. *Cell Death Differ*, Vol. 8, No.11, pp. 1052-1065, ISSN 1350-9047
- Khurts, S., Masutomi, K., Delgermaa, L., Arai, K., Oishi, N., Mizuno, H., Hayashi, N., Hahn, W. C. & Murakami, S., (2004). Nucleolin interacts with telomerase. *J Biol Chem*, Vol. 279, No.49, pp. 51508-51515, ISSN 0021-9258
- Kilian, A., Bowtell, D. D., Abud, H. E., Hime, G. R., Venter, D. J., Keese, P. K., Duncan, E. L., Reddel, R. R. & Jefferson, R. A., (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet*, Vol. 6, No.12, pp. 2011-2019, ISSN 0964-6906
- Kim, M. Y., Gleason-Guzman, M., Izbicka, E., Nishioka, D. & Hurley, L. H., (2003). The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures. *Cancer Res*, Vol. 63, No.12, pp. 3247-3256, ISSN 0008-5472
- Kim, M. Y., Vankayalapati, H., Shin-Ya, K., Wierzba, K. & Hurley, L. H., (2002). Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex. *J Am Chem Soc*, Vol. 124, No.10, pp. 2098-2099, ISSN 0002-7863
- Kim, S. H., Kaminker, P. & Campisi, J., (1999). TIN2, a new regulator of telomere length in human cells. *Nat Genet*, Vol. 23, No.4, pp. 405-412, ISSN 1061-4036
- Konishi, A. & de Lange, T., (2008). Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2. *Genes Dev*, Vol. 22, No.9, pp. 1221-1230, ISSN 0890-9369
- Kumari, S., Bugaut, A., Huppert, J. L. & Balasubramanian, S., (2007). An RNA G-quadruplex in the 5' UTR of the NRAS proto-oncogene modulates translation. *Nat Chem Biol*, Vol. 3, No.4, pp. 218-221, ISSN 1552-4450
- Lenain, C., Bauwens, S., Amiard, S., Brunori, M., Giraud-Panis, M. J. & Gilson, E., (2006). The Apollo 5' exonuclease functions together with TRF2 to protect telomeres from DNA repair. *Curr Biol*, Vol. 16, No.13, pp. 1303-1310, ISSN 0960-9822
- Li, B. & de Lange, T., (2003). Rap1 affects the length and heterogeneity of human telomeres. *Mol Biol Cell*, Vol. 14, No.12, pp. 5060-5068, ISSN 1059-1524
- Li, B., Oestreich, S. & de Lange, T., (2000). Identification of human Rap1: implications for telomere evolution. *Cell*, Vol. 101, No.5, pp. 471-483, ISSN 0092-8674
- Loayza, D., Parsons, H., Donigian, J., Hoke, K. & de Lange, T., (2004). DNA binding features of human POT1: a nonamer 5'-TAGGGTTAG-3' minimal binding site, sequence specificity, and internal binding to multimeric sites. *J Biol Chem*, Vol. 279, No.13, pp. 13241-13248, ISSN 0021-9258

- Londono-Vallejo, J. A., (2008). Telomere instability and cancer. *Biochimie*, Vol. 90, No.1, pp. 73-82, ISSN 0300-9084
- Londono-Vallejo, J. A., Der-Sarkissian, H., Cazes, L., Bacchetti, S. & Reddel, R. R., (2004). Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. *Cancer Res*, Vol. 64, No.7, pp. 2324-2327, ISSN 0008-5472
- Maizels, N., (2006). Dynamic roles for G4 DNA in the biology of eukaryotic cells. *Nat Struct Mol Biol*, Vol. 13, No.12, pp. 1055-1059, ISSN 1545-9993
- Makarov, V. L., Hirose, Y. & Langmore, J. P., (1997). Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell*, Vol. 88, No.5, pp. 657-666, ISSN 0092-8674
- Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A. & Hahn, W. C., (2003). Telomerase maintains telomere structure in normal human cells. *Cell*, Vol. 114, No.2, pp. 241-253, ISSN 0092-8674
- Mergny, J. L. & Helene, C., (1998). G-quadruplex DNA: a target for drug design. *Nat Med*, Vol. 4, No.12, pp. 1366-1367, ISSN 1078-8956
- Metcalf, J. A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P. J. & Taylor, A. M., (1996). Accelerated telomere shortening in ataxia telangiectasia. *Nat Genet*, Vol. 13, No.3, pp. 350-353, ISSN 1061-4036
- Mitchell, J. R., Wood, E. & Collins, K., (1999). A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*, Vol. 402, No.6761, pp. 551-555, ISSN 0028-0836
- Monchaud, D., Allain, C., Bertrand, H., Smargiasso, N., Rosu, F., Gabelica, V., De Cian, A., Mergny, J. L. & Teulade-Fichou, M. P., (2008). Ligands playing musical chairs with G-quadruplex DNA: a rapid and simple displacement assay for identifying selective G-quadruplex binders. *Biochimie*, Vol. 90, No.8, pp. 1207-1223, ISSN 0300-9084
- Morin, G. B., (1989). The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, Vol. 59, No.3, pp. 521-529, ISSN 0092-8674
- Muntoni, A. & Reddel, R. R., (2005). The first molecular details of ALT in human tumor cells. *Hum Mol Genet*, Vol. 14 Spec No. 2, pp. R191-196, ISSN 0964-6906
- Murnane, J. P., (2006). Telomeres and chromosome instability. *DNA Repair (Amst)*, Vol. 5, No.9-10, pp. 1082-1092, ISSN 1568-7864
- Neidle, S. & Parkinson, G. N., (2003). The structure of telomeric DNA. *Curr Opin Struct Biol*, Vol. 13, No.3, pp. 275-283, ISSN 0959-440X
- O'Connor, M. S., Safari, A., Xin, H., Liu, D. & Songyang, Z., (2006). A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. *Proc Natl Acad Sci U S A*, Vol. 103, No.32, pp. 11874-11879, ISSN 0027-8424
- Oganesian, L. & Bryan, T. M., (2007). Physiological relevance of telomeric G-quadruplex formation: a potential drug target. *Bioessays*, Vol. 29, No.2, pp. 155-165, ISSN 0265-9247
- Oikawa, S., Tada-Oikawa, S. & Kawanishi, S., (2001). Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochemistry*, Vol. 40, No.15, pp. 4763-4768, ISSN 0006-2960
- Olovnikov, A. M., (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol*, Vol. 41, No.1, pp. 181-190, ISSN 0022-5193

- Opresko, P. L., Mason, P. A., Podell, E. R., Lei, M., Hickson, I. D., Cech, T. R. & Bohr, V. A., (2005). POT1 stimulates RecQ helicases WRN and BLM to unwind telomeric DNA substrates. *J Biol Chem*, Vol. 280, No.37, pp. 32069-32080, ISSN 0021-9258
- Opresko, P. L., Otterlei, M., Graakjaer, J., Bruheim, P., Dawut, L., Kolvraa, S., May, A., Seidman, M. M. & Bohr, V. A., (2004). The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. *Mol Cell*, Vol. 14, No.6, pp. 763-774, ISSN 1097-2765
- Opresko, P. L., von Kobbe, C., Laine, J. P., Harrigan, J., Hickson, I. D. & Bohr, V. A., (2002). Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases. *J Biol Chem*, Vol. 277, No.43, pp. 41110-41119, ISSN 0021-9258
- Palm, W. & de Lange, T., (2008). How Shelterin Protects Mammalian Telomeres. *Annu Rev Genet*, Vol. pp. 0066-4197
- Paramasivan, S. & Bolton, P. H., (2008). Mix and measure fluorescence screening for selective quadruplex binders. *Nucleic Acids Res*, Vol. 36, No.17, pp. e106, ISSN 1362-4962
- Parkinson, G. N., Lee, M. P. & Neidle, S., (2002). Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature*, Vol. 417, No.6891, pp. 876-880, ISSN 0028-0836
- Pennarun, G., Granotier, C., Gauthier, L. R., Gomez, D., Hoffschir, F., Mandine, E., Riou, J. F., Mergny, J. L., Mailliet, P. & Boussin, F. D., (2005). Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands. *Oncogene*, Vol. 24, No.18, pp. 2917-2928, ISSN 0950-9232
- Pennarun, G., Granotier, C., Hoffschir, F., Mandine, E., Biard, D., Gauthier, L. R. & Boussin, F. D., (2008). Role of ATM in the telomere response to the G-quadruplex ligand 360A. *Nucleic Acids Res*, Vol. 36, No.5, pp. 1741-1754, ISSN 1362-4962
- Pennarun, G., Hoffschir, F., Revaud, D., Granotier, C., Gauthier, L. R., Mailliet, P., Biard, D. S. & Boussin, F. D., ATR contributes to telomere maintenance in human cells. *Nucleic Acids Res*, Vol. 38, No.9, pp. 2955-2963, ISSN 1362-4962
- Phan, A. T., Modi, Y. S. & Patel, D. J., (2004). Propeller-type parallel-stranded G-quadruplexes in the human c-myc promoter. *J Am Chem Soc*, Vol. 126, No.28, pp. 8710-8716, ISSN 0002-7863
- Phan, A. T. & Patel, D. J., (2003). Two-repeat human telomeric d(TAGGGTTAGGGT) sequence forms interconverting parallel and antiparallel G-quadruplexes in solution: distinct topologies, thermodynamic properties, and folding/unfolding kinetics. *J Am Chem Soc*, Vol. 125, No.49, pp. 15021-15027, ISSN 0002-7863
- Phatak, P. & Burger, A. M., (2007). Telomerase and its potential for therapeutic intervention. *Br J Pharmacol*, Vol. 152, No.7, pp. 1003-1011, ISSN 0007-1188
- Qin, Y. & Hurley, L. H., (2008). Structures, folding patterns, and functions of intramolecular DNA G-quadruplexes found in eukaryotic promoter regions. *Biochimie*, Vol. 90, No.8, pp. 1149-1171, ISSN 0300-9084
- Rahman, R., Forsyth, N. R. & Cui, W., (2008). Telomeric 3'-overhang length is associated with the size of telomeres. *Exp Gerontol*, Vol. 43, No.4, pp. 258-265, ISSN 0531-5565
- Rankin, S., Reszka, A. P., Huppert, J., Zloh, M., Parkinson, G. N., Todd, A. K., Ladame, S., Balasubramanian, S. & Neidle, S., (2005). Putative DNA quadruplex formation within the human c-kit oncogene. *J Am Chem Soc*, Vol. 127, No.30, pp. 10584-10589, ISSN 0002-7863
- Rha, S. Y., Izbicka, E., Lawrence, R., Davidson, K., Sun, D., Moyer, M. P., Roodman, G. D., Hurley, L. & Von Hoff, D., (2000). Effect of telomere and telomerase interactive

- agents on human tumor and normal cell lines. *Clin Cancer Res*, Vol. 6, No.3, pp. 987-993, ISSN 1078-0432
- Riou, J. F., Guittat, L., Mailliet, P., Laoui, A., Renou, E., Petitgenet, O., Megnin-Chanet, F., Helene, C. & Mergny, J. L., (2002). Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands. *Proc Natl Acad Sci U S A*, Vol. 99, No.5, pp. 2672-2677, ISSN 0027-8424
- Rizzo, A., Salvati, E., Porru, M., D'Angelo, C., Stevens, M. F., D'Incalci, M., Leonetti, C., Gilson, E., Zupi, G. & Biroccio, A., (2009). Stabilization of quadruplex DNA perturbs telomere replication leading to the activation of an ATR-dependent ATM signaling pathway. *Nucleic Acids Res*, Vol. 37, No.16, pp. 5353-5364, ISSN 1362-4962
- Rudd, M. K., Friedman, C., Parghi, S. S., Linardopoulou, E. V., Hsu, L. & Trask, B. J., (2007). Elevated rates of sister chromatid exchange at chromosome ends. *PLoS Genet*, Vol. 3, No.2, pp. e32, ISSN 1553-7404
- Salvati, E., Leonetti, C., Rizzo, A., Scarsella, M., Mottolise, M., Galati, R., Sperduti, I., Stevens, M. F., D'Incalci, M., Blasco, M., Chiorino, G., Bauwens, S., Horard, B., Gilson, E., Stoppacciaro, A., Zupi, G. & Biroccio, A., (2007). Telomere damage induced by the G-quadruplex ligand RHPS4 has an antitumor effect. *J Clin Invest*, Vol. 117, No.11, pp. 3236-3247, ISSN 0021-9738
- Schaffitzel, C., Berger, I., Postberg, J., Hanes, J., Lipps, H. J. & Pluckthun, A., (2001). In vitro generated antibodies specific for telomeric guanine-quadruplex DNA react with *Stylonychia lemnae* macronuclei. *Proc Natl Acad Sci U S A*, Vol. 98, No.15, pp. 8572-8577, ISSN 0027-8424
- Schaffitzel, C., Postberg, J., Paeschke, K. & Lipps, H. J., Probing telomeric G-quadruplex DNA structures in cells with in vitro generated single-chain antibody fragments. *Methods Mol Biol*, Vol. 608, pp. 159-181, ISSN 1940-6029
- Schoeftner, S. & Blasco, M. A., (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol*, Vol. 10, No.2, pp. 228-236, ISSN 1476-4679
- Sedivy, J. M., (2007). Telomeres limit cancer growth by inducing senescence: long-sought in vivo evidence obtained. *Cancer Cell*, Vol. 11, No.5, pp. 389-391, ISSN 1535-6108
- Sen, D. & Gilbert, W., (1990). A sodium-potassium switch in the formation of four-stranded G4-DNA. *Nature*, Vol. 344, No.6265, pp. 410-414, ISSN 0028-0836
- Sfeir, A. J., Chai, W., Shay, J. W. & Wright, W. E., (2005). Telomere-end processing the terminal nucleotides of human chromosomes. *Mol Cell*, Vol. 18, No.1, pp. 131-138, ISSN 1097-2765
- Shen, J. & Loeb, L. A., (2001). Unwinding the molecular basis of the Werner syndrome. *Mech Ageing Dev*, Vol. 122, No.9, pp. 921-944, ISSN 0047-6374
- Shiloh, Y., (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*, Vol. 3, No.3, pp. 155-168, ISSN 1474-175X
- Shin-ya, K., Wierzba, K., Matsuo, K., Ohtani, T., Yamada, Y., Furihata, K., Hayakawa, Y. & Seto, H., (2001). Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. *J Am Chem Soc*, Vol. 123, No.6, pp. 1262-1263, ISSN 0002-7863
- Shirude, P. S., Okumus, B., Ying, L., Ha, T. & Balasubramanian, S., (2007). Single-molecule conformational analysis of G-quadruplex formation in the promoter DNA duplex of the proto-oncogene c-kit. *J Am Chem Soc*, Vol. 129, No.24, pp. 7484-7485, ISSN 0002-7863

- Siddiqui-Jain, A., Grand, C. L., Bearss, D. J. & Hurley, L. H., (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc Natl Acad Sci U S A*, Vol. 99, No.18, pp. 11593-11598, ISSN 0027-8424
- Smogorzewska, A. & de Lange, T., (2004). Regulation of telomerase by telomeric proteins. *Annu Rev Biochem*, Vol. 73, pp. 177-208, ISSN 0066-4154
- Smogorzewska, A., Karlseder, J., Holtgreve-Grez, H., Jauch, A. & de Lange, T., (2002). DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. *Curr Biol*, Vol. 12, No.19, pp. 1635-1644, ISSN 0960-9822
- Stansel, R. M., de Lange, T. & Griffith, J. D., (2001). T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *EMBO J*, Vol. 20, No.19, pp. 5532-5540, ISSN 0261-4189
- Stewart, S. A., (2005). Telomere maintenance and tumorigenesis: an "ALT"ernative road. *Curr Mol Med*, Vol. 5, No.2, pp. 253-257, ISSN 1566-5240
- Stewart, S. A., Ben-Porath, I., Carey, V. J., O'Connor, B. F., Hahn, W. C. & Weinberg, R. A., (2003). Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet*, Vol. 33, No.4, pp. 492-496, ISSN 1061-4036
- Stewart, S. A. & Weinberg, R. A., (2000). Telomerase and human tumorigenesis *Semin Cancer Biol*, Vol. 10, No.6, pp. 399-406, ISSN 1044-579X
- Su, D. G., Fang, H., Gross, M. L. & Taylor, J. S., (2009). Photocrosslinking of human telomeric G-quadruplex loops by anti cyclobutane thymine dimer formation. *Proc Natl Acad Sci U S A*, Vol. 106, No.31, pp. 12861-12866, ISSN 1091-6490
- Sun, D., Guo, K., Rusche, J. J. & Hurley, L. H., (2005). Facilitation of a structural transition in the polypurine/polypyrimidine tract within the proximal promoter region of the human VEGF gene by the presence of potassium and G-quadruplex-interactive agents. *Nucleic Acids Res*, Vol. 33, No.18, pp. 6070-6080, ISSN 1362-4962
- Sun, D., Thompson, B., Cathers, B. E., Salazar, M., Kerwin, S. M., Trent, J. O., Jenkins, T. C., Neidle, S. & Hurley, L. H., (1997). Inhibition of human telomerase by a G-quadruplex-interactive compound. *J Med Chem*, Vol. 40, No.14, pp. 2113-2116, ISSN 0022-2623
- Tahara, H., Shin-Ya, K., Seimiya, H., Yamada, H., Tsuruo, T. & Ide, T., (2006). G-Quadruplex stabilization by telomestatin induces TRF2 protein dissociation from telomeres and anaphase bridge formation accompanied by loss of the 3' telomeric overhang in cancer cells. *Oncogene*, Vol. 25, No.13, pp. 1955-1966, ISSN 0950-9232
- Takai, H., Smogorzewska, A. & de Lange, T., (2003). DNA damage foci at dysfunctional telomeres. *Curr Biol*, Vol. 13, No.17, pp. 1549-1556, ISSN 0960-9822
- Tang, J., Kan, Z. Y., Yao, Y., Wang, Q., Hao, Y. H. & Tan, Z., (2008). G-quadruplex preferentially forms at the very 3' end of vertebrate telomeric DNA. *Nucleic Acids Res*, Vol. 36, No.4, pp. 1200-1208, ISSN 1362-4962 ISSN
- Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J. H. & Ohyashiki, K., (2003). Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. *Oncogene*, Vol. 22, No.34, pp. 5338-5347, ISSN 0950-9232
- Ting, N. S., Yu, Y., Pohorelic, B., Lees-Miller, S. P. & Beattie, T. L., (2005). Human Ku70/80 interacts directly with hTR, the RNA component of human telomerase. *Nucleic Acids Res*, Vol. 33, No.7, pp. 2090-2098, ISSN 1362-4962

- Todd, A. K., Haider, S. M., Parkinson, G. N. & Neidle, S., (2007). Sequence occurrence and structural uniqueness of a G-quadruplex in the human c-kit promoter. *Nucleic Acids Res*, Vol. 35, No.17, pp. 5799-5808, ISSN 1362-4962
- Todd, A. K., Johnston, M. & Neidle, S., (2005). Highly prevalent putative quadruplex sequence motifs in human DNA. *Nucleic Acids Res*, Vol. 33, No.9, pp. 2901-2907, ISSN 1362-4962
- Tomlinson, R. L., Ziegler, T. D., Supakorndej, T., Terns, R. M. & Terns, M. P., (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol Biol Cell*, Vol. 17, No.2, pp. 955-965, ISSN 1059-1524
- van Overbeek, M. & de Lange, T., (2006). Apollo, an Artemis-related nuclease, interacts with TRF2 and protects human telomeres in S phase. *Curr Biol*, Vol. 16, No.13, pp. 1295-1302, ISSN 0960-9822
- van Steensel, B., Smogorzewska, A. & de Lange, T., (1998). TRF2 protects human telomeres from end-to-end fusions. *Cell*, Vol. 92, No.3, pp. 401-413, ISSN 0092-8674
- Veldman, T., Etheridge, K. T. & Counter, C. M., (2004). Loss of hPot1 function leads to telomere instability and a cut-like phenotype. *Curr Biol*, Vol. 14, No.24, pp. 2264-2270, ISSN 0960-9822
- Verdun, R. E., Crabbe, L., Haggblom, C. & Karlseder, J., (2005). Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. *Mol Cell*, Vol. 20, No.4, pp. 551-561, ISSN 1097-2765
- Verdun, R. E. & Karlseder, J., (2006). The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. *Cell*, Vol. 127, No.4, pp. 709-720, ISSN 0092-8674
- Verdun, R. E. & Karlseder, J., (2007). Replication and protection of telomeres. *Nature*, Vol. 447, No.7147, pp. 924-931, ISSN 1476-4687
- von Zglinicki, T., (2000). Role of oxidative stress in telomere length regulation and replicative senescence. *Ann N Y Acad Sci*, Vol. 908, pp. 99-110, ISSN 0077-8923
- Wang, Q., Liu, J. Q., Chen, Z., Zheng, K. W., Chen, C. Y., Hao, Y. H. & Tan, Z., (2010) G-quadruplex formation at the 3' end of telomere DNA inhibits its extension by telomerase, polymerase and unwinding by helicase. *Nucleic Acids Res*, Vol. pp. 1362-4962
- Wang, R. C., Smogorzewska, A. & de Lange, T., (2004). Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell*, Vol. 119, No.3, pp. 355-368, ISSN 0092-8674
- Wang, Y. & Patel, D. J., (1993). Solution structure of a parallel-stranded G-quadruplex DNA. *J Mol Biol*, Vol. 234, No.4, pp. 1171-1183, ISSN 0022-2836
- Ward, R. J. & Autexier, C., (2005). Pharmacological telomerase inhibition can sensitize drug-resistant and drug-sensitive cells to chemotherapeutic treatment. *Mol Pharmacol*, Vol. 68, No.3, pp. 779-786, ISSN 0026-895X
- Williamson, J. R., Raghuraman, M. K. & Cech, T. R., (1989). Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell*, Vol. 59, No.5, pp. 871-880, ISSN 0092-8674
- Wu, P., van Overbeek, M., Rooney, S. & de Lange, T., (2010). Apollo contributes to G overhang maintenance and protects leading-end telomeres. *Mol Cell*, Vol. 39, No.4, pp. 606-617, ISSN 1097-4164

- Xin, H., Liu, D., Wan, M., Safari, A., Kim, H., Sun, W., O'Connor, M. S. & Songyang, Z., (2007). TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature*, Vol. 445, No.7127, pp. 559-562, ISSN 1476-4687
- Xu, Y., Kaminaga, K. & Komiyama, M., (2008). Human telomeric RNA in G-quadruplex structure. *Nucleic Acids Symp Ser (Oxf)*, Vol. No.52, pp. 175-176, ISSN 1746-8272
- Xu, Y., Noguchi, Y. & Sugiyama, H., (2006). The new models of the human telomere d[AGGG(TTAGGG)₃] in K⁺ solution. *Bioorg Med Chem*, Vol. 14, No.16, pp. 5584-5591, ISSN 0968-0896
- Xu, Y., Suzuki, Y., Ito, K. & Komiyama, M., Telomeric repeat-containing RNA structure in living cells. *Proc Natl Acad Sci U S A*, Vol. 107, No.33, pp. 14579-14584, ISSN 1091-6490
- Yang, D. & Hurley, L. H., (2006). Structure of the biologically relevant G-quadruplex in the c-MYC promoter. *Nucleosides Nucleotides Nucleic Acids*, Vol. 25, No.8, pp. 951-968, ISSN 1525-7770
- Yang, Q., Xiang, J., Yang, S., Zhou, Q., Li, Q., Tang, Y. & Xu, G., (2009). Verification of specific G-quadruplex structure by using a novel cyanine dye supramolecular assembly: I. recognizing mixed G-quadruplex in human telomeres. *Chem Commun (Camb)*, Vol. No.9, pp. 1103-1105, ISSN 1359-7345
- Yang, Q., Zheng, Y. L. & Harris, C. C., (2005). POT1 and TRF2 cooperate to maintain telomeric integrity. *Mol Cell Biol*, Vol. 25, No.3, pp. 1070-1080, ISSN 0270-7306
- Ye, X., Zerlanko, B., Zhang, R., Somaiah, N., Lipinski, M., Salomoni, P. & Adams, P. D., (2007). Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. *Mol Cell Biol*, Vol. 27, No.7, pp. 2452-2465, ISSN 0270-7306
- Ye, J., Lenain, C., Bauwens, S., Rizzo, A., Saint-Leger, A., Poulet, A., Benarroch, D., Magdinier, F., Morere, J., Amiard, S., Verhoeyen, E., Britton, S., Calsou, P., Salles, B., Bizard, A., Nadal, M., Salvati, E., Sabatier, L., Wu, Y., Biroccio, A., Londono-Vallejo, A., Giraud-Panis, M. J. & Gilson, E., (2010). TRF2 and apollo cooperate with topoisomerase 2alpha to protect human telomeres from replicative damage. *Cell*, Vol. 142, No.2, pp. 230-242, ISSN 1097-4172
- Zahler, A. M., Williamson, J. R., Cech, T. R. & Prescott, D. M., (1991). Inhibition of telomerase by G-quartet DNA structures. *Nature*, Vol. 350, No.6320, pp. 718-720, ISSN 0028-0836
- Zakian, V. A., (1995). Telomeres: beginning to understand the end. *Science*, Vol. 270, No.5242, pp. 1601-1607, ISSN 0036-8075
- Zaug, A. J., Podell, E. R. & Cech, T. R., (2005). Human POT1 disrupts telomeric G-quadruplexes allowing telomerase extension in vitro. *Proc Natl Acad Sci U S A*, Vol. 102, No.31, pp. 10864-10869, ISSN 0027-8424
- Zhong, Z., Shiue, L., Kaplan, S. & de Lange, T., (1992). A mammalian factor that binds telomeric TTAGGG repeats in vitro. *Mol Cell Biol*, Vol. 12, No.11, pp. 4834-4843, ISSN 0270-7306
- Zhu, X. D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J. H. & de Lange, T., (2003). ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell*, Vol. 12, No.6, pp. 1489-1498, ISSN 1097-2765
- Zou, L., Liu, D. & Elledge, S. J., (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci U S A*, Vol. 100, No.24, pp. 13827-13832, ISSN 0027-8424

Pharmacogenetics of Cancer and DNA Repair Enzymes

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1. Introduction

Pharmacogenetics is focused on finding associations between drug response and the genetic background of a patient.³⁸ Resequencing of the human genome revealed that nucleotide variation between individuals exists in 0.1% of the genome, which corresponds to 3 million differences. These variations occur in more than 1% of a population and are designated as single nucleotide polymorphisms (SNP), normally not causing any disease. Functional variants caused by SNPs in drug related genes (such as metabolism enzymes, transporters and receptors) have become of interest more and more in recent years.^{21,22,40,73} Variations that occur less frequent than 1% are designated as mutations and could be disease causing. Pharmacogenetic research has been expanded dramatically, with 1334 publications in the past century starting from 1961, while 7654 papers have been published since the year 2000 (pubmed accession date: November 18, 2010). The ultimate goal of pharmacogenetic research is the establishment of personalized medicine, aiming in prescribing the best choice of drug with the optimal concentration.⁶⁷ Even the route of administration could be considered. At present, pharmacogenetics is not applied widely in clinical practice as a diagnostic tool, but is mainly restricted to research, which is, finding associations between drug response and genetic background within a group of patients. Much research is being performed in order to achieve a more beneficial cancer therapy, although pharmacogenetic research is also active in other fields like rheumatoid arthritis,^{7,42,52} transplantation^{50,84} and diabetes.^{19,51}

The application of whole genome techniques for predicting patients' sensitivity or resistance to a drug is the definition of pharmacogenomics.^{27,68}

At present, most pharmacogenetic research is focused on enzymes that control the metabolism and uptake of many clinically used drugs. Most of these drugs are metabolized by Cytochrome P450 of which variant alleles are common that affects drug effectiveness.^{15,24,33} Roche diagnostics has developed an array to screen for the most important SNPs in Cytochrome P450 isoenzyme 2D6.⁷² This array is the first one that is approved by FDA for diagnostic testing. Other interesting genes with relatively high frequency of variant alleles are transporters such as MDR1.^{5,41,70} Affymetrix had developed an array (DMET) to screen for 1936 SNPs in genes that are involved in drug metabolism, uptake and detoxification.¹⁶

Although screening for SNPs in DNA mismatch repair enzymes is a standard method for diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC),^{45,61} pharmacogenetics of DNA repair enzymes is only applied in research settings and is discussed later in this chapter.

2. Genetic characteristics of tumor cells

When a normal cell loses the ability to regulate its own cell division but instead continuously divides, then this cell has become a tumor cell (reviewed by Hanahan and Weinberg²⁵). Benign tumors result from minor imbalances in tissue whereby too many genetically stable cells are produced. These cells grow expansively and since they are often encapsulated, they can be removed by surgery. Since blood cells travel throughout the body, clonal expansion of these cells never result in benign tumors, but always in malignant tumors. Malignant tumors are genetically instable, metastasize easily and are causing cancer. These type of tumors can only be removed by surgery if they are caught in early development, but many solid tumors smaller than 1 cm have already been metastasized. Due to their genetic instability, tumor cells have different genetic characteristics and consequently a heterogeneous phenotype. In such a heterogeneous population, the cells that are best adapted to their environment survive better than the less malignant ones. Genetic alterations consist of “loss of heterozygosity”, translocations, mutations and disturbance in methylation.

3. Platinum drugs for cancer treatment

The treatment of cancer depends fully on the type of tumor and often several approaches are made to kill the tumor. Antibodies are frequently used to block receptors that are over expressed in the tumor, or to scavenge growth stimulating factors. For example the epidermal growth factor receptor (EGFR) is essential for normal cellular function, however, increased levels of EGFR mRNA are associated with metastasis and aggressive tumor growth.³⁹ EGFR is over expressed in tumor cells and many therapies are focused on blocking this receptor using antibodies such as cetuximab or panitumumab.^{39,56,63} On the other hand, platinum-drugs intercalate in the tumor DNA thereby inhibiting DNA replication and thus inducing cell death.^{8,9} Platinum containing drugs such as cisplatin, carboplatin or oxaliplatin, have a broad range of activity in malignant disease and are used to threat many types of cancer. In general, the antitumor effect of platinum drugs is the result of intercalation of platinum in the DNA helix, causing the formation of platinum-DNA cross-links which ultimately leads to programmed cell death.

Cisplatin was the first platinum drug approved for the treatment of both ovarian and testicular cancer in 1978.²⁹ At present, more than 80% of patients with testicular cancer can be cured with cisplatin-based chemotherapy.³¹ It was also applied to threat other solid tumors such as cervical, head and neck, lung and bladder cancer. Unfortunately, neither of these cancer types could be treated with a similar efficiency as accounts for testicular cancer.⁶⁴ Cisplatin was the most commonly used chemotherapy drug but its use is limited by severe side effects such as gastrointestinal and renal toxicities. For that reason, an analogue with less toxicity was developed that replaced cisplatin in many chemotherapeutic regiments. This second-generation platinum drug was carboplatin which has equivalent

activity, is more stable, and is less toxic than cisplatin. Especially neurotoxicity is less frequently observed when compared with cisplatin.²⁶ Additionally, loss of hearing is less frequently observed in carboplatin-treated patients than is seen in patients treated with cisplatin.^{10,83} Carboplatin has a different spectrum of toxicity, as its primary toxic effects are hematological.⁷⁸ A third generation-platinum compound was oxaliplatin. Oxaliplatin shows no cross resistance with cisplatin and carboplatin which is an important benefit for the treatment of colorectal cancer. Colorectal cancer appeared extremely insensitive to cisplatin and carboplatin. Another advantage is the toxicity profile which is much less frequent, although neurotoxicity is still observed.²⁶

Cisplatin and carboplatin, that share the same mechanism of action, are fully cross resistant and form identical lesions in DNA. The mechanism of action of oxaliplatin is different and oxaliplatin does not share cross resistance.^{17,44}

4. Platinum induced DNA damage

As mentioned above, the cytotoxic property of platinum (Pt)-drugs is the intercalation in cellular DNA, forming Pt-adducts, that consequently inhibit DNA replication and thus induce cell death.⁹ Although Pt-based drugs are the most widely used in cancer treatment, many tumors are completely resistant to these drugs. The difference in clinical response is thought to be due, in part, to the pharmacokinetics of these drugs, as summarized by Marsh et al.⁵³ Once Pt is inside the cell, Pt-adducts are formed within the DNA and a cellular defense is activated (Figure 1). DNA is the preferential and cytotoxic target for platinating agents. Three different types of lesions can be formed: monoadducts, intrastrand crosslinks and interstrand crosslinks (Figure 2). Monoadducts are first formed, but almost all monoadducts then react to form crosslinks of which the majority is intrastrand crosslinks. Cisplatin- and carboplatin-induced crosslinks bend the DNA double helix by 32-35° toward the major groove, whereas oxaliplatin induced crosslinks bend the double helix even more.¹⁸

Oxaliplatin adducts are bulkier and more hydrophobic than those formed by cisplatin and carboplatin, leading to different effects in the cell.^{55,65} Interstrand crosslinks induce more steric changes in DNA and are therefore considered to be more toxic. Cisplatin and oxaliplatin have been found to form the same types of adducts at the same sites on the DNA.^{35,58} Both cisplatin and oxaliplatin form approximately 60-65% intrastrand GG, 25-30% intrastrand AG, 5-10% intrastrand GNG, and 1-3% interstrand GG diadducts.²⁰

5. DNA repair mechanisms

Platinum adducts are recognized by the cellular DNA repair system and resistance to platinum chemotherapy is achieved by activity of either the nucleotide excision repair (NER), mismatch repair (MMR) or homologous recombination (HR) pathways. On the other hand, mutations in key enzymes of these pathways result in sensitivity to platinum drugs. The nucleotide excision repair system deals with helix-distorting lesions that interfere with base pairing and obstruct transcription and normal replication. Therefore, NER is the most important pathway involved in the efficacy of platinum chemotherapeutic therapy.⁶⁴ NER consists of two sub-pathways, global genome NER (GG-NER) that screens the entire genome for damage, and transcription coupled repair (TCR) that screens for lesions that might block elongating RNA polymerases.⁷⁶ Specific protein complexes are involved in the

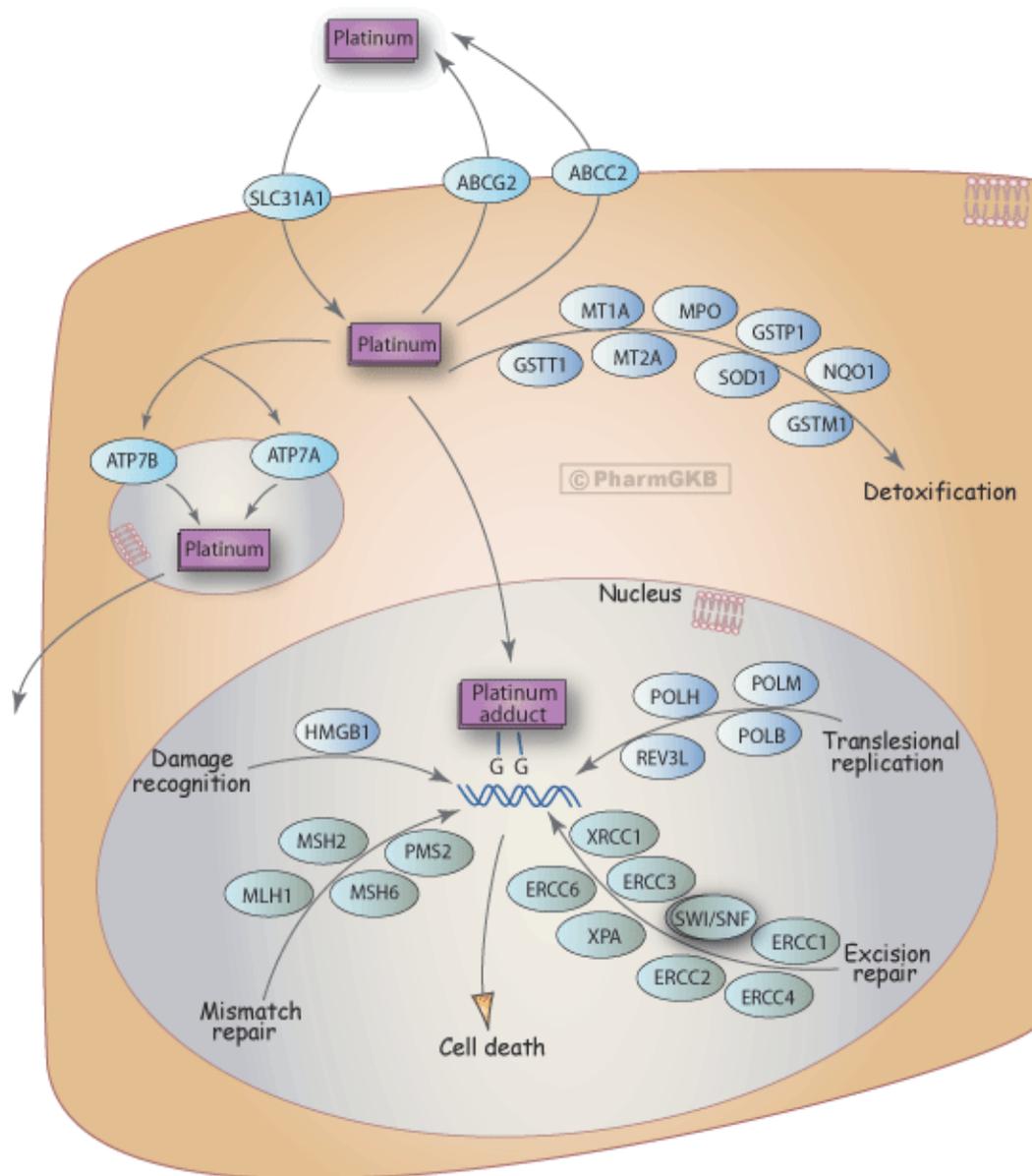


Fig. 1. Cellular response to platinum exposure. Figure taken from <http://www.pharmgkb.org/do/serve?objId=PA150642262&objCls=Pathway>. XPC, sensor for DNA damage, has not been depicted in this figure.

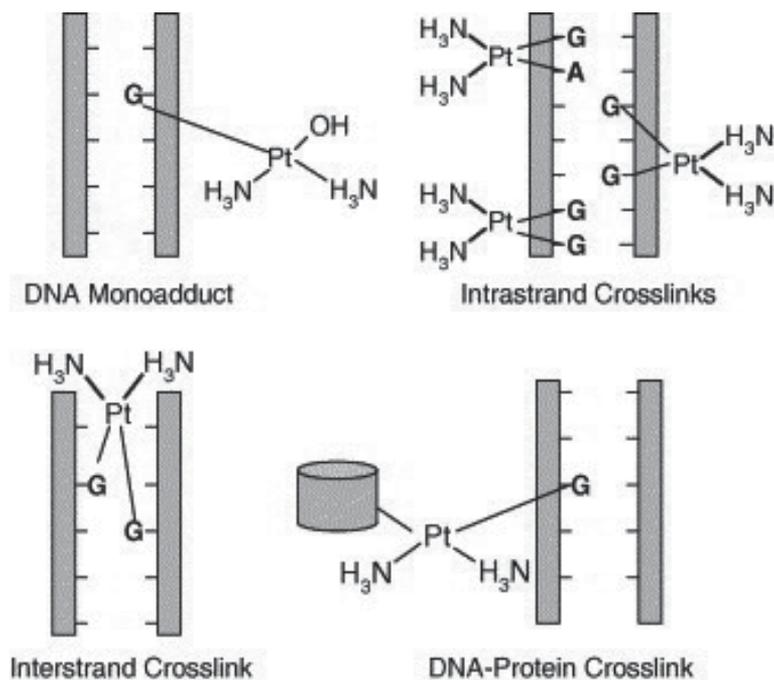


Fig. 2. Platinating agent adducts on DNA. Platinating agents are able to react with DNA to form monoadducts, intrastrand crosslinks, interstrand crosslinks and DNA-protein crosslinks. Figure taken from Rabik and Dolan.⁶⁴

first step of DNA damage recognition of GG-NER and TC-NER. Nomenclature of key enzymes involved in NER is listed in table 1. In GG-NER, XPC-hHR23B screens first on the basis of disrupted base pairing instead of lesions,⁷⁵ explaining why mildly distorted damage is poorly repaired.⁷⁵ In TCR, two specific enzymes are necessary to displace the stalled polymerase to make the lesion accessible for repair, CSA and CSB.⁴⁶ Next steps in the NER system are identical (Figure 3). In general, the helix structure of about 30 base pairs around the damaged site, is opened by helicase XPD (part of the transcription factor TFIIH). Replication protein A binds to the undamaged site to stabilize the open DNA strands. Next, XPG and ERCC1/XPF cleave the DNA strand on both sides of the damaged site. The DNA replication machinery then completes the repair by filling the gap. In total, more than 25 proteins participate in NER and for almost all NER factors, mouse mutants have been generated¹⁴ some of which show features of premature ageing. In general, mutations in key enzymes of NER (such as XPD, XPB, XPG and ERCC1) compromise NER and cause developmental delay and affect transcription.⁸⁰ The incidence of sun induced skin cancer is >1000 fold increased, whereas frequency of internal tumors is modestly elevated.

The presence of dinucleotide repeats in the human genome is quite common and form unstable motifs in some cancers.³⁶ This phenotype of microsatellite instability is caused by defects in MMR in the hereditary nonpolyposis colorectal cancer (HNPCC) and in a variety of sporadic cancers. Replication slippage of repetitive sequences introduce mispairing of nucleotides. These errors are removed by MMR and defects in MMR increase mutation rates leading to the development of tumors. The MMR system consists of four stages: 1) mismatch

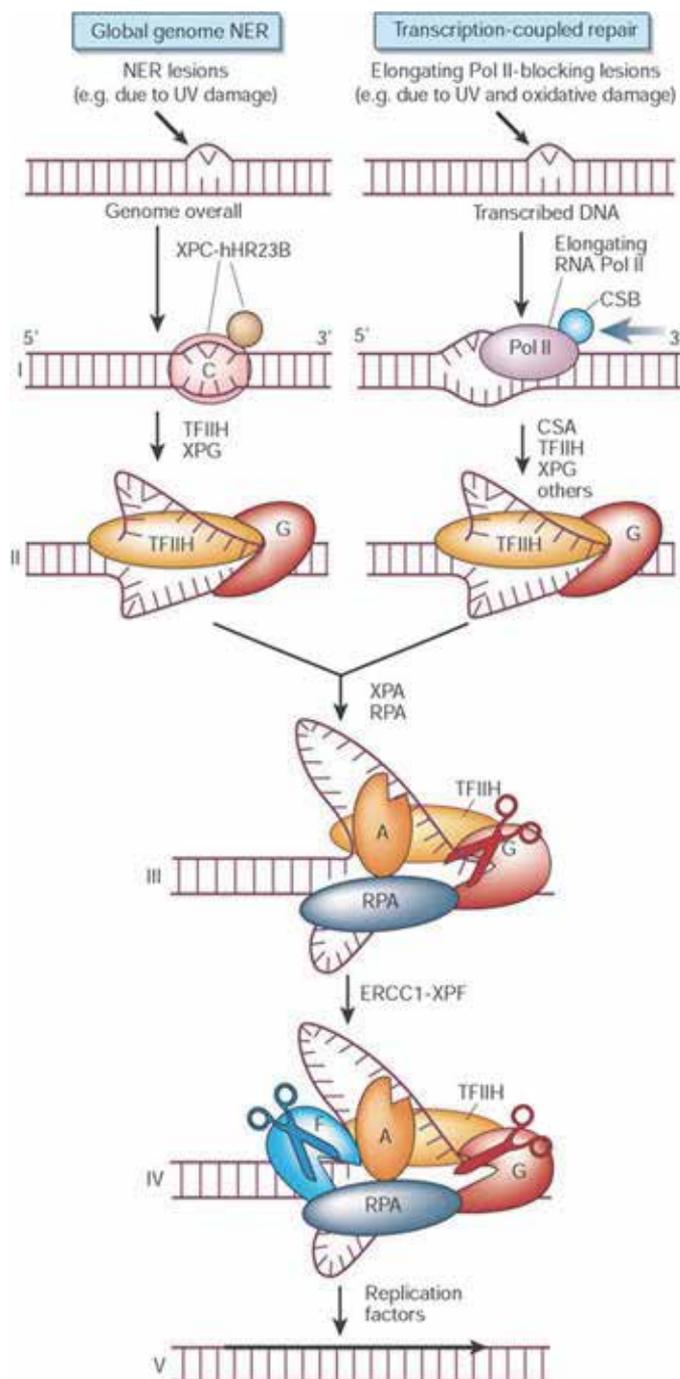


Fig. 3. Two NER subpathways exist with partly distinct substrate specificity: global genome NER (GG-NER) surveys the entire genome for distorting injury, and transcription-coupled repair (TCR) focuses on damage that blocks elongating RNA polymerases. Figure taken from Hoeijmakers³⁰

recognition, 2) recruitment of MMR factors, 3) identification and degradation of the mismatch containing strand, and 4) synthesis of the new strand (Figure 4). HNPCC is an inherited disease caused, in 60% of cases, by germline mutations in *hMLH1* and *hMSH2* genes. Defects in *hMSH6* cause late-onset HNPCC. Mice that are completely MMR deficient show a normal development²⁸ but are cancer-prone. Homologous recombination (HR) has been proposed to play a role in repairing double-strand breaks as a result of cisplatin-induced crosslinks.²³ HR is not discussed in this chapter but different DNA repair systems are discussed in an excellent review by Hoeijmakers.³⁰

Gene name	other name	Function
ERCC1	ERCC1	Forms complex with XPF for 5'-incision
XPA	XPA	Verifies DNA damage
XPB	ERCC3	3'->5' helicase
XPC	XPC	Sensor for DNA damage
XPB	ERCC2	5'->3' helicase
XPE	DDB2	Damage specific binding
XPF	ERCC4	Forms complex with ERCC1 for 5'-incision
XPG	ERCC5	3'-incision
CSA	ERCC8	Forms complex with CSB in TCR
CSB	ERCC6	Binds stalled polymerase II in TCR

Table 1. Key enzymes in NER

6. Cellular response to platinum-DNA adducts

As described above, cells possess several DNA repair mechanisms for removing Pt-DNA adducts. The tumor specificity of oxaliplatin and cisplatin is not fully understood. Although both drugs cause DNA damage, the DNA repair mechanism of the host cell responds differently to these drugs. For example, damage caused by oxaliplatin is restored by the nucleotide excision repair mechanism, whereas the mismatch repair mechanism is also active to restore damage caused by cisplatin.^{10,54} So, what is the underlying mechanism that makes certain tumors more sensitive to cisplatin than to oxaliplatin? Resistance to platinum anticancer agents can result from decreased accumulation, increased inactivation by glutathione, or an increased ability of cells to tolerate Pt-DNA adducts.¹ The ability of cells to repair platinum-induced DNA lesions is known to be an important factor in cisplatin cytotoxicity.¹³

Interestingly, oxaliplatin induced DNA damage is as effectively repaired as cisplatin-induced damage, as shown in plasmid reactivation experiments.⁷¹ The nucleotide excision repair has an extremely broad specificity, therefore it is not surprising that NER does not discriminate between oxaliplatin and cisplatin DNA adducts.⁶⁶ The MMR system, however, is a crucial element in the repair of cisplatin-induced damage as this system does not appear

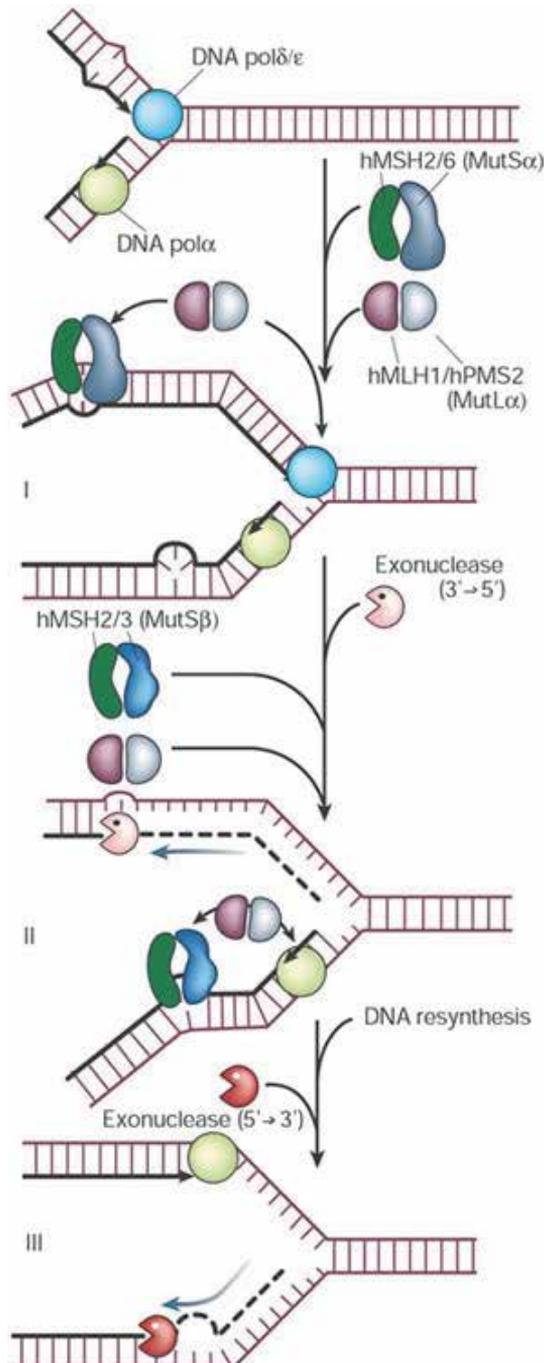


Fig. 4. Four principal steps in MMR can be delineated: (1) mismatch recognition; (2) recruitment of additional MMR factors; (3) search for a signal that identifies the wrong (newly synthesized) strand, followed by degradation past the mismatch; and (4) resynthesis of the excised tract. Figure taken from Hoeijmakers³⁰

to recognize diaminocyclohexane-containing platinum⁶⁴ DNA-adducts such as those caused by oxaliplatin. Although cells definitely respond differently to oxaliplatin and cisplatin, the mechanism behind that has still to be elucidated. Many studies that investigate the role of DNA repair mechanism in repairing DNA crosslinks, use cisplatin or mitomycin C as a model to induce such crosslinks.⁵⁷ For instance the human ovarian cell line A2780 is sensitive to cisplatin but resistant to oxaliplatin.⁴⁸ In this study it was shown that ERCC1 (a key enzyme in NER) mRNA is upregulated after exposure to cisplatin. Whether ERCC1 mRNA levels are upregulated after exposure to oxaliplatin, is not known.

7. The role of DNA repair enzymes in platinum resistance

There is growing evidence that activity of DNA repair enzymes contributes to the interindividual differences in the anti-tumor effect of platinum drugs. Activity of DNA repair system is affected by functional SNPs in key enzymes, but also expression levels of such enzymes might differ between individuals. High levels of ERCC1 mRNA are associated with worse outcome in patients with bladder cancer treated with oxaliplatin.³ An SNP in exon 4 of *ERCC1* (rs11615) was associated with better survival in non-small cell lung carcinoma treated with cisplatin,³⁴ as was in colorectal cancer patients treated with oxaliplatin.^{62,81} The *ERCC2* gene (*XPB*) is located at the same chromosome as *ERCC1* and multiple non-synonymous SNPs in *ERCC2* have been found associated with diminished DNA repair capacity.⁷⁴ Since rs11615 is a silent SNP (not causing amino acid change) the association with diminished DNA repair capacity is suggested to be due to low ERCC1 expression caused by a linked SNP (rs3212986) in its 3'NTR region.³⁴ In vitro studies to test this hypothesis have not been performed. Interestingly, another gene in a reverse orientation, overlaps with ERCC1 3'NTR, and a role for this gene in DNA repair has been suggested.⁶⁹ This gene was identified in 1999 as a CD3 ϵ binding protein in T-cells and was therefore named CAST (CD3 ϵ -associated signal transducer).⁸⁶ Later, this gene was identified as a subunit of RNA Polymerase I.⁶⁰ While the exact function of this Pol I-specific subunit is unknown, in mammalian cells the interaction with the activator of Pol I transcription, UBF, suggests a role for this subunit in facilitating the transition from transcription initiation to elongation at promoter escape.⁵⁹ In another model it has been proposed that conformational changes in the Pol I enzyme complex through CAST convert the Pol I into a processive enzyme complex.⁶⁰ Since the 3' NTR of ERCC1 overlaps with CAST open reading frame, rs3212986 causes an amino acid change in CAST in a putative nuclear localization signal. It is likely that this SNP affects CAST function instead of ERCC1 expression. The hypothesis that CAST function is related with DNA repair is strengthened by its chromosomal location, between *ERCC1* and *ERCC2* (Figure 5).

An effect of platinum-based drugs on RNA polymerase I transcription is not without precedent. Cisplatin-induced platinum adducts interact with proteins that contain high-mobility-group (HMG) domains, such as UBF, an activator of Pol I transcription. UBF was able to bind cisplatin-modified DNA more avidly than unmodified DNA¹² and caused a redistribution of UBF in the nucleolus and a block of rRNA synthesis in human cells.³⁷ Furthermore, UBF expression could increase the cell sensitivity to the chemotherapeutic reagent cis-diaminedichloroplatinum,³² perhaps by inhibiting repair of the DNA adducts. In addition, SNPs in CAST gene are associated with adult onset glioma^{11,85} and treatment outcome in multiple myeloma patients undergoing bone marrow transplantation.⁷⁹ In view of the model where Pol I transcription of the rRNA genes is a sensor for DNA damage and

of the intricate association of CAST with Pol I, rs3212986 could result in a CAST-Pol I complex with a decreased capacity to elicit a DNA damage response. In those cells that carry this particular SNP, damage is therefore not efficiently repaired and this might result in increased cytotoxicity to platinum drugs, impairment of tumour growth and hence an increased patient survival.

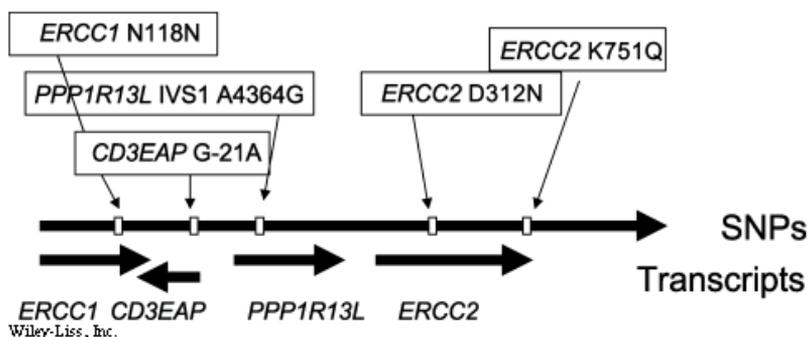


Fig. 5. Graphical presentation of the studied SNPs on chromosome 19. The *ERCC1* polymorphism is silent and does not result in an amino acid substitution. The *CD3EAP* polymorphism is G to A transversion in the 5' untranslated region of the mRNA. The *PPP1R13L* polymorphism is in intron 1 of the *PPP1R13L* gene. The *ERCC2* D312N polymorphism gives rise to the Asp to Asn amino acid substitution in position 312 of *ERCC2*. *ERCC2* K751Q gives rise to the Lys to Gln substitution in position 751 in *ERCC2*. Figure taken from Vangsted et al⁷⁹

8. Pharmacogenetic research of DNA repair genes in practice

Cisplatin pharmacogenetics have been studied in esophageal cancer patients where several SNPs in NER were investigated. Two SNPs in *ERCC1* and the SNP in *ERCC1* 3'-NTR (or CAST as discussed above) were found associated with improved overall survival.⁶ In testicular cancer cells, cisplatin sensitivity has been associated with low expression of *ERCC1*, however no SNPs were investigated in this study.⁷⁷ Low expression of *ERCC1* was also associated with oxaliplatin cytotoxicity in colorectal cancer cell lines when cetuximab (monoclonal antibody raised against EGFR) was co-administered.² Platinum resistant ovarian cancer patients receive carboplatin together with gemcitabine⁴⁹ that appeared to inhibit carboplatin induced interstrand crosslink repair.^{4,47} The role of *ERCC1* in platinum treatment of non-small cell lung cancer with emphasis on carboplatin, has been reviewed by Vilmar and Sorensen.⁸² Recently, an explorative study has been performed to identify novel candidate genes related to oxaliplatin efficacy and toxicity in colorectal cancer patients, using a DNA repair array.⁴³ Only *ERCC5* (another factor in NER) and *ATM* (general responder to DNA damage) were found associated with clinical response. Discrepancies in association with SNPs in other DNA repair enzymes, present on the array, are discussed in this paper.

9. Summary

Since the entire human genome has been sequenced and 3,000,000 (1 in 1000 nucleotides) SNPs have been identified, more and more research is being performed to test effects of

specific SNPs. Pharmacogenetic research is particularly focused on SNPs with a high allele frequency and an altered response to drugs. Many association studies have been performed and SNPs in genes encoding for drug transport, uptake, metabolism, detoxification and DNA repair, are found to be related to drug response. SNPs in coding regions may alter gene function, however, associated SNPs are not causal per se but may be linked to another, yet unidentified, linked SNP. The ultimate goal of pharmacogenetic research is to predict an individuals' response to drug therapy and subsequently adapt the therapeutic strategy. Peripheral blood can be taken to isolate chromosomal DNA and to genotype for specific SNPs, although tumor DNA might contain more mutations. In addition, expression levels of key enzymes may differ in tumor cells compared to normal cells. An approach to measure mRNA expression or to genotype tumor DNA could be the isolation of circulating tumor cells from peripheral blood. Whether this is feasible and cost effective for diagnostic healthcare should be investigated. In conclusion, more research is needed before any of the associated SNPs in DNA repair enzymes could be used to predict an individual response to a specific platinum-drug.

10. References

- [1] Arnould, S., I. Hennebelle, P. Canal, R. Bugat, and S. Guichard. 2003. Cellular determinants of oxaliplatin sensitivity in colon cancer cell lines. *Eur.J.Cancer* 39:112-119.
- [2] Balin-Gauthier, D., J. P. Delord, M. J. Pillaire, P. Rochaix, J. S. Hoffman, R. Bugat, C. Cazaux, P. Canal, and B. C. Allal. 2008. Cetuximab potentiates oxaliplatin cytotoxic effect through a defect in NER and DNA replication initiation. *Br.J.Cancer*. 98:120-128.
- [3] Bellmunt, J., L. Paz-Ares, M. Cuello, F. L. Cecere, S. Albiol, V. Guillem, E. Gallardo, J. Carles, P. Mendez, J. J. de la Cruz, M. Taron, R. Rosell, and J. Baselga. 2007. Gene expression of ERCC1 as a novel prognostic marker in advanced bladder cancer patients receiving cisplatin-based chemotherapy. *Ann.Oncol.* 18:522-528.
- [4] Bergman, A. M., v. H. Ruiz, V. G. Veerman, C. M. Kuiper, and G. J. Peters. 1996. Synergistic interaction between cisplatin and gemcitabine in vitro. *Clin.Cancer Res.* 2:521-530.
- [5] Borst, P., R. Evers, M. Kool, and J. Wijnholds. 2000. A family of drug transporters: the multidrug resistance-associated proteins. *J.Natl.Cancer Inst.* 92:1295-1302.
- [6] Bradbury, P. A., M. H. Kulke, R. S. Heist, W. Zhou, C. Ma, W. Xu, A. L. Marshall, R. Zhai, S. M. Hooshmand, K. Asomaning, L. Su, F. A. Shepherd, T. J. Lynch, J. C. Wain, D. C. Christiani, and G. Liu. 2009. Cisplatin pharmacogenetics, DNA repair polymorphisms, and esophageal cancer outcomes. *Pharmacogenet.Genomics.* 19:613-625.
- [7] Brinker, R. R. and P. Ranganathan. 2010. Methotrexate pharmacogenetics in rheumatoid arthritis. *Clin.Exp.Rheumatol.* 28:S33-S39.
- [8] bu-Surrah, A. S. 2007. Development and current status of unconventional platinum anticancer complexes. *Mini.Rev.Med.Chem.* 7:203-211.
- [9] Cepeda, V., M. A. Fuertes, J. Castilla, C. Alonso, C. Quevedo, and J. M. Perez. 2007. Biochemical mechanisms of cisplatin cytotoxicity. *Anticancer Agents Med.Chem.* 7:3-18.

- [10] Chaney, S. G., S. L. Campbell, E. Bassett, and Y. Wu. 2005. Recognition and processing of cisplatin- and oxaliplatin-DNA adducts. *Crit Rev.Oncol.Hematol.* 53:3-11.
- [11] Chen, P., J. Wiencke, K. Aldape, A. Kesler-Diaz, R. Miike, K. Kelsey, M. Lee, J. Liu, and M. Wrensch. 2000. Association of an ERCC1 polymorphism with adult-onset glioma. *Cancer Epidemiol.Biomarkers Prev.* 9:843-847.
- [12] Codony-Servat, J., R. Gimeno, C. Gelpi, J. L. Rodriguez-Sanchez, and C. Juarez. 1996. The two isoforms of the 90-kDalton nucleolus organizer region autoantigen (upstream binding factor) bind with different avidity to DNA modified by the antitumor drug cisplatin. *Biochem.Pharmacol.* 51:1131-1136.
- [13] Damia, G., G. Guidi, and M. D'Incalci. 1998. Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. *Eur.J.Cancer* 34:1783-1788.
- [14] de, B. J. and J. H. Hoeijmakers. 1999. Cancer from the outside, aging from the inside: mouse models to study the consequences of defective nucleotide excision repair. *Biochimie.* 81:127-137.
- [15] de, L. J. 2006. AmpliChip CYP450 test: personalized medicine has arrived in psychiatry. *Expert.Rev.Mol.Diagn.* 6:277-286.
- [16] Deeken, J. 2009. The Affymetrix DMET platform and pharmacogenetics in drug development. *Curr.Opin.Mol.Ther.* 11:260-268.
- [17] Desoize, B. and C. Madoulet. 2002. Particular aspects of platinum compounds used at present in cancer treatment. *Crit Rev.Oncol.Hematol.* 42:317-325.
- [18] Di Francesco, A. M., A. Ruggiero, and R. Riccardi. 2002. Cellular and molecular aspects of drugs of the future: oxaliplatin. *Cell Mol.Life Sci.* 59:1914-1927.
- [19] Distefano, J. K. and R. M. Watanabe. 2010. Pharmacogenetics of Anti-Diabetes Drugs. *Pharmaceuticals.(Basel).* 3:2610-2646.
- [20] Eastman, A. 1987. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol.Ther.* 34:155-166.
- [21] Evans, W. E. and J. A. Johnson. 2001. Pharmacogenomics: the inherited basis for interindividual differences in drug response. *Annu.Rev.Genomics Hum.Genet.* 2:9-39.:9-39.
- [22] Evans, W. E. and M. V. Relling. 1999. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science.* 286:487-491.
- [23] Frankenberg-Schwager, M., D. Kirchermeier, G. Greif, K. Baer, M. Becker, and D. Frankenberg. 2005. Cisplatin-mediated DNA double-strand breaks in replicating but not in quiescent cells of the yeast *Saccharomyces cerevisiae*. *Toxicology.* 212:175-184.
- [24] Han, X. M. and H. H. Zhou. 2000. Polymorphism of CYP450 and cancer susceptibility. *Acta Pharmacol.Sin.* 21:673-679.
- [25] Hanahan, D. and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57-70.
- [26] Helbekkmo, N., S. H. Sundstrom, U. Aasebo, P. F. Brunsvig, P. C. von, H. H. Hjelde, O. K. Garpestad, A. Bailey, and R. M. Bremnes. 2007. Vinorelbine/carboplatin vs gemcitabine/carboplatin in advanced NSCLC shows similar efficacy, but different impact of toxicity. *Br.J.Cancer.* 97:283-289.
- [27] Hess, P. and D. Cooper. 1999. Impact of pharmacogenomics on the clinical laboratory. *Mol.Diagn.* 4:289-298.

- [28] Heyer, J., K. Yang, M. Lipkin, W. Edelmann, and R. Kucherlapati. 1999. Mouse models for colorectal cancer. *Oncogene*. 20:5325-5333.
- [29] Higby, D. J., H. J. Wallace, Jr., D. J. Albert, and J. F. Holland. 1974. Diaminodichloroplatinum: a phase I study showing responses in testicular and other tumors. *Cancer*. 33:1219-5.
- [30] Hoeijmakers, J. H. 2001. Genome maintenance mechanisms for preventing cancer. *Nature* 411:366-374.
- [31] Horwich, A., J. Shipley, and R. Huddart. 2006. Testicular germ-cell cancer. *Lancet*. 367:754-765.
- [32] Huang, R., T. Wu, L. Xu, A. Liu, Y. Ji, and G. Hu. 2002. Upstream binding factor up-regulated in hepatocellular carcinoma is related to the survival and cisplatin-sensitivity of cancer cells. *FASEB J*. 16:293-301.
- [33] Ingelman-Sundberg, M. and S. C. Sim. 2010. Pharmacogenetic biomarkers as tools for improved drug therapy; emphasis on the cytochrome P450 system. *Biochem.Biophys.Res.Commun*. 396:90-94.
- [34] Isla, D., C. Sarries, R. Rosell, G. Alonso, M. Domine, M. Taron, G. Lopez-Vivanco, C. Camps, M. Botia, L. Nunez, M. Sanchez-Ronco, J. J. Sanchez, M. Lopez-Brea, I. Barneto, A. Paredes, B. Medina, A. Artal, and P. Lianes. 2004. Single nucleotide polymorphisms and outcome in docetaxel-cisplatin-treated advanced non-small-cell lung cancer. *Ann.Oncol*. 15:1194-1203.
- [35] Jennerwein, M. M., A. Eastman, and A. Khokhar. 1989. Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexaneplatinum(II) complexes. *Chem.Biol.Interact*. 70:39-49.
- [36] Jiricny, J. 1998. Replication errors: challenging the genome. *EMBO J*. 17:6427-6436.
- [37] Jordan, P. and M. Carmo-Fonseca. 1998. Cisplatin inhibits synthesis of ribosomal RNA in vivo. *Nucleic Acids Res*. 26:2831-2836.
- [38] Kalow, W. 1965. Contribution of hereditary factors to the response to drugs. *Fed.Proc*. 24:1259-1265.
- [39] Kim, E. S., F. R. Khuri, and R. S. Herbst. 2001. Epidermal growth factor receptor biology (IMC-C225). *Curr.Opin.Oncol*. 13:506-513.
- [40] Kleyn, P. W. and E. S. Vesell. 1998. Genetic variation as a guide to drug development. *Science*. 281:1820-1821.
- [41] Komoto, C., T. Nakamura, T. Sakaeda, D. L. Kroetz, T. Yamada, H. Omatsu, T. Koyama, N. Okamura, I. Miki, T. Tamura, N. Aoyama, M. Kasuga, and K. Okumura. 2006. MDR1 haplotype frequencies in Japanese and Caucasian, and in Japanese patients with colorectal cancer and esophageal cancer. *Drug Metab Pharmacokinet*. 21:126-132.
- [42] Kooloos, W. M., T. W. Huizinga, H. J. Guchelaar, and J. A. Wessels. 2010. Pharmacogenetics in treatment of rheumatoid arthritis. *Curr.Pharm.Des*. 16:164-175.
- [43] Kweekel, D. M., N. F. Antonini, J. W. Nortier, C. J. Punt, H. Gelderblom, and H. J. Guchelaar. 2009. Explorative study to identify novel candidate genes related to oxaliplatin efficacy and toxicity using a DNA repair array. *Br.J.Cancer*. 101:357-362.
- [44] Kweekel, D. M., H. Gelderblom, and H. J. Guchelaar. 2005. Pharmacology of oxaliplatin and the use of pharmacogenomics to individualize therapy. *Cancer Treat.Rev*. 31:90-105.

- [45] Lagerstedt, R. K., T. Liu, J. Vandrovcova, B. Halvarsson, M. Clendenning, T. Frebourg, N. Papadopoulos, K. W. Kinzler, B. Vogelstein, P. Peltomaki, R. D. Kolodner, M. Nilbert, and A. Lindblom. 2007. Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. *J.Natl.Cancer Inst.* 99:291-299.
- [46] Le, P. F., E. E. Kwoh, A. Avrutskaya, A. Gentil, S. A. Leadon, A. Sarasin, and P. K. Cooper. 2000. Transcription-coupled repair of 8-oxoguanine: requirement for XPG, TFIIH, and CSB and implications for Cockayne syndrome. *Cell.* 101:159-171.
- [47] Ledermann, J. A., H. Gabra, G. C. Jayson, V. J. Spanswick, G. J. Rustin, M. Jitlal, L. E. James, and J. A. Hartley. 2010. Inhibition of carboplatin-induced DNA interstrand cross-link repair by gemcitabine in patients receiving these drugs for platinum-resistant ovarian cancer. *Clin.Cancer Res.* 16:4899-4905.
- [48] Li, Q., K. Gardner, L. Zhang, B. Tsang, F. Bostick-Bruton, and E. Reed. 1998. Cisplatin induction of ERCC-1 mRNA expression in A2780/CP70 human ovarian cancer cells. *J.Biol.Chem.* 273:23419-23425.
- [49] Lund, B., O. P. Hansen, K. Theilade, M. Hansen, and J. P. Neijt. 1994. Phase II study of gemcitabine (2',2'-difluorodeoxycytidine) in previously treated ovarian cancer patients. *J.Natl.Cancer Inst.* 86:1530-1533.
- [50] MacPhee, I. A. 2010. Use of pharmacogenetics to optimize immunosuppressive therapy. *Ther.Drug Monit.* 32:261-264.
- [51] Malandrino, N. and R. J. Smith. 2011. Personalized medicine in diabetes. *Clin.Chem.* 57:231-240.
- [52] Marsal, S. and A. Julia. 2010. Rheumatoid arthritis pharmacogenomics. *Pharmacogenomics.* 11:617-619.
- [53] Marsh, S., H. McLeod, E. Dolan, S. J. Shukla, C. A. Rabik, L. Gong, T. Hernandez-Boussard, X. J. Lou, T. E. Klein, and R. B. Altman. 2009. Platinum pathway. *Pharmacogenet.Genomics.* 19:563-564.
- [54] Martin, L. P., T. C. Hamilton, and R. J. Schilder. 2008. Platinum resistance: the role of DNA repair pathways. *Clin.Cancer Res.* 14:1291-1295.
- [55] Misset, J. L., H. Bleiberg, W. Sutherland, M. Bekradda, and E. Cvitkovic. 2000. Oxaliplatin clinical activity: a review. *Crit Rev.Oncol.Hematol.* 35:75-93.
- [56] Morrow, P. K. and E. S. Kim. 2005. New biological agents in the treatment of advanced non-small cell lung cancer. *Semin.Respir.Crit Care Med.* 26:323-332.
- [57] Niedernhofer, L. J., G. A. Garinis, A. Raams, A. S. Lalai, A. R. Robinson, E. Appeldoorn, H. Odijk, R. Oostendorp, A. Ahmad, W. van Leeuwen, A. F. Theil, W. Vermeulen, G. T. van der Horst, P. Meinecke, W. J. Kleijer, J. Vijg, N. G. Jaspers, and J. H. Hoeijmakers. 2006. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. *Nature* 444:1038-1043.
- [58] Page, J. D., I. Husain, A. Sancar, and S. G. Chaney. 1990. Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality. *Biochemistry.* 29:1016-1024.
- [59] Panov, K. I., J. K. Friedrich, J. Russell, and J. C. Zomerdijk. 2006. UBF activates RNA polymerase I transcription by stimulating promoter escape. *EMBO J.* 25:3310-3322.
- [60] Panov, K. I., T. B. Panova, O. Gadal, K. Nishiyama, T. Saito, J. Russell, and J. C. Zomerdijk. 2006. RNA polymerase I-specific subunit CAST/hPAF49 has a role in the activation of transcription by upstream binding factor. *Mol.Cell Biol.* 26:5436-5448.

- [61] Papadopoulos, N. and A. Lindblom. 1997. Molecular basis of HNPCC: mutations of MMR genes. *Hum.Mutat.* 10:89-99.
- [62] Pare, L., E. Marcuello, A. Altes, R. E. del, L. Sedano, J. Salazar, A. Cortes, A. Barnadas, and M. Baiget. 2008. Pharmacogenetic prediction of clinical outcome in advanced colorectal cancer patients receiving oxaliplatin/5-fluorouracil as first-line chemotherapy. *Br.J.Cancer.* 99:1050-1055.
- [63] Pirker, R. and M. Filipits. 2010. Monoclonal antibodies against EGFR in non-small cell lung cancer. *Crit Rev.Oncol.Hematol.*
- [64] Rabik, C. A. and M. E. Dolan. 2007. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat.Rev.* 33:9-23.
- [65] Raymond, E., S. Faivre, S. Chaney, J. Woynarowski, and E. Cvitkovic. 2002. Cellular and molecular pharmacology of oxaliplatin. *Mol.Cancer Ther.* 1:227-235.
- [66] Reardon, J. T., A. Vaisman, S. G. Chaney, and A. Sancar. 1999. Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. *Cancer Res.* 59:3968-3971.
- [67] Roses, A. D. 2000. Pharmacogenetics and the practice of medicine. *Nature.* 405:857-865.
- [68] Rusnak, J. M., R. M. Kisabeth, D. P. Herbert, and D. M. McNeil. 2001. Pharmacogenomics: a clinician's primer on emerging technologies for improved patient care. *Mayo Clin.Proc.* 76:299-309.
- [69] Russell, J. and J. C. Zomerdijk. 2005. RNA-polymerase-I-directed rDNA transcription, life and works. *Trends Biochem.Sci.* 30:87-96.
- [70] Sakaeda, T., T. Nakamura, M. Horinouchi, M. Kakumoto, N. Ohmoto, T. Sakai, Y. Morita, T. Tamura, N. Aoyama, M. Hirai, M. Kasuga, and K. Okumura. 2001. MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects. *Pharm.Res.* 18:1400-1404.
- [71] Scheeff, E. D., J. M. Briggs, and S. B. Howell. 1999. Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin. *Mol.Pharmacol.* 56:633-643.
- [72] Seufferlein, T. and B. O. Boehm. 2002. The impact of pharmacogenomics on gastrointestinal cancer therapy. *Pharmacogenomics.* 3:625-633.
- [73] Severino, G., C. Chillotti, M. E. Stochino, and Z. M. Del. 2003. Pharmacogenomics: state of the research and perspectives in clinical application. *Neurol.Sci.* 24 Suppl 2:S146-8.:S146-S148.
- [74] Spitz, M. R., X. Wu, Y. Wang, L. E. Wang, S. Shete, C. I. Amos, Z. Guo, L. Lei, H. Mohrenweiser, and Q. Wei. 2001. Modulation of nucleotide excision repair capacity by XPD polymorphisms in lung cancer patients. *Cancer Res.* 61:1354-1357.
- [75] Tang, J. Y., B. J. Hwang, J. M. Ford, P. C. Hanawalt, and G. Chu. 2000. Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. *Mol.Cell.* 5:737-744.
- [76] Tornaletti, S. and P. C. Hanawalt. 1999. Effect of DNA lesions on transcription elongation. *Biochimie.* 81:139-146.
- [77] Usanova, S., A. Piee-Staffa, U. Sied, J. Thomale, A. Schneider, B. Kaina, and B. Koberle. 2010. Cisplatin sensitivity of testis tumour cells is due to deficiency in interstrand-crosslink repair and low ERCC1-XPF expression. *Mol.Cancer.* 9:248.:248.

- [78] van Glabbeke, M., J. Renard, H. M. Pinedo, F. Cavalli, J. Vermorken, C. Sessa, R. Abele, M. Clavel, and S. Monfardini. 1988. Iproplatin and carboplatin induced toxicities: overview of phase II clinical trial conducted by the EORTC Early Clinical Trials Cooperative Group (ECTG). *Eur.J.Cancer Clin.Oncol.* 24:255-262.
- [79] Vangsted, A., P. Gimsing, T. W. Klausen, B. A. Nexø, H. Wallin, P. Andersen, P. Hokland, S. T. Lillevang, and U. Vogel. 2007. Polymorphisms in the genes ERCC2, XRCC3 and CD3EAP influence treatment outcome in multiple myeloma patients undergoing autologous bone marrow transplantation. *Int.J.Cancer* 120:1036-1045.
- [80] Vermeulen, W., S. Rademakers, N. G. Jaspers, E. Appeldoorn, A. Raams, B. Klein, W. J. Kleijer, L. K. Hansen, and J. H. Hoeijmakers. 2001. A temperature-sensitive disorder in basal transcription and DNA repair in humans. *Nat.Genet.* 27:299-303.
- [81] Viguier, J., V. Boige, C. Miquel, M. Pocard, B. Giraudeau, J. C. Sabourin, M. Ducreux, A. Sarasin, and F. Praz. 2005. ERCC1 codon 118 polymorphism is a predictive factor for the tumor response to oxaliplatin/5-fluorouracil combination chemotherapy in patients with advanced colorectal cancer. *Clin.Cancer Res.* 11:6212-6217.
- [82] Vilmar, A. and J. B. Sorensen. 2009. Excision repair cross-complementation group 1 (ERCC1) in platinum-based treatment of non-small cell lung cancer with special emphasis on carboplatin: a review of current literature. *Lung Cancer.* 64:131-139.
- [83] Wang, D. and S. J. Lippard. 2005. Cellular processing of platinum anticancer drugs. *Nat.Rev.Drug Discov.* 4:307-320.
- [84] Ware, N. and I. A. MacPhee. 2010. Current progress in pharmacogenetics and individualized immunosuppressive drug dosing in organ transplantation. *Curr.Opin.Mol.Ther.* 12:270-283.
- [85] Wrensch, M., K. T. Kelsey, M. Liu, R. Miike, M. Moghadassi, J. D. Sison, K. Aldape, A. McMillan, J. Wiemels, and J. K. Wiencke. 2005. ERCC1 and ERCC2 polymorphisms and adult glioma. *Neuro.-oncol.* 7:495-507.
- [86] Yamazaki, T., Y. Hamano, H. Tashiro, K. Itoh, H. Nakano, S. Miyatake, and T. Saito. 1999. CAST, a novel CD3epsilon-binding protein transducing activation signal for interleukin-2 production in T cells. *J.Biol.Chem.* 274:18173-18180.

Part 3

DNA Repair and Aging

Involvement of Histone PTMs in DNA Repair Processes in Relation to Age-Associated Neurodegenerative Disease

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1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), are becoming common in the world with increasing number of aged people in the population. Many investigations have been performed in an attempt to elucidate the mechanisms of neurodegenerative diseases. However, no single etiopathological factor was found to be responsible for such diseases, and therefore no effective therapeutic strategy could be designed.

Aging is regarded as the greatest risk factor for the development of neurodegenerative diseases. Upon aging, reactive oxygen species (ROS) accumulation induces damage to DNA as well as protein and lipid, thus resulting in a progressive loss in the functional efficiency of the brain. Recently, it has been demonstrated that hundreds of proteins including KIN-19, a homolog of mammalian casein kinase 1 isoform alpha (CK1a), become more insoluble with age in *Caenorhabditis elegans* and its over-expression could enhance polyglutamine-repeat pathology (David et al., 2010). Such discovery indicated that aging process itself could be a causative factor for protein aggregation. Increasing aggregation of proteins, such as amyloid beta peptide, could also promote the generation of ROS, DNA damage and thus accelerate neurodegenerative events (Butterfield, 2002). In addition, redox-active metals Cu and Fe could also generate ROS. Normal aging resulted in an elevation of Cu and Fe in the brain, and further interruption of metal homeostasis was noted in AD (Tabner et al., 2010).

Oxidative DNA lesions, such as 8-oxoguanine (8-oxoG) and 8-hydroxyguanosine (8-OHG), were increased dramatically in patients with PD (Nakabeppu et al., 2007; Lovell et al., 1999). Statistically significant elevation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was detected on DNA of AD subjects, even at the early stage of AD (Lovell et al., 1999; Markesbery & Lovell, 2006). At the same time, DNA repair deficiency in aged or neurodegenerative brain was observed. Both base excision repair (BER) and non-homologous end joining (NHEJ) pathway were deficient in AD subjects (Shackelford, 2006; Weissman et al., 2007). The animals with deficiencies in DNA repair exhibited neurological abnormalities or severe postnatal neurodegeneration and shortened life span (Laposa & Cleaver, 2001; Best, 2009; Dollé et al., 2006), that proved the role of DNA repair deficiency in neurodegeneration. DNA damage combined with inefficient repair mechanism could induce the apoptosis of

brain cells, as well as transcriptional inhibition of the vulnerable genes involved in learning, memory and neuronal survival (Hetman et al., 2010). All these contribute to the pathogenesis of age related neurodegenerative diseases.

Now, histone posttranslational modifications (PTMs) have become an emerging discipline of research, exploring various physiological and pathological processes. Histone PTMs take part in diverse biological processes by inducing chromatin remodelling and regulating gene expression. Increasing evidence suggests that altered patterns of histone PTMs are central to many human diseases. The involvement of histone PTMs in normal nervous system development has been demonstrated and the aberrant PTMs patterns were detected in neurodegenerative disorders (Büttner et al., 2010; Mattson, 2001; Wang et al., 2010; Penner et al., 2010).

It has been widely accepted that histone modifications play important roles in response to DNA damage and in DNA repair (Méndez-Acuña et al., 2010). In the cellular repair machinery, these modified histone sites not only signal the presence of damage, but also provide a landing platform for necessary repair/signaling proteins. Moreover, different PTMs could work together during DNA damage response (Van Attikum & Gasser, 2009). Here, we summarize the abnormal histone PTMs patterns in nuclear DNA damage response (DDR). The role of abnormally expressed histone PTMs in age-associated neurodegenerative disease and their mechanisms are also proposed.

2. Histone PTMs in DNA damage and repair

The core unit of chromatin is the nucleosome which consists of 147 bp of DNA wrapped around histone octamer containing two of each of the core histone proteins H2A, H2B, H3 and H4. The residues at the histone N-terminal tails and globular domains are subjected to PTMs. There are at least nine different types of covalent modifications found in histone proteins, including acetylation, methylation, phosphorylation, deimination, ubiquitination, sumoylation, ADP-ribosylation, proline isomerization and O-linked β -N-acetylglucosamination. Méndez-Acuña has summarized four types of these histone PTMs related to DNA damage response (Méndez-Acuña et al., 2010). Here, we will focus on histone PTMs which are related to DNA damage response during aging and neurodegeneration.

2.1 Phosphorylation

Histone H2A has four variants including H2A1, H2A2, H2AX, and H2AZ. Among them, H2AX is the histone guardian of the genome (Fernandez-Capetillo et al., 2004b). H2AX-/- mice showed the phenomena of radiation sensitivity, growth retardation and immune deficiency (Celeste et al., 2002). Phosphorylation of histone H2AX at serine 139, named gamma-H2AX, is the most characterized PTM at DNA double strand breaks (DSBs). It is catalyzed by the kinases of the PI3-family (ATM, ATR and DNA-PK), especially by ATM (Kinner et al., 2008). It appeared rapidly after the exposure to ionizing radiation and half-maximal amounts were reached by 1 min and maximal amounts by 10 min (Rogakou et al., 1998). Therefore, gamma-H2AX is the first step in recruiting and localizing DNA repair proteins after damage. Presently, gamma-H2AX is regarded as a novel biomarker for DNA breaks and for early stage of apoptosis. Moreover, its expression in a wide range of eukaryotic organisms has indicated its conserved function (Foster & Downs, 2005).

Gamma-H2AX was suggested as a molecular marker of aging and diseases (Mah et al., 2010). The level of H2AX in astrocytes and neurons was found to significantly decrease with the age of participants (Simpson et al., 2010). Meanwhile, the incidence of endogenous gamma-H2AX foci was increased with age (Sedelnikova et al., 2008). The number of gamma H2AX-immunopositive nuclei was significantly increased in the astrocytes of both gray and white matter, and consistently in the cornu ammonis (CA) regions of the hippocampus of AD patients compared to those of control cases (Myung et al., 2008). However, the rate of recruitment of DSB repair proteins to gamma-H2AX foci was correlated inversely with age for both normal and premature aging disease donors (Sedelnikova et al., 2008).

Nuclear localization of DNA repair enzyme DNA-PK and of damaged base 8-OHdG reflect different aspects of the cell response to DNA damage. DNA-PK is required for the NHEJ pathway of DNA repair, whereas, 8-OHdG indicates DNA lesion caused by oxidative damage. Simpson and his colleagues found that the localization of H2AX and DNA-PK demonstrated a good correlation, whereas 8-OHdG localization expression demonstrated a weak correlation with DNA-PK and no significant correlation with H2AX (Simpson et al., 2010). This indicates that H2AX preferentially detects DSBs and is involved in NHEJ repair.

Besides gamma-H2AX, other histone serine phosphorylation events also take place during DDR. Yeast H2A is phosphorylated at serine 122 (threonine 119 in higher eukaryotes) upon DNA damage. Such serine residue was essential for cell survival in the presence of DNA damaging agents (Harvey et al., 2005). The phosphorylation at serine 14 of histone H2B (H2BS14ph) and the phosphorylation at serine 1 of histone H4 (H4S1ph) also occurred in response to DNA damage, and H4S1ph has been shown to be required for an efficient DSB repair by NHEJ (Fernandez-Capetillo et al., 2004a; Cheung et al., 2005). However, the expression of all these PTM sites in aged or neurodegenerative brain has not been reported yet.

Recently, histone phosphorylation at tyrosine was found to be involved in aging, such as phosphorylation of histone H2AX on tyrosine 142 (H2AXY142ph) and histone H3 on tyrosine 99 (H2AXY99ph) (Singh & Gunjan, 2011). Histone H2AXY142 was phosphorylated by the WICH complex and dephosphorylated by the EYA1/3 phosphatases, determining the relative recruitment of either DNA repair or pro-apoptotic factors to DNA damage sites (Stucki, 2009). In contrast to H2AX serine 139 phosphorylation, tyrosine 142 appeared to be constitutively phosphorylated in undamaged cells, but was gradually dephosphorylated in chromatin bearing unrepaired DSBs (Stucki, 2009).

2.2 Methylation

Along the histone polypeptide chain, one, two, or three methyl groups could be added onto the lysine residues by histone methyltransferases (HMT) and could also be removed by histone demethyltransferase (HDMT). Histone methylation is generally associated with transcriptional repression with the exception that several methylation sites are involved in transcriptional activation.

Tri-methylated histone H3 at position lysine 4 (H3K4me₃), an important modification associated with transcriptional regulation, showed different epigenome in neuron cells comparing with non-neuronal cells (Cheung et al., 2010). During aging, a significant decrease in H3K4me₃ was observed at migrated neural progenitor double cortin gene promoters, indicating the possibility of H3K4me₃ in the mechanism of aging-dependent hippocampal dysfunction (Kuzumaki et al., 2010). Other investigation also found that H3K4me₃ was related to DDR and could be detected at newly created DSB. In budding

yeast cells, H3K4me3 was important for a proper response to DNA damaging agents, and the cells that cannot methylate H3K4 displayed a defect in DSB repair by NHEJ (Faucher & Wellinger, 2010). During meiosis, H3K4me3 was critical for the formation of the programmed DSB that initiated homologous recombination (Kniewel & Keeney, 2009).

Histone H3 is constitutively methylated at lysine 79 (H3K79me) in both mammalian and yeast cells. In yeast, H3K79 methylation played an important role in the activation of the G1 and intra S-phase DNA damage checkpoint (Wysocki et al., 2005). It was also detected in the brains of senescence-accelerated prone 8 mice and increased with aging (Wang et al., 2010). Lysine 79 locates in a loop connecting the first and the second α helices in H3 structure (Luger et al., 1997). This region is exposed and adjacent to the interface between H3/H4 tetramer and H2A/H2B dimer, which could influence the access of molecules to the interface. Therefore, the added methyl group on H3K79 might alter the properties of the nucleosome and play an important role in regulating the access of other DNA binding factors to chromatin (Feng et al., 2002). However, the expression level of H3K79 methylation was found unchanged in response to DNA damage. Therefore, methylated H3K79 site might change the higher-order chromatin structure and expose the binding site to DNA damage and repair factors, e.g. the exposure of 53BP1 binding site (Huyen et al., 2004). In addition, H3K79me worked together with phosphorylated H2A serine 129 for the recruitment of budding yeast homolog Rad9 to the DNA damage sites (Huyen et al., 2004; Toh et al. 2006).

Previous investigation has shown that methylated histone H4 at lysine 20 (H4K20me) increased in kidneys and liver of the old-aged rat (Sarg et al., 2002). Except for transcriptional regulation, H4K20me is another reported methylation site that related to DDR. DNA breakage might cause exposure of methylated H4K20 previously buried within the chromosome. At the same time, the level of H4K20 methylation increased locally upon the induction of DSBs by the enzyme named histone methyltransferase MMSET (also known as NSD2 or WHSC1) in mammals (Pei et al., 2011).

2.3 Acetylation

During neurodegeneration, the degree of acetylation balance in brain was greatly impaired (Saha & Pahan, 2006). The inhibition of histone deacetylation induced the sprouting of dendrites, an increased number of synapses, learning behavior reinstatement and long-term memories in bi-transgenic CK-p25 Tg mice (Fischer et al., 2007). All these changes are mainly caused by transcriptional regulation of histone acetylation and/or deacetylation. Actually, DNA damage response regulated by histone acetylation and deacetylation state is also important in neurodegenerative diseases. Moreover, histone deacetylases (HDAC) 1- and 2-depleted cells were hypersensitive to DNA-damaging agents and showed defective DSB repair, particularly NHEJ repair pathway (Miller et al., 2010).

It was found that the acetylation of histone H3 lysine 56 (H3K56ac) was involved in DDR. Mutation of K56 site made the cells sensitive to genotoxic agents (Masumoto et al., 2005). After DNA damage, H3K56ac co-localized with gamma-H2AX and other proteins, involved in DNA damage signaling pathways, such as phospho-ATM, CHK2, and p53, at the sites of DNA repair (Vempati et al., 2010). Furthermore, GCN5, histone acetyltransferase (HAT) for H3K56 was shown to have an important role in maintaining genome stability (Burgess & Zhang, 2010). Knocking down of GCN5 resulted in impaired recruitment of NER factors to sites of damage and inefficient DNA repair (Guo et al., 2011). Histone deacetylases HDAC1 and HDAC2 could be rapidly recruited to DNA-damage sites and promote hypoacetylation

of H3K56. However, HDAC1/2 depletion or inhibition did not affect the amount of DNA damage produced by DSB-inducing agents but impaired DNA repair particularly through NHEJ (Miller et al., 2010)

Histone H4 is acetylated at lysine 16 (H4K16ac) by a human MOF gene encoded protein. Reduced level of H4K16ac correlated with a defective DDR and DSB repair after exposure to ionizing radiation (IR). MOF depletion greatly decreased DSB repair by both NHEJ and homologous recombination (HR) (Sharma et al., 2010). Its specific deacetylase Sir2 was recruited to the HO lesion during HR repair process in budding yeast cells (Tamburini & Tyler, 2005). In response to DNA damage, SIRT1, a mammalian homologue of yeast Sir2, re-localized to DNA breaks to promote repair, resulting in transcriptional changes that parallel those in the aging mouse brain (Oberdoerffer et al., 2008).

Acetylation of H2AX on lysine 36 (H2AXK36Ac) also plays a key role in DSB repair pathway. This modification site is constitutively acetylated by the CBP/p300 acetyltransferase. Though its level was not increased by DNA damage, this modification was required for cells to survive in IR exposure. However, H2AXK36Ac did not affect phosphorylation of H2AX or the formation of DNA damage foci, indicating that H2AXK36Ac was a novel, constitutive histone modification regulating radiation sensitivity independently of H2AX phosphorylation (Jiang et al., 2010).

2.4 Ubiquitination

Increased level of monoubiquitinated histone H2A and decreased level of monoubiquitinated H2B were found to be involved in transcriptional repression during HD, and these two ubiquitylation states inhibited methylation of histone H3K9 and histone H3K4, respectively (Kim et al., 2008). Interestingly, histone acetylation could affect monoubiquitination of histone H2A (Sadri-Vakili et al., 2007), whereas monoubiquitinated histone H2B controlled histone methylation (Sun and Allis, 2002). Thus, histone monoubiquitylation provided a potential bridge between histone acetylation and methylation, which led to the change of gene expression in neurodegenerative diseases (Kim et al., 2008).

Same as other modifications, histone ubiquitination is also involved in DNA damage response. Monoubiquitination of histone H2B, known for its involvement in transcription, was also important for a proper response of budding yeast cells to DNA damaging agents (Faucher & Wellinger, 2010). In human cells, DSBs induced monoubiquitination of histone H2B on lysine 120, and this monoubiquitination was required for timely repair of DSBs (Moyal et al., 2011).

2.5 Poly ADP-ribosylation

Poly ADP-ribosylation of histones is carried out by poly ADP-ribose polymerases (PARPs). Poly ADP-ribosylated histones could stimulate local chromatin relaxation to facilitate the repair process (Monks et al., 2006). Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that contributed to both neuronal death and survival under stress conditions (Kauppinen & Swanson, 2007). PARPs were activated in AD in response to oxidative damage to DNA (Love et al., 1999). PARP-1 activation enhanced core histone acetylation, and the acetylated histone H4 facilitated ADP-ribosylation of histones (Cohen-Armon et al., 2007; Boulikas, 1990). 3-aminobenzamide was found to inhibit poly (ADP-ribose) polymerase as well as histone H3 phosphorylation (Tikoo et al., 2001). Therefore, histone H3

phosphorylation was often coupled to poly-(ADP-ribosylation) during ROS-induced cell death. However, the appearance of histone ADP-ribosylation preceded histone H3 phosphorylation after DNA damage (Monks et al., 2006).

3. How are PTMs involved in DNA damage response and repair?

Several histone modifications are associated with DNA damage and repair by directly regulating activation or repression of DNA repair genes. For example, the expression of manganese-dependent superoxide dismutase (Mn-SOD) was regulated by the acetylation of histones H3 and H4 at Mn-SOD proximal promoter (Maehara et al., 2002). In addition, decreased level of dimethyl H3K4 and acetylated H3K9 also regulated the expression of SOD2 gene (Hitchler et al., 2008). SODs are a group of critical enzymes in counteracting the superoxide toxicity. Altered expression and activity of SOD are all associated with oxidative DNA damage. It was found that *SOD1* mutation could cause familial amyotrophic lateral sclerosis (ALS) (Li et al., 2010). High level of MnSOD was detected in hippocampus of AD patients (Marcus et al., 2005).

On the other hand, these PTM signals are directly read to initiate DNA repair process. 53BP1 is found as one key point protein connecting the PTM signals with other repair molecules. It is a conserved checkpoint protein with properties of a DSB sensor. DNA damage-induced PTMs could change higher-order structure of chromatin and then expose the 53BP1-binding site. In response to DNA damage, 53BP1 is recruited to DSBs sites by binding to gamma-H2AX. A region of 53BP1 upstream of tandem tudor domains bound gamma-H2AX in vitro (Ward et al., 2003). Moreover, 53BP1 was recruited to the sites of DSBs by binding of its tandem tudor domain to methylated histones, such as H4K20me1/2, H3K79me1/2 (Huyen et al., 2004; Pei et al., 2011).

However, the binding of 53BP1 to DNA damage sites is not the first step in DDR. The upstream molecules could regulate such binding activity, such as mediator of DNA damage checkpoint protein 1 (MDC1) and E3 ubiquitin-protein ligase RNF8. MDC1 is a cell cycle checkpoint protein, activated in response to DNA damage. Through its BRCT motifs, MDC1 interacted with gamma-H2AX at sites near DDR within minutes after exposure to ionizing radiation, which facilitated the recruitment of ATM kinase to DNA damage foci (Stewart et al., 2003). Then RNF8 was rapidly assembled at DSBs via interaction of its FHA domain with the phosphorylated adaptor protein MDC1 (Mailand et al., 2007). After that, ubiquitinated H2A and H2AX by RNF8 made the translocation of 53BP1 to the sites of DNA damage (Yan & Jetten, 2008). At the same time, phosphorylated histone methyltransferase MMSET (at Ser 102 site) was also recruited with the interaction to MDC1 BRCT domain, which induced H4K20 methylation around DSBs and also facilitates 53BP1 recruitment (Pei et al., 2011).

What about the mechanism of histone acetylation in DDR? Evidence has indicated that acetylated histones and histone acetyltransferases were involved in DDR by recruiting DNA repair proteins as well as chromatin remodeling factors to DSB sites (Tamburini & Tyler, 2005; Ogiwara et al., 2011). For example, the recruitment of CBP and p300 to the DSB sites induced the acetylation of lysine 18 within histone H3, and lysines 5, 8, 12, and 16 within histone H4, which facilitated the recruitment of KU70 and KU80 for NHEJ. At the same time, BRM, a catalytic subunit of the SWI/SNF complex, was also recruited at DSB sites to establish a relaxed chromatin environment for DNA damage repair. During homologous recombinational repair, histone acetyltransferases GCN5 and Esa1 and histone deacetylases Rpd3, Sir2 and Hst1 were recruited to the HO lesion (Tamburini & Tyler, 2005). Dynamic

changes in histone acetylation were detectable at DSB sites, which might represent important signal for cells indicating that chromosomal repair was complete and might be required to turn off the DNA damage or chromatin structure checkpoint (Tamburini & Tyler, 2005).

Recently, researchers explained the mechanism of ubiquitination of histone H2A at lysine 119 (H2AK119ub) in DDR (Ginjala et al., 2011). ATM, phosphorylated H2AX and RNF8 firstly recruited polycomb protein BMI1 to sites of DNA damage. BMI1 then catalyzed ubiquitination of histone H2AK119 and activated homologous recombination.

Kauppinen and Swanson have described the possible mechanism of poly ADP-ribosylation in DDR (Kauppinen & Swanson, 2007). Poly ADP-ribosylation of histones induced local relaxation of the chromatin structure, which in turn facilitated access of repair proteins to damaged DNA. In addition, the binding of PARP induced the synthesis of a poly ADP-ribose chain (PAR), which worked as a signal for the other DNA repair enzymes, such as DNA ligase III (LigIII) and DNA polymerase beta (pol β), which were necessary for BER process. Regrettably, over-activation of PARP might induce a progressive ATP depletion and finally result in cell death.

4. Specificity of DNA damage and repair in age-associated neurodegenerative diseases

DNA damage could be caused by both exogenous and endogenous damaging agents. However, some external agents, such as UV light, are unlikely to affect neuronal cells because they are never exposed to sunlight. Endogenous DNA damaging agents, such as ROSs, is the predominant DNA damaging agent in age-associated neurodegenerative diseases. The DNA damage and repair occurring in neurodegenerative diseases have been summarized in a number of reviews (Fishel et al., 2007; Martin, 2008). The main forms of damage detected in AD brains as well as in other neurodegenerative diseases are DNA single-strand breaks (SSBs) and DSBs, which are intermediates in repair of oxidative DNA damage. SSBs are primarily removed by BER pathway and nucleotide excision repair (NER) pathway. Neuronal cells also have the capacity to repair DNA lesions by direct repair and mismatch repair (MMR) and have the ability to repair DSBs through homologous recombination (HR) and NHEJ mode. However, the cohesive end joining activity decreased with age of the animal (Vyjayanti & Rao, 2006).

Interestingly, different types of DNA damage had different distribution patterns in neurodegenerative brain, and different cells showed various fates (Barzilai et al., 2008). Most of DNA damage was found in the hippocampus and cortex during aging, which was consistent with the decline in memory and cognitive capacity as the early features of neurodegenerative disease. Astrocytes and neurons, but not microglia, were associated with the presence of DNA damage-associated molecules (H2AX, DNA-PK and 8-OHdG). PARP protein recognizes SSBs sites. Most of the cells containing poly (ADP-ribose), end-product of PARP, were neurons, such as small pyramidal neurons in cortex and some astrocytes, but not microglia (Love et al., 1999). Interestingly, accumulation of poly (ADP-ribose) was not detectable in the cells containing tangles and relatively low accumulation occurred within plaques, which were caused by tau or amyloid beta protein (Love et al., 1999). This might imply that the damage form caused by tau or amyloid beta protein might be DSBs rather than SSBs.

The destiny of different brain cells in response to the same type of DNA damage was diverse, which led researchers to propose that brain cells had different thresholds to

DNA damage (Barzilai et al., 2008). Certain types of neurons, such as hippocampal, pyramidal and granule cells as well as cerebellar granule cells, suffer from an age-associated accumulation of DNA damage but do not reduce in number during aging. Other types of neurons, such as cerebellar Purkinje cells, reduce cell number during aging, but remaining cells show no age-associated accumulation of DNA damage.

In conclusion, DDR differs between various neuronal cells. Therefore certain histone PTM types and their role in repair pathways might be specific to certain age-associated neurodegenerative diseases. However, we should note that some conclusions on histone PTMs involving in DDR came from the investigations on budding yeast cells or non-human mammalian cells. Therefore, further work focusing on human neuronal cells is needed.

5. PTMs in cell cycle re-entry

It is commonly believed that neurons are postmitotic cells, which remains in G0 phase of the cell cycle indefinitely. Actually, neurons in the adult human brain are able to re-enter the cell-division cycle. Schwartz and colleagues have demonstrated cell cycle re-entry phenomenon in neurons (Schwartz et al., 2007). They found that subtoxic concentrations of H₂O₂ induced the formation of repairable DSBs associated with the activation of cyclin D1, G1 cell cycle component, and the phosphorylation of retinoblastoma tumor suppressor protein (pRB) at serine 795, a marker of G0-G1 transition, was also significantly elevated. In addition, DNA helicase subunit minichromosome maintenance (Mcm) protein 2, which is strongly down regulated in quiescent, terminally differentiated or senescent cells, was higher in H₂O₂-treated cells. Nuclear antigen Ki-67 positive neurons, a marker of cells in G1, S, G2 and mitosis, were also increased after treatment. Except for the expression of several cell cycle proteins (Nagy et al., 1997), H3 phosphorylation at serine 10, as a marker for mitosis and transcriptional activation, was also significantly increased in the cytoplasm of neurons in the hippocampus of AD cases (Ogawa et al., 2003).

It seemed that neuronal cells also need to re-enter the cell cycle to activate DNA repair and/or contribute to apoptosis, which shared the same function with proliferating cells (Kruman, 2004). The transition from G0 to G1 was required for NHEJ repair in neurons (Tomashevski et al., 2010). In the brains of AD patients, the cell cycle of neurons could progress as far as the G2 phase, which was also required for repairing DNA lesions (Obulesu & Rao, 2010).

Dynamic change of histone PTMs during cell cycle has been analyzed in HeLa cells (Bonenfant et al., 2007). For example, phosphorylated histone H3 at serine 10 and threonine 3 was only detected in G2/M phase and acetylation on histone H2A and H2B was reduced in G2/M phase, while histone H3K79me showed no change during the cell cycle. Such studies provide us with valuable information for understanding the roles of histone PTMs in cell cycle regulation. However, several recent investigations have reported the roles of histone PTMs in cell cycle re-entry under DNA damage stress (see above), which may be more important in terminally differentiated neurons and likely represent a new research area in the future.

6. Conclusions

Oxidative stress-induced DNA damage has been proposed as pathological mechanism contributing to age-associated neurodegenerative diseases. The modified histone sites not

only signal the presence of damage, but also provide a landing platform for necessary repair/signaling proteins in cellular repair machinery. Moreover, histone residues covalently modified alone or in combination could provide distinct docking sites for multiple nuclear proteins, and thus regulate the expression of genes in response to oxidative stress or other extracellular signals. Therefore, both direct and indirect participation of histone PTMs in DNA damage recognition and repair might play a critical role in the pathological process of age-related neurodegenerative diseases.

Except for nuclear DNA damage, mitochondrial DNA (mtDNA) damage is also proposed to play a critical role in aging and in the pathogenesis of several neurological disorders (Yang et al., 2008). According to previous studies, no histone proteins were found in mitochondria. DNA damage detection and DNA repair processes that involve histone PTMs might not be suitable for mtDNA damage. However, Katherine and her group recently found the positive signal of histone H3 in mitochondrial extracts from *Brassica oleracea* by western blot (Katherine et al., 2010). Regretfully, the function of mitochondrial H3 has not been demonstrated by experimental data. More evidence in other species had not been reported. The question whether there is a relationship between PTMs and mtDNA damage or not needs more hard experimental work to be answered.

In summary, studies of the mechanisms involving histone PTMs will enrich our basic knowledge of the role of histone PTMs in DNA damage response and thus will benefit the strategies for clinical interventions in age-related neurodegenerative diseases in the future.

7. Abbreviations

8-oxoG, 8-oxoguanine; 8-OHG, 8-hydroxyguanosine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; BER, base excision repair; DDR, DNA damage response; DSBs, DNA double strand breaks; HMT, histone methyltransferases; HDMT, histone demethyltransferase; HDAC, histone deacetylases; HAT, histone acetyltransferase; HR, homologous recombination; IR, ionizing radiation; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARPs, poly ADP-ribose polymerase; PTMs, histone post translational modifications; ROS, reactive oxygen species; SSBs, single-strand breaks.

8. References

- Barzilai, A., Biton, S. & Shiloh, Y. (2008). The role of the DNA damage response in neuronal development, organization and maintenance. *DNA Repair (Amst)*, 7(7), 1010-27.
- Best, BP. (2009). Nuclear DNA damage as a direct cause of aging. *Rejuvenation Res*, 12(3), 199-208.
- Bonenfant, D., Towbin, H., Coulot, M., Schindler, P., Mueller, DR. & van Oostrum, J. (2007). Analysis of dynamic changes in post-translational modifications of human histones during cell cycle by mass spectrometry. *Mol Cell Proteomics*, 6(11), 1917-32.
- Boulikas, T. (1990). Poly (ADP-ribosylated) histones in chromatin replication. *J Biol Chem*. 265(24), 14638-47.
- Butterfield, DA. (2002). Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. *Free Radic Res*, 36(12), 1307-13.

- Büttner, N., Johnsen, SA., Kügler, S. & Vogel, T. (2010). Af9/Mllt3 interferes with Tbr1 expression through epigenetic modification of histone H3K79 during development of the cerebral cortex. *Proc Natl Acad Sci U S A*, 107(15), 7042-7.
- Celeste, A., Petersen, S., Romanienko, PJ., Fernandez-Capetillo, O., Chen, HT., Sedelnikova, OA., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, MJ., Redon, C., Pilch, DR., Olaru, A., Eckhaus, M., Camerini-Otero, RD., Tessarollo, L., Livak, F., Manova, K., Bonner, WM., Nussenzweig, MC. & Nussenzweig, A. (2002). Genomic instability in mice lacking histone H2AX. *Science*, 296(5569), 922-7.
- Cheung, I., Shulha, HP., Jiang, Y., Matevossian, A., Wang, J., Weng, Z. & Akbarian, S. (2010). Developmental regulation and individual differences of neuronal H3K4me3 epigenomes in the prefrontal cortex. *Proc Natl Acad Sci U S A*, 107(19), 8824-9.
- Cheung, WL., Turner, FB., Krishnamoorthy, T., Wolner, B., Ahn, SH., Foley, M., Dorsey, JA., Peterson, CL., Berger, SL. & Allis, CD. (2005). Phosphorylation of histone H4 serine 1 during DNA damage requires casein kinase II in *S. cerevisiae*. *Curr Biol*, 15(7), 656-60.
- Cohen-Armon, M., Visochek, L., Rozensal, D., Kalal, A., Geistrikh, I., Klein, R., Bendetz-Nezer, S., Yao, Z. & Seger, R. (2007). DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. *Mol Cell*, 25(2), 297-308.
- David, DC., Ollikainen, N., Trinidad, JC., Cary, MP., Burlingame, AL. & Kenyon, C. (2010). Widespread protein aggregation as an inherent part of aging in *C. elegans*. *PLoS Biol*, 8(8), e1000450.
- Dollé, ME., Busuttill, RA., Garcia, AM., Wijnhoven, S., van Drunen, E., Niedernhofer, LJ., van der Horst, G., Hoeijmakers, JH., van Steeg, H. & Vijg, J. (2006). Increased genomic instability is not a prerequisite for shortened lifespan in DNA repair deficient mice. *Mutat Res*, 596(1-2), 22-35.
- Faucher, D. & Wellinger, RJ. (2010). Methylated H3K4, a transcription-associated histone modification, is involved in the DNA damage response pathway. *PLoS Genet*, 6(8), pii: e1001082.
- Feng, Q., Wang, H., Ng, HH., Erdjument-Bromage, H., Tempst, P., Struhl, K. & Zhang, Y. (2002). Methylation of H3-Lysine 79 Is Mediated by a New Family of HMTases without a SET Domain. *Current Biology*, 12(12), 1052-8.
- Fernandez-Capetillo, O., Allis, CD. & Nussenzweig, A. (2004a). Phosphorylation of histone H2B at DNA double-strand breaks. *J Exp Med*, 199(12), 1671-7.
- Fernandez-Capetillo, O., Lee, A., Nussenzweig, M. & Nussenzweig, A. (2004b). H2AX: the histone guardian of the genome. *DNA Repair (Amst)*, 3(8-9), 959-67.
- Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M. & Tsai LH. (2007). Recovery of learning and memory is associated with chromatin remodelling. *Nature*, 447 (7141), 178-182.
- Fishel, ML., Vasko, MR. & Kelley, MR. (2007). DNA repair in neurons: so if they don't divide what's to repair? *Mutat Res*, 614(1-2), 24-36.
- Foster, ER. & Downs, JA. (2005). Histone H2A phosphorylation in DNA double-strand break repair. *FEBS J*, 272(13), 3231-40.
- Ginjala, V., Nacerddine, K., Kulkarni, A., Oza, J., Hill, SJ., Yao, M., Citterio, E., van Lohuizen, M. & Ganesan, S. (2011). BMI1 is recruited to DNA breaks and contributes to DNA damage induced H2A ubiquitination and repair. *Mol Cell Biol*, Doi: 10.1128/MCB.00981-10.

- Harvey, AC., Jackson, SP. & Downs, JA. (2005). *Saccharomyces cerevisiae* histone H2A Ser122 facilitates DNA repair. *Genetics*, 170(2), 543-53.
- Hetman, M., Vashishta, A. & Rempala, G. (2010). Neurotoxic mechanisms of DNA damage: focus on transcriptional inhibition. *J Neurochem*, 114(6), 1537-49.
- Hitchler, MJ., Oberley, LW. & Domann, FE. (2008). Epigenetic silencing of SOD2 by histone modifications in human breast cancer cells. *Free Radic Biol Med*, 45(11), 1573-80.
- Huyen, Y., Zgheib, O., Ditullio, RA Jr., Gorgoulis, VG., Zacharatos, P., Petty, TJ., Sheston, EA., Mellert, HS., Stavridi, ES. & Halazonetis, TD. (2004). Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature*, 432(7015), 406-11.
- Jiang, X., Xu, Y., & Price, BD. (2010). Acetylation of H2AX on lysine 36 plays a key role in the DNA double-strand break repair pathway. *FEBS Lett*, 584(13), 2926-30.
- Katherine, B., Zanin, M., Donohue, JM., & Everitt, BA. (2010). Evidence that core histone H3 is targeted to the mitochondria in Brassica oleracea. *Cell Biol Int*, 34(10), 997-1003.
- Kauppinen, TM. & Swanson, RA. (2007). The role of poly (ADP-ribose) polymerase-1 in CNS disease. *Neuroscience*, 145(4), 1267-72.
- Kim, MO., Chawla, P., Overland, RP., Xia, E., Sadri-Vakili, G. & Cha, JH. (2008). Altered histone monoubiquitylation mediated by mutant huntingtin induces transcriptional dysregulation. *J Neurosci*, 28(15), 3947-57.
- Kinner, A., Wu, W., Staudt, C. & Iliakis, G. (2008). Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res*, 36(17), 5678-94.
- Kniewel, R. & Keeney, S. (2009). Histone methylation sets the stage for meiotic DNA breaks. *EMBO J*, 28(2), 81-3.
- Kruman, II. (2004). Why do neurons enter the cell cycle? *Cell Cycle*, 3(6), 769-73.
- Kuzumaki, N., Ikegami, D., Tamura, R., Sasaki, T., Niikura, K., Narita, M., Miyashita, K., Imai, S., Takeshima, H., Ando, T., Igarashi, K., Kanno, J., Ushijima, T., Suzuki, T., & Narita, M. (2010). Hippocampal epigenetic modification at the doublecortin gene is involved in the impairment of neurogenesis with aging. *Synapse*, 64(8), 611-6.
- Laposa, RR. & Cleaver, JE. (2001). DNA repair on the brain. *Proc Natl Acad Sci U S A*, 98(23), 12860-2.
- Li, Q., Vande Velde, C., Israelson, A., Xie, J., Bailey, AO., Dong, MQ., Chun, SJ., Roy, T., Winer, L., Yates, JR., Capaldi, RA., Cleveland, DW. & Miller, TM. (2010). ALS-linked mutant superoxide dismutase 1 (SOD1) alters mitochondrial protein composition and decreases protein import. *Proc Natl Acad Sci U S A*, 107(49), 21146-51.
- Love, S., Barber, R. & Wilcock, GK. (1999). Increased poly(ADP-ribosyl)ation of nuclear proteins in Alzheimer's disease. *Brain*, 122 (Pt 2), 247-53.
- Lovell, MA., Gabbita, SP. & Markesbery, WR. (1999). Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. *J Neurochem*, 72(2), 771-6.
- Luger, K., Mäder, AW., Richmond, RK., Sargent, DF. & Richmond, TJ. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, 389(6648), 251-260.
- Maehara, K., Uekawa, N. & Isobe K. (2002). Effects of histone acetylation on transcriptional regulation of manganese superoxide dismutase gene. *Biochem Biophys Res Commun*, 295(1), 187-92.

- Mah, L.J., El-Osta, A. & Karagiannis, T.C. (2010). GammaH2AX as a molecular marker of aging and disease. *Epigenetics*, 5(2), 129-36.
- Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C., Lukas, J., Marcus, D.L., Strafaci, J.A. & Freedman, M.L. (2006). Differential neuronal expression of manganese superoxide dismutase in Alzheimer's disease. *Med Sci Monit*, 12(1), BR8-14.
- Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C. & Lukas, J. (2007). RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell*, 131(5), 887-900.
- Markesbery, W.R. & Lovell, M.A. (2006). DNA oxidation in Alzheimer's disease. *Antioxid Redox Signal*, 8(11-12), 2039-45.
- Martin, L.J. (2008). DNA damage and repair: relevance to mechanisms of neurodegeneration. *J Neuropathol Exp Neurol*, 67(5), 377-87.
- Mattson, M.P. (2003). Methylation and acetylation in nervous system development and neurodegenerative disorders. *Ageing Res Rev*, 2(3), 329-42.
- Méndez-Acuña, L., Di Tomaso, M.V., Palitti, F. & Martínez-López, W. (2010) Histone post-translational modifications in DNA damage response. *Cytogenet Genome Res*, 128(1-3), 28-36.
- Monks, T.J., Xie, R., Tikoo, K. & Lau, S.S. (2006). Ros-induced histone modifications and their role in cell survival and cell death. *Drug Metab Rev*, 38(4), 755-67.
- Moyal, L., Lerenthal, Y., Gana-Weisz, M., Mass, G., So, S., Wang, S.Y., Eppink, B., Chung, Y.M., Shalev, G., Shema, E., Shkedy, D., Smorodinsky, N.I., van Vliet, N., Kuster, B., Mann, M., Ciechanover, A., Dahm-Daphi, J., Kanaar, R., Hu, M.C., Chen, D.J., Oren, M. & Shiloh, Y. (2011) Requirement of ATM-dependent monoubiquitylation of histone H2B for timely repair of DNA double-strand breaks. *Mol Cell*, 41(5), 529-42.
- Myung, N.H., Zhu, X., Kruman, I.I., Castellani, R.J., Petersen, R.B., Siedlak, S.L., Perry, G., Smith, M.A. & Lee, H.G. (2008). Evidence of DNA damage in Alzheimer disease: phosphorylation of histone H2AX in astrocytes. *Age (Dordr)*, 30(4), 209-15.
- Nakabeppu, Y., Tsuchimoto, D., Yamaguchi, H. & Sakumi, K. (2007). Oxidative damage in nucleic acids and Parkinson's disease. *J Neurosci Res*, 85(5), 919-34.
- Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S.K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P., Wright, S.M., Mills, K.D., Bonni, A., Yankner, B.A., Scully, R., Prolla, T.A., Alt, F.W. & Sinclair, D.A. (2008). SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. *Cell*, 135(5), 907-18.
- Obulesu, M. & Rao, D.M. (2010). DNA damage and impairment of DNA repair in Alzheimer's disease. *Int J Neurosci*, 120(6), 397-403.
- Ogawa, O., Zhu, X., Lee, H.G., Raina, A., Obrenovich, M.E., Bowser, R., Ghanbari, H.A., Castellani, R.J., Perry, G. & Smith, M.A. (2003). Ectopic localization of phosphorylated histone H3 in Alzheimer's disease: a mitotic catastrophe? *Acta Neuropathol*, 105(5), 524-8.
- Ogiwara, H., Ui, A., Otsuka, A., Satoh, H., Yokomi, I., Nakajima, S., Yasui, A., Yokota, J. & Kohno, T. (2011). Histone acetylation by CBP and p300 at double-strand break sites facilitates SWI/SNF chromatin remodeling and the recruitment of non-homologous end joining factors. *Oncogene*, Doi:10.1038/onc.2010.592.

- Pei, H., Zhang, L., Luo, K., Qin, Y., Chesi, M., Fei, F., Bergsagel, PL., Wang, L., You, Z. & Lou, Z. (2011). MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature*, 470(7332), 124-8.
- Penner, MR., Roth, TL., Barnes, CA. & Sweatt, JD. (2010). An epigenetic hypothesis of aging-related cognitive dysfunction. *Front Aging Neurosci*, Doi: 10.3389/fnagi.2010.00009.
- Rogakou, EP., Pilch, DR., Orr, AH., Ivanova, VS. & Bonner, WM. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*, 273(10), 5858-68.
- Sadri-Vakili, G., Bouzou, B., Benn, CL., Kim, MO., Chawla, P., Overland, RP., Glajch, KE., Xia, E., Qiu, Z., Hersch, SM., Clark, TW., Yohrling, GJ. & Cha, JH. (2007) Histones associated with downregulated genes are hypo-acetylated in Huntington's disease models. *Hum Mol Genet*, 16(11), 1293-306.
- Saha, RN. & Pahan, K. (2006). HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. *Cell Death Differ*, 13(4), 539-50.
- Sarg, B., Koutzamani, E., Helliger, W., Rundquist, I. & Lindner, HH. (2002) Postsynthetic trimethylation of histone H4 at lysine 20 in mammalian tissues is associated with aging. *J Biol Chem*, 277(42), 39195-201.
- Schwartz, EI., Smilenov, LB., Price, MA., Osredkar, T., Baker, RA., Ghosh, S., Shi, FD., Vollmer, TL., Lencinas, A., Stearns, DM., Gorospe, M. & Kruman, II. (2007). Cell cycle activation in postmitotic neurons is essential for DNA repair. *Cell Cycle*, 6(3), 318-29.
- Sedelnikova, OA., Horikawa, I., Redon, C., Nakamura, A., Zimonjic, DB., Popescu, NC. & Bonner, WM. (2008). Delayed kinetics of DNA double-strand break processing in normal and pathological aging. *Aging Cell*, 7(1), 89-100.
- Shackelford, DA. (2006). DNA end joining activity is reduced in Alzheimer's disease. *Neurobiol Aging*, 27(4), 596-605.
- Sharma, GG., So, S., Gupta, A., Kumar, R., Cayrou, C., Avvakumov, N., Bhadra, U., Pandita, RK., Porteus, MH., Chen, DJ., Cote, J. & Pandita, TK. (2010). MOF and histone H4 acetylation at lysine 16 are critical for DNA damage response and double-strand break repair. *Mol Cell Biol*, 30(14), 3582-95.
- Simpson, JE., Ince, PG., Haynes, LJ., Theaker, R., Gelsthorpe, C., Baxter, L., Forster, G., Lace, GL., Shaw, PJ., Matthews, FE., Savva, GM., Brayne, C. & Wharton, SB. (2010). MRC cognitive function and ageing neuropathology study group. population variation in oxidative stress and astrocyte DNA damage in relation to Alzheimer-type pathology in the ageing brain. *Neuropathol Appl Neurobiol*, 36(1), 25-40.
- Singh, RK. & Gunjan, A. (2011). Histone tyrosine phosphorylation comes of age. *Epigenetics*, 6(2), 153-60.
- Stewart, GS., Wang, B., Bignell, CR., Taylor, AM. & Elledge, SJ. (2003). MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature*, 421(6926), 961-6.
- Stucki, M. (2009). Histone H2A.X Tyr142 phosphorylation: a novel sWItCH for apoptosis? *DNA Repair (Amst)*, 8(7), 873-6.
- Sun, ZW. & Allis, CD. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature*, 418(6893), 104-8.
- Tabner, BJ., Mayes, J. & Allsop, D. (2010). Hypothesis: soluble A β oligomers in association with redox-active metal ions are the optimal generators of reactive oxygen species in Alzheimer's disease. *Int J Alzheimers Dis*, 2011, 546380.

- Tamburini, BA. & Tyler, JK. (2005). Localized histone acetylation and deacetylation triggered by the homologous recombination pathway of double-strand DNA repair. *Mol Cell Biol*, 25(12), 4903-13.
- Tikoo, K., Lau, SS. & Monks, TJ. (2001). Histone H3 phosphorylation is coupled to poly-(ADP-ribosylation) during reactive oxygen species-induced cell death in renal proximal tubular epithelial cells. *Mol Pharmacol*, 60(2), 394-402.
- Toh, GW., O'Shaughnessy, AM., Jimeno, S., Dobbie, IM., Grenon, M., Maffini, S., O'Rorke, A. & Lowndes, NF. (2006). Histone H2A phosphorylation and H3 methylation are required for a novel Rad9 DSB repair function following checkpoint activation. *DNA Repair (Amst)*, 5(6), 693-703.
- Tomashevski, A., Webster, DR., Grammas, P., Gorospe, M. & Kruman, II. (2010). Cyclin-C-dependent cell-cycle entry is required for activation of non-homologous end joining DNA repair in postmitotic neurons. *Cell Death Differ*, 17(7), 1189-98.
- Van Attikum, H. & Gasser, SM. (2009). Crosstalk between histone modifications during the DNA damage response. *Trends Cell Biol*, 19(5), 207-17.
- Vyjayanti, VN. & Rao, KS. (2006). DNA double strand break repair in brain: reduced NHEJ activity in aging rat neurons. *Neurosci Lett*, 393(1): 18-22.
- Wang, CM., Tsai, SN., Yew, TW., Kwan, YW. & Ngai, SM. (2010). Identification of histone methylation multiplicities patterns in the brain of senescence-accelerated prone mouse 8. *Biogerontology*, 11(1), 87-102.
- Ward, IM., Minn, K., Jorda, KG. & Chen, J. (2003). Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J Biol Chem*, 278(22): 19579-82.
- Weissman, L., Jo, DG., Sørensen, MM., de Souza-Pinto, NC., Markesbery, WR., Mattson, MP. & Bohr, VA. (2007). Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnesic mild cognitive impairment. *Nucleic Acids*, 35(16), 5545-55.
- Wysocki, R., Javaheri, A., Allard, S., Sha, F., Côté, J. & Kron, SJ. (2005). Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. *Mol Cell Biol*, 25(19), 8430-43.
- Yan, J. & Jetten, AM. (2008). RAP80 and RNF8, key players in the recruitment of repair proteins to DNA damage sites. *Cancer Lett*, 271(2), 179-90.
- Yang, JL., Weissman, L., Bohr, VA. & Mattson, MP. (2008). Mitochondrial DNA damage and repair in neurodegenerative disorders. *DNA Repair (Amst)*, 7(7), 1110-20.

Transcriptional Functions of DNA Repair Proteins Involved in Premature Aging

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1. Introduction

Premature aging diseases or progerias are rare genetical disorders displaying symptoms of the aging body early in life or even in childhood. They are called segmental because they show some, but not all features of aging. There is the progeria of the adult, Werner syndrome and, more severe because limiting lifespan to the first or second decade, the progerias of the childhood, Cockayne syndrome, trichothiodystrophy and Hutchinson-Gilford syndrome. With the exception of Hutchinson-Gilford syndrome, the progerias are caused by recessive mutations. Cockayne syndrome and trichothiodystrophy are polygenic disorders- the recessive mutation in five respective three different genes can cause the same devastating phenotype. All genes of a polygenic disorder may function in a critical redundant pathway. The identification of these pathways is topic of intensive labour in different laboratories for more than one reason. First, the identification of the molecular defects will help us to treat these diseases. Second, as these diseases mimic the normal aging process, understanding these diseases will strengthen our understanding of aging in general. Third, as these disorders display accelerated aging, the underlying pathways may be critical for the rate of aging and may help us to slow aging respectively allow us to affect healthy aging. Aging is believed to be due to the accumulation of molecular and macromolecular damage (Kirkwood, 2010), thus accelerated aging might be caused by a higher damage rate or by an impairment of counteracting pathways as repair mechanisms. The later assumption is generally believed to be the explanation for the accelerated aging seen in progerias, defects in macromolecular repair pathways, especially in DNA-repair pathways are generally considered as being causal for accelerated aging. Although there is ample evidence that aging is accompanied by macromolecular damage and DNA damage in particular, the causal connection between DNA damage and tissue or organismal aging is far from understood. Here the investigation of progerias is able to fuel our understanding of the mechanisms of aging as most of the involved genes play roles in different DNA repair pathways. But it is not so simple because all the progeria genes display multiple functions in the cells and are also involved in the regulation of gene expression by acting as basal transcription factors or as chromatin modifying enzymes. The discovery of the transcriptional function of DNA repair factors was accompanied by the hypothesis that accelerated aging could also be caused by alterations in gene expression mechanisms (Drapkin et al, 1994, Guzder et al, 1994). Since then the "transcriptional" versus the "DNA

repair” hypothesis were intensively discussed and today there is a lot of evidence for the involvement of both pathways in the pathogenesis of premature aging (Chalut et al,1994, Winkler and Hoeijmakers,1998, Bergmann and Egly 2001, deBoer et al 2002). Transcription of DNA by the RNA polymerases serves as a DNA damage sensor and recruits DNA repair proteins to sites of DNA damage. Moreover, hitherto as pure DNA repair factors recognized proteins turned out to be involved in chromatin remodeling and epigenetic modulation of gene expression of undamaged DNA (Schmitz et al, 2009, LeMay et al, 2010). Thus repair of DNA and gene expression at the level of transcription are intimately structurally and functionally linked. Here we review the current knowledge about transcriptional functions of DNA repair proteins involved in the pathogenesis of progerias.

2. Premature aging syndromes

2.1 Werner syndrome

Werner syndrome (WS) or the progeria of the adult, is an autosomal recessive genetic instability and cancer predisposition syndrome that mimics premature aging. WS patients lack the pubertal growth spurt and develop bilateral cataracts, premature graying and loss of hair and scleroderma-like skin changes already beginning in the second decade of life. Patients have an elevated risk of age-associated diseases as atherosclerotic cardiovascular disease, diabetes mellitus, osteoporosis and cancer. Life expectancy is shortened to 47 years. The WS gene WRN encodes a member of the RecQ helicase protein family and possesses an additional 3'-5' exonuclease domain. WRN is involved in different DNA metabolizing pathways as DNA repair and replication, telomere maintenance and transcription (Rossi et al, 2010, Chu and Hickson, 2009, Ding and Shen 2008). WRN deficient cells display a telomere lagging strand replication defect and karyotypic instability associated with short telomeres (Crabbe et al, 2004, Crabbe et al, 2007). Moreover, premature aging in mice deficient for WRN is dependent on telomere shortening in the telomerase RNA subunit TERC deficient background (Chang et al, 2004). These double knockout mice also show a hallmark of cells derived from Werner syndrome patients- premature senescence. Whereas fibroblasts from normal donors enter the stage of irreversible division stop, replicative senescence, after 60 population doublings, cells from Werner syndrome patients enter senescence after 15-20 population doublings (Faragher et al, 1993). Thus it seems that the role of the WRN protein at the telomeres might be critical to protect us from premature aging. Nonetheless, there are host of questions arising. It is not clear if replicative senescence is responsive for aging pathologies, although markers of replicative senescence accumulate in aged skin of baboons (Herbig et al 2006). Studies on telomere length dynamics in Werner syndrome cells revealed that telomeres did not erode faster than in normal cells (Baird et al, 2004). Expression of telomerase in Werner syndrome cells can overcome premature senescence (Wyllie et al, 2000, Choi et al, 2001), but inhibition of the stress-activated kinase p38 by the compound PD203580 also extended the replicative lifespan of WS cells to that observed in normal fibroblasts (Davis et al 2005). These observations substantiate that our knowledge about the pathophysiology of Werner syndrome is still limited and that alternative concepts also deserve attention.

The first indication of the involvement of RecQ helicases in gene expression came from studies of the yeast WRN homolog *sgs1*. As in WS, the *sgs1* deletion decreases the average life span of cells and accelerates aging (Sinclair et al, 1997). Conditional mutation of *sgs1* in a yeast strain lacking the helicase *srs2* is followed by a drastical inhibition of DNA replication

and RNA polymerase I transcription. The authors concluded that the replication defect could contribute to the genomic instability of WS, whereas impaired ribosomal RNA chain elongation may render RNA polymerase I prone to pausing that could trigger the formation of double strand breaks. Repair by nonhomologous end-joining could result in the accumulation of deletions in the genomic rDNA and contribute to premature aging in WS (Lee et al, 1999). Shiratori and co-workers showed that in human WS cells RNA polymerase I transcription is reduced and can be restored by wildtype WRN. Moreover, nucleolar localization of WRN is dependent on ongoing RNA polymerase I transcription. WRN can be co-immunoprecipitated with RNA polymerase I. In humans, the decreased transcriptional rate of rRNA could be the primary molecular defect causing the premature aging phenotype in WS patients, the authors speculated (Shiratori et al, 2002). Another study with human WS cells described, that the stimulation of RNA polymerase I transcription by some growth factors is impaired in WS cells. Moreover, WRN acts as a transcription factor and stimulates the step of promoter clearance of RNA polymerase I transcription. Chromatin-immunoprecipitations revealed that WRN binds to quiescent and unmethylated rDNA, implicating a role of WRN in epigenetic regulation of RNA polymerase I transcription. Taken together, this study implicated that WRN acts as a growth factor dependent transcription factor of RNA polymerase I and may prevent inactivation of rDNA genes in the absence of growth factors (Lutomska et al, 2008). These mechanisms may contribute to the lack of the pubertal growth spurt, impaired wound healing and premature aging in WS patients.

Beside RNA polymerase I transcription several publications show that WRN influences gene expression by RNA polymerase II. One study from 1999 describes a significant reduction of RNA polymerase II transcription in WS cells. This is reflected in *in vitro* transcription and can be rescued by addition of wildtype but not mutant WRN. Moreover a 27aa repeated sequence in the WRN gene was identified as a strong transcriptional activator domain. The transcription defect in WS cells may be global or may affect certain genes or categories of genes within the genome (Balajee et al, 1999). The later hypothesis is supported by a report showing that the transcriptional activator function of p53 is stimulated by WRN (Blander et al, 1999). Gene expression profiling comparing cells from young donors and cells from old donors with WS cells unravelled that mutation of WRN affects the expression of certain genes within the genome. Moreover transcription alterations in WS were strikingly similar to those in normal aging (Kyng et al, 2003). These findings validated WS as a model disease for aging research and established, that WRN influences the expression of certain genes within the genome. To investigate if the observed changes in the transcriptome are due to the direct loss of WRN or are secondary consequences of genomic instability, Turaga et al used short-term siRNA based knockdown of WRN. This was sufficient to trigger an expression profile resembling fibroblasts established from old donor patients and identified genes involved in 14 distinct biological pathways to be affected by loss of WRN. It is conceivable that WRN might associate with chromatin and affect the activity of classical transcription factors (Turaga et al, 2009). A recent report also used microarray expression analysis to investigate if RecQ helicases and WRN in particular regulate genes enriched in G-quadruplex DNA, a family of non-canonical nucleic acids structures formed by certain G-rich sequences. RecQ helicases can unwind these structures *in vitro* and *in vivo*. In fact the authors found significant associations between loci that are regulated in WS and loci containing potential G-quadruplex forming sequences. These findings indicate that WRN can regulate transcription globally by targeting G-quadruplex DNA (Johnson et al, 2010).

Taken together there is ample evidence that the WRN protein influences transcription by RNA polymerase I and II and is involved in gene regulation of certain genes that are also regulated throughout the normal aging process and in the globally regulation of genes with G-quadruplex forming sequences.

2.2 Cockayne syndrome

Cockayne syndrome is an autosomal recessive neurodegenerative disorder characterized by progressive growth failure, retinal degeneration, cataracts, sensorineural deafness, mental retardation, and photosensitivity (Nance and Berry, 1992, Laugel et al, 2009). Cataracts, loss of retinal cells, neurological degeneration and cachexia are prominent premature aging symptoms of this disorder followed by infant death. Cockayne syndrome is caused by mutations in *CSA* and *CSB* genes and rare combinations with the cancer prone skin disease xeroderma pigmentosum and complementation groups *XPB*, *XPD* and *XPG* have been described. All five genes are involved in repair of helix-distorting lesions of DNA by the nucleotide excision repair pathway (NER). Damage recognition mechanisms differ in the subpathways transcription-coupled repair (TCR) in which *CSA* and *CSB* are involved and global genomic repair (GGR). Both pathways of damage recognition flow into a common DNA repair mechanism. Premature aging in Cockayne syndrome is commonly attributed to defective transcription coupled repair (Hoeijmakers, 2009), although this view raises a plethora of questions. There are multiple mutations in NER proteins described (*XPA*, *XPB*, *XPD*, *XPF*, *XPG*) that completely impair both branches of this DNA repair pathway, but are not followed by premature aging but cause the severe cancer prone skin disease xeroderma pigmentosum. Xeroderma pigmentosum patients, when shed from UV-light do not develop the premature aging traits of Cockayne syndrome although the same type of DNA lesions remain unrepaired and should accumulate and disturb cellular fidelity. Thus transcription coupled repair might also be responsive for the repair of hitherto undefined DNA lesions that compromise transcription (Laugel et al, 2009). It is conceivable that all five genes that, when mutated cause Cockayne syndrome, are involved in a critical redundant function that protects us from accelerated aging. This function is not identified yet. Transcription-coupled repair is responsible for the recruitment of the repair machinery to the transcribed strand of active genes. It has been shown, that nearly all factors involved in transcription coupled repair are coincidental participating in basal transcription mechanisms thus raising the possibility that premature aging might be due to aberrant transcription. This hypothesis is discussed since two decades without a definitive answer. Here, we would like to review the current knowledge about the transcriptional functions of the five proteins involved in the pathogenesis of Cockayne syndrome.

2.2.1 Cockayne syndrome protein A (CSA)

As the *CSA* gene was identified, the interaction of the corresponding protein with *CSB* and the TFIIH subunit p44 were described. TFIIH, beside being essential for Nucleotide Excision Repair (NER) of UV damaged DNA, is a general transcription factor of RNA polymerase II (see below). Thus the authors proposed a transcriptional function of *CSA* (Henning et al, 1995). In vitro transcription studies with a template bearing oxidative lesions showed a reduced RNA polymerase II transcription in nuclear extracts of *CSA* cells, that could be rescued by the overexpression of *CSA* indicating a transcriptional function of *CSA* (Dianov et al, 1997). However, microinjection of *CSA* antibodies in cells reduced the repair capacity

of the cells, but did not influence the rate of transcription by RNA polymerase II (van Gool et al, 1997). Since then additional evidence for a direct involvement of CSA in transcription was not found.

2.2.2 Cockayne syndrome protein B (CSB)

Mutations in the *ERCC6/CSB* gene are responsible for 62% of Cockayne syndrome cases (Laugel et al, 2009). The *ERCC6* gene product belongs to the family of SWI/SNF chromatin remodeling enzymes (Troelstra et al, 1992). ATP-dependent chromatin remodeling enzymes coordinate changes in chromatin structure to help regulate transcription. This structure of CSB implies a regulatory role in transcription. The first study investigating a transcriptional role of CSB used *in vivo* labeling and permeabilisation of cells to show that CSB mutant cells exhibit a severely reduced RNA polymerase II transcription that could be restored by addition of CSB. As transcription of chromatin from permeabilized cells represent the elongation activity of RNA polymerase II, the authors proposed a role of CSB in transcription elongation (Balajee et al, 1997). Using microinjection of antibodies against CSB, van Gool et al did not detect an inhibition of transcription by all three RNA polymerases measured by labelled thymidine incorporation. However, CSB cofractionates with RNA polymerase II over chromatographic columns and RNA polymerase II can be coimmunoprecipitated with CSB (van Gool et al, 1997). As these complexes do not contain initiation factors of RNA polymerase II, the authors speculated that CSB might be involved in a non-essential step of transcription elongation. Another study confirmed the interaction of RNA polymerase II with CSB and could convincingly show, that CSB stimulates elongation by RNA polymerase II *in vitro* (Selby and Sancar, 1997). If this function of CSB is relevant for transcription elongation *in vivo*, the study by van Gool et al should have detected a reduction of transcription by microinjection of CSB antibodies. The recruitment of CSB to RNA polymerase II elongation complexes *in vitro* was also demonstrated by other investigators (Tantin et al, 1997) but the *in vivo* relevance of this interaction was not studied. CSB mutant cells were found to exhibit metaphase fragility of highly transcribed genes of RNA polymerase II and III that are coding for structural RNAs. The authors proposed that CSB might play a role in transcription elongation of these genes and lack of CSB would be followed by stalled polymerases inducing metaphase fragility (Yu et al, 2000). ATP-dependent chromatin remodeling activity of CSB was substantiated in another study, thus implicating that CSB may play a role in facilitating transcription by RNA polymerase II through pause sites on natural chromatin templates *in vivo* by modulating nucleosome structure on DNA. It is possible that defective chromatin rearrangements during DNA repair or transcription may contribute to the severe clinical symptoms of CS patients (Citterio et al, 2000). A study performed in *Saccharomyces cerevisiae* provided *in vivo* evidence for a role of *rad26*, the counterpart of the *CSB* gene, in transcription elongation by RNA polymerase II. Under conditions requiring rapid synthesis of new mRNAs, growth is considerably reduced in cells lacking *rad26*. These findings implicate a role for CSB in transcription elongation, and they strongly suggest that impaired transcription elongation is the underlying cause of the developmental problems in CS patients (Lee et al, 2001).

The same authors showed that the CSB homolog *rad26* plays a role in promoting transcription by RNA polymerase II through bases damaged by the alkylating agent MMS. Transcription through these bases is severely inhibited in *rad26Δ* cells lacking both the NER (nucleotide excision repair) and BER (base excision repair) pathways required for the removal of these lesions (Lee et al, 2002). This report demonstrates a transcriptional function

of CSB independent from DNA repair and, moreover, fit to the observation, that nuclear extracts from CS cells of three complementation groups exhibit reduced RNA polymerase II *in vitro* transcription only on oxidised template (Dianov et al, 1997).

Microarray analysis of gene expression profiles did not identify significant differences in gene expression between CSB deficient and transfected cells indicating that CSB does not function as a gene specific transcription factor (Selzer et al, 2002). Bradsher et al reported a novel aspect of CSB as a component of RNA polymerase I transcription in the nucleolus. CSB was localized to nucleoli and isolated in a complex with RNA polymerase I, transcription initiation factors of RNA polymerase I, TFIIB and XPG. CS mutations in TFIIB subunits rendered this complex unstable and stability of this transcription competent complex was speculated to contribute to Cockayne syndrome phenotype. RNA polymerase I transcription was reduced in CSB mutant cells and restored by transfection of CSB (Bradsher et al, 2002). This report is inasmuch interesting as it describes a functional complex of 4 from 5 proteins that, when mutated cause Cockayne syndrome, indicating that rDNA transcription by RNA polymerase I might be the redundant function of the CS proteins whose failure causes premature aging.

Microarray analysis after oxidative stress revealed that there is a bundle of genes that is differently regulated after H₂O₂ treatment of CSB deficient and CSB competent cells. If the identified genes are directly regulated by CSB was not further specified (Kyng et al, 2003).

Confocal microscopy and quantitative digital image analysis of different photobleaching (FRAP) procedures showed transient interactions of CSB with the transcription machinery, which are prolonged when RNA polymerases are arrested at sites of DNA damage. Active RNA polymerase II could be immunopurified with CSB, but no transcription factors were found to be associated (van dem Boom et al, 2003). The CSB function in transcription was to this timepoint always linked to transcription elongation. A novel study discovered functions of CSB upstream of transcription initiation. The authors unveiled the crucial role played by CSB in the transcription initiation of a certain set of protein coding genes after UV irradiation. CSB cells cannot transcribe even nondamaged genes if the cells were previously UV irradiated. The recruitment of TBP, which is supposed to initiate transcription, was severely decreased; also, the recruitment of TFIIB was almost absent. Furthermore, histone H4 acetylation does not occur properly, highlighting a defect in one of the earlier events of the transcriptional process. The fact that CSB associates mainly with the unphosphorylated RNA pol IIA and the serine 5 phosphorylated RNA pol IIO, strongly supports a role for CSB during the first phases of the transcription reaction (Proietti-Di-Santis et al, 2006). Although earlier microarray analysis did not yield gene expression differences in CS-cells (Selzer et al, 2002), refined methodology using microarrays in combination with a unique method for comparative expression analysis found many genes regulated by CSB. Remarkably, many of the genes regulated by CSB are also affected by inhibitors of histone deacetylase and DNA methylation, as well as by defects in poly(ADP-ribose)-polymerase function and RNA polymerase II elongation. This data indicate a general role for CSB protein in maintenance and remodeling of chromatin structure and suggest that CS is a disease of transcriptional deregulation caused by misexpression of growth-suppressive, inflammatory, and proapoptotic pathways (Newman et al, 2006). *In vitro* transcription analysis using a reconstituted transcription system showed, that bypass of different oxidative lesions in the template requires elongation factors like CSB, thus again evaluating the initial observation by Dianov et al, that CSB deficient cells exhibit reduced RNA polymerase II transcription on oxidised template (Charlet-Berguerant et al, 2006).

Extending the findings of Newman et al, that CSB influences chromatin remodeling to transcription of RNA polymerase I, Yuan et al demonstrated in an intricate analysis that transcription activation of RNA polymerase I is dependent on CSB. CSB is recruited to active rDNA repeats by TTF-I bound to the promoter-proximal terminator T₀. Depletion of CSB by siRNA impairs the formation Pol I preinitiation complexes and inhibits rDNA transcription. CSB recruits G9a that methylates histone H3 on lysine 9 (H3K9) in the pre-rRNA coding region. The results demonstrate that the functional cooperation between CSB and G9a is important for efficient pre-rRNA synthesis (Yuan et al, 2007). This study integrates findings of several above mentioned publications. A gene specific regulatory function upstream of transcription initiation was mechanistically deciphered and a chromatin organisation mode of CSB was described in detail.

Analysis of in vitro transcription by RNA polymerase I revealed that CSB plays a role as an elongation factor in rDNA transcription and that truncated CSB still localizes to the rDNA repeats in vivo. Truncated CSB actively represses in vitro transcription of RNA polymerase I thus providing an explanation for the observation that a null mutation in CSB is not necessarily followed by CS (Horibata et al, 2004) whereas truncating mutations are devastating (Lebedev et al, 2008).

CSB is also a critical mediator of the hypoxic response and influences binding of the general transcription factors and RNA polymerase II in a gene-specific manner in response to hypoxia as demonstrated by chromatin-immunoprecipitation analysis. CSB binds to p53 and might also influence its transcriptional activity, the authors speculated (Filippi et al, 2008). Thus it becomes evident that CSB is not only an elongation factor of RNA polymerase II but exhibits gene regulatory functions in a gene-specific manner.

The reviewed studies clearly show that CSB is an elongation factor of transcription by RNA polymerase I and II and that CSB facilitates transcription through damaged bases. Additional, CSB functions upstream of initiation by RNA polymerase I and II by recruiting chromatin modifying cofactors or by remodeling chromatin itself in a gene specific manner.

2.2.3 TFIIH

TFIIH is a multisubunit complex composed of ten subunits. It harbors three enzymatic activities, two ATP dependent helicases of opposite orientation, XPB and XPD and the cyclin dependent kinase cdk7. Mutations in the XPB and XPD helicases are followed by the skin cancer prone xeroderma pigmentosum syndrome but also by the premature aging syndromes Cockayne and trichothiodystrophy. Mutations in the recently discovered tenth subunit p8/TTDA destabilize TFIIH and is followed by trichothiodystrophy. Xeroderma pigmentosum is characterized by a 1000fold elevated skin cancer risk, the german term "Mondscheinkinder" translated "moonshine-children" denominates the fact that the failure of Nucleotide excision repair (NER) renders the skin of affected children so sensitive to UV-induced DNA damage and consecutive development of skin destructive cancers that they need to be shed totally from UV-light by special clothing. Then they develop normally. The ATPase of XPB and XPD helicase activity of TFIIH are necessary for the unwinding of the damaged DNA strand that can then be cleaved and resynthesized. Thus highly mutagenic DNA lesions persist in the genome when the helicase functions of TFIIH are reduced or inactivated by mutations. The second main function of TFIIH is as a general transcription factor of RNA polymerase II. General or basal transcription factors are needed at every protein coding gene for bending of the promoter, positioning of the polymerase or promoter opening as through the ATPase activity of the XPB subunit of TFIIH (Kim et al, 2000,

Douziech et al, 2000). TFIIF has also been reported to play a postinitiation role in transcription by RNA polymerase I, the key step of ribosomal biogenesis, that accounts for up to 60% of ongoing transcription in a growing cell (Iben et al, 2002).

Xeroderma pigmentosum is due to unrepaired DNA damage, whereas premature aging in Cockayne syndrome and trichothiodystrophy might be caused by transcriptional deficiencies. Several studies addressed this hypothesis and an unequivocal answer to the question "is it repair or transcription?" has not been delivered yet.

Trichothiodystrophy mice with a mutation in XPD reflect to a remarkable extent the human disorder, including brittle hair, developmental abnormalities, reduced life span, UV sensitivity, and skin abnormalities. The cutaneous symptoms are associated with reduced transcription of a skin-specific gene strongly supporting the concept of TTD as a human disease due to inborn defects in basal transcription. To explain the characteristic hair and skin abnormalities of TTD, TTD-type XPD mutations may alter the XPD conformation and in this way affect the stability of the TFIIF complex. Under normal conditions, *de novo* synthesis of TFIIF is thought to compensate for the reduced half-life. However, in terminal differentiating tissues where *de novo* synthesis gradually declines, the mutated TFIIF might get exhausted before the transcriptional program has been completed (de Boer et al, 1998). These authors describe that late in the differentiation pathway and thus gene-specific transcription is severely disturbed in trichothiodystrophy in contrast to general transcription deficiencies (whole genome).

A study comparing XP versus TTD mutations in the helicase XPD showed the following: all XPD mutations, regardless of causing Xeroderma pigmentosum or trichothiodystrophy are detrimental for XPD helicase activity, thus explaining the NER defect. TFIIF from TTD patients, but not from XP patients, exhibits a significant *in vitro* basal transcription defect in addition to a reduced intracellular concentration. Moreover, when XPD mutations prevent interaction with the p44 subunit of TFIIF, transactivation directed by certain nuclear receptors is inhibited, regardless of TTD versus XP phenotype, thus explaining the overlapping symptoms (Dubaele et al, 2003). TTD can also be caused by mutations in the TTDA subunit of TFIIF. Although this subunit is dispensable for the transcriptional activity of TFIIF in RNA polymerase II transcription, it nonetheless stabilizes TFIIF allowing expression of late acting genes and thus performs a specific gene expression activity (Hashimoto and Egly, 2009 and references therein).

Asking if TTD might be a transcription syndrome, the authors of the next study used microarrays to detect transcriptional differences between TTD and XP cells from the XP-D complementation group. They compared gene expression profiles in cultured fibroblasts from normal, XP and TTD donors and concluded that there are minimal differences in gene expression in proliferating fibroblasts from TTD, XP-D and normal donors (Offmann et al, 2008) thus arguing against transcriptional deficiencies being causal for trichothiodystrophy. Mutations in XPB and XPD subunits of TFIIF can also cause a combination of xeroderma pigmentosum and Cockayne syndrome. As the failure of the DNA repair function of TFIIF explains the cancer susceptibility of xeroderma pigmentosum, additional functions of TFIIF might be responsible for the premature aging phenotype of Cockayne syndrome.

An optimized cell-free *in vitro* RNA polymerase II transcription assay was used to analyze transcription activity of XP-B and XP-D as well as XPB/CS and XPD/CS. Although the growth rate was normal, the XP-B and XP-D cells contained reduced amounts of TFIIF. Extracts prepared from XP-B and XP-D lymphoblastoid cells exhibited similar transcription activity from the adenovirus major late promoter when compared to that in extracts from

normal cells. Thus, the authors concluded that the XP-B and XP-D lymphoblastoid cells do not have impaired RNA transcription activity. They considered the possible consequences of the reduced cellular content of TFIIH for the clinical symptoms in XP-B or XP-D patients, and discuss a 'conditional phenotype' that may involve an impairment of cellular function only under certain growth conditions (Satoh and Hanawald, 1997).

Subsequent another study investigated mutant TFIIH in a reconstituted RNA polymerase II transcription assay. Mutations in XP-B/Cockayne syndrome patients decrease the transcriptional activity of the corresponding TFIIH by preventing promoter opening of RNA polymerase II. The XP-B patient with the most severe symptoms was the patient with the lowest TFIIH transcription activity *in vitro*. These findings point out that the severity of the clinical symptoms observed within the XP-B patients is a function of the TFIIH activity in transcription rather than in NER. Both XPB mutations result in an almost total inhibition (~95%) of NER. Western blot analysis and enzymatic assays indicate that XPD mutations affect the stoichiometric composition of TFIIH due to a weakness in the interaction between XPD-CAK complex and the core TFIIH, resulting in a partial reduction of transcription activity. The authors concluded that XP-B and XP-D patients are more likely to suffer from transcription repair syndromes rather than DNA repair disorders (Coin et al, 1999). This report identified failures of mutant TFIIH acting on the adenovirus-major-late promoter representative for all RNA polymerase II genes. Thus it describes a general deficiency in gene expression rather than a gene specific effect expected to be causative for Cockayne syndrome. TFIIH does influence gene-specific transcription by the interaction with transcriptional regulators or by phosphorylation of transcription factors like nuclear receptors (reviewed in Zurita and Merino, 2003).

2.2.4 XPG in transcription

XPG also called ERCC5 is an endonuclease that excises the 3' end of an unwinded damaged DNA single strand in nucleotide excision repair (NER). Endonuclease inactivating mutations are followed by xeroderma pigmentosum, whereas truncating mutations in XPG are followed by a severe form of Cockayne syndrome. The authors hypothesised that XPG exhibits a second function critical for the development of Cockayne syndrome (Noussipikel et al, 1997). That XPG as well as its yeast counterpart RAD2 are biochemically isolatable in a complex with TFIIH and interact with multiple subunits of this DNA repair/basal transcription factor was early recognized (Iyer et al, 1996, Habraken et al, 1996). Genetic studies in yeast cells, knocking out the yeast counterparts of XPG and CSB, Rad2 and Rad26, unravelled an involvement of both proteins in transcription by RNA polymerase II.

The authors provide evidence for the involvement of RAD2 in Pol II-dependent transcription. Interestingly, they found that both transcription and growth are more severely inhibited in the *rad2Δ rad26Δ* double mutant than in the *rad2Δ* and *rad26Δ* single mutants. These results indicate that RAD2 and RAD26 provide alternate means for efficient transcription, and further, they implicate transcriptional defects as the underlying cause of growth impairment that occurs in the *rad2Δ*, *rad26Δ*, and *rad2Δ rad26Δ* mutant strains under conditions that would require the synthesis of new mRNAs. From these studies, they infer that CS is likely a transcription syndrome and that growth and developmental defects in CS could result from defects in transcription (Lee et al, 2002). In a report studying the involvement of CSB in RNA polymerase I transcription, functional XPG was identified in a complex with CSB, TFIIH, RNA polymerase I initiation factor TIF-IB and RNA polymerase I indicating that XPG might play a role in ribosomal transcription by RNA polymerase I

(Bradsher et al, 2002). Thorel and coworkers described a mild case of Cockayne syndrome characterised by a XPG with nuclease activity that lost the interaction domain with TFIIH. This interaction might be critical for the development of the disease (Thorel et al, 2004).

Another level of complexity was added by the description of an epigenetic function of XPG. DNA methylation is an epigenetic modification that is essential for gene silencing and genome stability in many organisms. The authors show that Gadd45a (growth arrest and DNA-damage-inducible protein 45 alpha), a nuclear protein involved in maintenance of genomic stability, DNA repair and suppression of cell growth, has a key role in active DNA demethylation. Active demethylation occurs by DNA repair and Gadd45a interacts with and requires the DNA repair endonuclease XPG. They concluded that Gadd45a relieves epigenetic gene silencing by promoting DNA repair, which erases methylation marks (Barreto et al, 2007).

XPG forms a stable complex with TFIIH, which is active in transcription and NER. Mutations in XPG found in XP-G/CS patient cells that prevent the association with TFIIH also resulted in the dissociation of CAK and XPD from the core TFIIH. As a consequence, the phosphorylation and transactivation of nuclear receptors were disturbed in XP-G/CS as well as *xpg(-/-)* MEF cells and could be restored by expression of wild-type XPG. These results provide an insight into the role of XPG in the stabilization of TFIIH and the regulation of gene expression and provide an explanation of some of the clinical features of XP-G/CS. (Ito et al, 2007). This is the first report indicating that XPG serves a gene-specific regulatory function in transcription. An involvement of XPG in the regulation of RNA polymerase I transcription described a mechanism that seems to be conserved between RNA polymerase I and II. In both cases, Gadd45a recruits the NER proteins including XPG to demethylate and activate epigenetic silenced promoter regions. The results reveal a mechanism that recruits the DNA repair machinery to the promoter of active genes, keeping them in a hypomethylated state (Barreto et al, 2007; Schmitz et al, 2009). An intimate functional link between Nucleotide excision repair (NER) and transcription by RNA polymerase II was unravelled in the groundbreaking study by Egly and co-workers.

Upon gene activation, they found that RNA polymerase II transcription machinery assembles sequentially with the nucleotide excision repair (NER) factors at the promoter. This recruitment occurs in absence of exogenous genotoxic attack, is sensitive to transcription inhibitors, and depends on the XPC protein. The presence of these repair proteins at the promoter of activated genes is necessary in order to achieve optimal DNA demethylation and histone posttranslational modifications (H3K4/H3K9 methylation, H3K9/14 acetylation) and thus efficient RNA synthesis. Deficiencies in some NER factors impede the recruitment of others and affect nuclear receptor transactivation. This data suggest that there is a functional difference between the presence of the NER factors at the promoters (which requires XPC) and the NER factors at the distal regions of the gene (which requires CSB). While the latter may be a repair function, the former is a function with respect to transcription (LeMay et al, 2010).

2.3 Hutchinson-Gilford progeria syndrome

Hutchinson-Gilford progeria syndrome (HGPS) is a very rare genetic disorder with an estimated incidence rate of 1 in 8 million. Taken in consideration misdiagnosed or unreported cases, the true figure might be closer to 1 in 4 million (Pollex et al, 2004). HGPS was first described by Dr. Jonathan Hutchinson in 1886 and Dr. Hastings Gilford in 1897 and ever since just over 100 cases of HGPS have been reported. Like all progeria HGPS is

characterised by segmental premature aging. Children with this disease appear normal at birth but manifestations of HGPS appear at the age between 12-24 months. Characteristic features include delayed dentition, micrognathia, loss of subcutaneous fat, growth retardation, midface hypoplasia, alopecia, atherosclerosis and generalised osteodysplasia with osteolysis and pathologic fractures (www.progeriaresearch.org). The median age at death is about 13 years, and at least 90% of all patients die from progressive atherosclerosis of the coronary and cerebrovascular arteries (Baker et al. 1981).

HGPS is caused by mutations in *LMNA* which encodes lamins A and C. Most patients (80%) reveal a de novo heterozygous point mutation (G608G: GGC→GGT) in exon 11 of *LMNA* gene. (Eriksson et al, 2003; De Sandre-Giovannoli et al, 2003). Lamins A and C are type V intermediate filaments which are major components of the nuclear lamina, a protein scaffold at the inner nuclear membrane, which also extends as a network throughout the nucleus.

However, the *LMNA* G608G mutation responsible for most cases of HGPS does not cause an amino acid change, but activates a cryptic splice site leading to a truncated variant of lamin A (progerin) with an in-frame deletion of 50 amino acids near the carboxy terminus (Eriksson et al, 2003; De Sandre-Giovannoli et al, 2003). Due to the loss of 50 amino acids, progerin is lacking an important endoprotease cleavage site required for excision of the farnesylcystein methyl ester. Thus, the HGPS mutation causes the accumulation of permanently farnesylated progerin in the cell nucleus.

One of the most apparent outcomes of the accumulation of progerin is the morphological change of nuclei. HGPS is characterised by significant changes in nuclear size and shape, including lobulation of the nuclear envelope, wrinkle formation, thickening of the the nuclear lamina and clustering of nuclear pores (Goldman et al, 2004.; Eriksson et al, 2003; De Sandre-Giovannoli et al, 2003; Scaffidi et al, 2005; Lammerding et al, 2005).

Another aspect widely discussed in literature is the potential regulatory role of progerin in gene expression as lamins are also interacting with chromatin. Chromatin in *Zmpste24*^{-/-} MEFs, which are also accumulating farnesylated prelamin A (progerin), aggregates at discrete regions in a balloon shape and further analysis showed a variety of chromosomal abnormalities (aneuploidy, ring structures, chromosome instability and DNA breaks) (Liu et al, 2005). Further studies revealed that HGPS cells show significant changes in epigenetic control of heterochromatin (Goldman et al, 2004; Shumaker et al, 2006). Heterochromatin markers such as histone H3 trimethylated on lysine 27 (H3K27me3), for facultative heterochromatin, as well as H3 trimethylated on lysine 9 (H3K9me3), for pericentric constitutive heterochromatin, are lost in HGPS cells (Shumakers et al, 2006). These changes could be directly link to progerin expression and are detectable even before changes in shapes of nuclei occur, suggesting that progerin changes gene regulation and silencing even at low levels.

It is suggested that lamin A has diverse roles in DNA metabolism, including DNA replication and transcription and also gene expression. Genome expression profiling of HGPS revealed differentially expressed genes in HGPS fibroblasts compared to age matched control cell lines, which play a role in a variety of biological processes. The most prominent categories encode transcription factors and extracellular matrix proteins, many of which are known to function in the tissues severely affected in HGPS. The most affected gene was *MEOX/GAX*, a homeobox gene that functions as a negative regulator of mesodermal tissue proliferation (Csoka et al, 2004). Microarray analysis from another study showed significant changes in 352 genes of which 306 were down regulated and 46 up regulated in HGPS cells. Functional analysis indicated that most of the genes are important for lipid metabolism, cell

growth and differentiation, cell cycle, DNA replication and repair as well as cardiovascular system development (Marji et al, 2010). The only altered expressed gene encoding a protein known to directly interact with A-type lamins has been *Rb1*. Rb plays an important role in cell cycle control and also regulates differentiation. The level of Rb expression at the mRNA and protein level was down regulated in cells derived from HGPS patients and also downstream targets of *Rb1* were affected (Marji et al, 2010). There is also evidence for a significant reduction of hyperphosphorylated Rb in HGPS fibroblasts (Dechat et al, 2007). Based on these observations, decreased Rb expression and reduction of hyperphosphorylated Rb in HGPS cells may lead to deregulation of proliferation (Marji et al, 2010). Similar observation have been reported in cells derived from *Lmna*^{-/-} mice implicating that absence or mutation of lamin A lead to unstable Rb and a altered lamin A/C-Rb signaling in HGPS cells (Johnson et al, 2004).

Transcription is also in part regulated by the nuclear scaffold which regulates the association and organisation of genes and transcription factors. Certain observations implicate that active transcription complexes are bound to the nuclear lamina and transcription factors as well as active genes are reported to be enriched in nuclear matrix preparations (Jackson et al, 1985; Stein et al, 1995). The contribution of nuclear lamins in transcription has been suggested by different studies. Loss of function mutation of lamin (*Dm*₀) in *Drosophila* disrupts the directed outgrowth of cytoplasmic extensions from terminal cells of the tracheal system, and oocytes from germ line mutants show improper localization of mRNA in the cytoplasm (Guillemín et al., 2001). These results confirm the requirement of nuclear lamin for cytoplasmic as well as nuclear organization. Lamin associated protein 2B (LAP 2B) is a lamin binding protein and has been shown to mediate transcriptional repression (Mancini et al, 1994). Another study revealed that during vertebrate development, changes in the expression of lamins correlates with the beginning of transcription and cell differentiation (Moir et al, 1995). Additionally, disruption of normal lamin organization in active embryonic nuclei from *Xenopus* leads to inhibition of RNA polymerase II activity. The authors suggested that lamins may act as a scaffold upon which the basal transcription factors required for RNA polymerase II transcription are organized (Spann et al, 2002). A recent study by Osorio et al. also demonstrate reduction of RNA polymerase I transcription in *Zmpst24* deficient mice. This decrease is due to hypermethylation and hypoacetylation of rDNA leading to a more compact, silent and dysfunctional rDNA gene activity (Osorio et al, 2010). It is also reported that DNA replication can be regulated by the lamina scaffold. Nuclei from *Xenopus* eggs lost their ability to synthesise DNA after immunodepletion of lamins (Newport et al, 1990). Furthermore, mutation in lamina in *Xenopus* blocks DNA replication at the transition from the initiation to the elongation phase of DNA replication (Moir et al, 2000).

3. Conclusion

Transcriptional alterations as the driving force behind premature aging- the implications are vast. The dispute of the competing hypothesis if its primarily unrepaired, accumulating DNA damage or if there is a kind of genetic program that drives premature and normal aging is not solved yet. As both pathways, the DNA damage response and transcriptional regulation are intimately connected by bi- or multifunctional proteins, the separation of progerias in DNA-repair or transcriptional syndromes might turn out to be artificial. The gene expression profiles, the transcriptomes of the aging body might be initiated by DNA

damage but executed by specific transcription factors that respond to DNA damage. Mutations in these specific transcription factors could initiate a gene expression profile that resembles the normal answer to accumulating DNA damage. To prove these hypothesis there is a lot of exciting work ahead.

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5. References

- Baird, D.M., Davis, T., Rowson, J., Jones, C.J. and Kipling, D. (2004) Normal telomere erosion rates at the single cell level in Werner syndrome fibroblast cells. *Hum Mol Genet*, 13, 1515-1524.
- Baker, P.B., Baba, N. and Boesel, C.P. (1981) Cardiovascular abnormalities in progeria. Case report and review of the literature. *Arch Pathol Lab Med*, 105, 384-386.
- Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S.K., Handa, V., Doderlein, G., Maltry, N., Wu, W., Lyko, F. and Niehrs, C. (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature*, 445, 671-675.
- Bergmann, E. and Egly, J.M. (2001) Trichothiodystrophy, a transcription syndrome. *Trends Genet*, 17, 279-286.
- Blander, G., Kipnis, J., Leal, J.F., Yu, C.E., Schellenberg, G.D. and Oren, M. (1999) Physical and functional interaction between p53 and the Werner's syndrome protein. *J Biol Chem*, 274, 29463-29469.
- Chalut, C., Moncollin, V. and Egly, J.M. (1994) Transcription by RNA polymerase II: a process linked to DNA repair. *Bioessays*, 16, 651-655.
- Chang, S., Multani, A.S., Cabrera, N.G., Naylor, M.L., Laud, P., Lombard, D., Pathak, S., Guarente, L. and DePinho, R.A. (2004) Essential role of limiting telomeres in the pathogenesis of Werner syndrome. *Nat Genet*, 36, 877-882.
- Charlet-Berguerand, N., Feuerhahn, S., Kong, S.E., Ziserman, H., Conaway, J.W., Conaway, R. and Egly, J.M. (2006) RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors. *Embo J*, 25, 5481-5491.
- Chu, W.K. and Hickson, I.D. (2009) RecQ helicases: multifunctional genome caretakers. *Nat Rev Cancer*, 9, 644-654.
- Coin, F., Bergmann, E., Tremeau-Bravard, A. and Egly, J.M. (1999) Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH. *Embo J*, 18, 1357-1366.
- Crabbe, L., Jauch, A., Naeger, C.M., Holtgreve-Grez, H. and Karlseder, J. (2007) Telomere dysfunction as a cause of genomic instability in Werner syndrome. *Proc Natl Acad Sci U S A*, 104, 2205-2210.
- Crabbe, L., Verdun, R.E., Haggblom, C.I. and Karlseder, J. (2004) Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. *Science*, 306, 1951-1953.
- Csoka, A.B., English, S.B., Simkevich, C.P., Ginzinger, D.G., Butte, A.J., Schatten, G.P., Rothman, F.G. and Sedivy, J.M. (2004) Genome-scale expression profiling of

- Hutchinson-Gilford progeria syndrome reveals widespread transcriptional misregulation leading to mesodermal/mesenchymal defects and accelerated atherosclerosis. *Aging Cell*, 3, 235-243.
- de Boer, J., Andressoo, J.O., de Wit, J., Huijman, J., Beems, R.B., van Steeg, H., Weeda, G., van der Horst, G.T., van Leeuwen, W., Themmen, A.P., Meradji, M. and Hoeijmakers, J.H. (2002) Premature aging in mice deficient in DNA repair and transcription. *Science*, 296, 1276-1279.
- De Sandre-Giovannoli, A., Bernard, R., Cau, P., Navarro, C., Amiel, J., Boccaccio, I., Lyonnet, S., Stewart, C.L., Munnich, A., Le Merrer, M. and Levy, N. (2003) Lamin A truncation in Hutchinson-Gilford progeria. *Science*, 300, 2055.
- Dechat, T., Shimi, T., Adam, S.A., Rusinol, A.E., Andres, D.A., Spielmann, H.P., Sinensky, M.S. and Goldman, R.D. (2007) Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging. *Proc Natl Acad Sci U S A*, 104, 4955-4960.
- Dianov, G.L., Houle, J.F., Iyer, N., Bohr, V.A. and Friedberg, E.C. (1997) Reduced RNA polymerase II transcription in extracts of cockayne syndrome and xeroderma pigmentosum/Cockayne syndrome cells. *Nucleic Acids Res*, 25, 3636-3642.
- Douziech, M., Coin, F., Chipoulet, J.M., Arai, Y., Ohkuma, Y., Egly, J.M. and Coulombe, B. (2000) Mechanism of promoter melting by the xeroderma pigmentosum complementation group B helicase of transcription factor IIIH revealed by protein-DNA photo-cross-linking. *Mol Cell Biol*, 20, 8168-8177.
- Drapkin, R., Reardon, J.T., Ansari, A., Huang, J.C., Zavel, L., Ahn, K., Sancar, A. and Reinberg, D. (1994) Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature*, 368, 769-772.
- Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P., Dutra, A., Pak, E., Durkin, S., Csoka, A.B., Boehnke, M., Glover, T.W. and Collins, F.S. (2003) Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature*, 423, 293-298.
- Faragher, R.G., Kill, I.R., Hunter, J.A., Pope, F.M., Tannock, C. and Shall, S. (1993) The gene responsible for Werner syndrome may be a cell division "counting" gene. *Proc Natl Acad Sci U S A*, 90, 12030-12034.
- Filippi, S., Latini, P., Frontini, M., Palitti, F., Egly, J.M. and Proietti-De-Santis, L. (2008) CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response. *Embo J*, 27, 2545-2556.
- Goldman, R.D., Shumaker, D.K., Erdos, M.R., Eriksson, M., Goldman, A.E., Gordon, L.B., Gruenbaum, Y., Khuon, S., Mendez, M., Varga, R. and Collins, F.S. (2004) Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci U S A*, 101, 8963-8968.
- Guillemin, K., Williams, T. and Krasnow, M.A. (2001) A nuclear lamin is required for cytoplasmic organization and egg polarity in *Drosophila*. *Nat Cell Biol*, 3, 848-851.
- Guzder, S.N., Sung, P., Bailly, V., Prakash, L. and Prakash, S. (1994) RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. *Nature*, 369, 578-581.

- Habraken, Y., Sung, P., Prakash, S. and Prakash, L. (1996) Transcription factor TFIIH and DNA endonuclease Rad2 constitute yeast nucleotide excision repair factor 3: implications for nucleotide excision repair and Cockayne syndrome. *Proc Natl Acad Sci U S A*, 93, 10718-10722.
- Hashimoto, S. and Egly, J.M. (2009) Trichothiodystrophy view from the molecular basis of DNA repair/transcription factor TFIIH. *Hum Mol Genet*, 18, R224-230.
- Henning, K.A., Li, L., Iyer, N., McDaniel, L.D., Reagan, M.S., Legerski, R., Schultz, R.A., Stefanini, M., Lehmann, A.R., Mayne, L.V. and Friedberg, E.C. (1995) The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. *Cell*, 82, 555-564.
- Herbig, U., Ferreira, M., Condell, L., Carey, D. and Sedivy, J.M. (2006) Cellular senescence in aging primates. *Science*, 311, 1257.
- Hoeijmakers, J.H. (2009) DNA damage, aging, and cancer. *N Engl J Med*, 361, 1475-1485.
- Horibata, K., Iwamoto, Y., Kuraoka, I., Jaspers, N.G., Kurimasa, A., Oshimura, M., Ichihashi, M. and Tanaka, K. (2004) Complete absence of Cockayne syndrome group B gene product gives rise to UV-sensitive syndrome but not Cockayne syndrome. *Proc Natl Acad Sci U S A*, 101, 15410-15415.
- Iben, S., Tschochner, H., Bier, M., Hoogstraten, D., Hozak, P., Egly, J.M. and Grummt, I. (2002) TFIIH plays an essential role in RNA polymerase I transcription. *Cell*, 109, 297-306.
- Ito, S., Kuraoka, I., Chymkowitz, P., Compe, E., Takedachi, A., Ishigami, C., Coin, F., Egly, J.M. and Tanaka, K. (2007) XPG stabilizes TFIIH, allowing transactivation of nuclear receptors: implications for Cockayne syndrome in XP-G/CS patients. *Mol Cell*, 26, 231-243.
- Iyer, N., Reagan, M.S., Wu, K.J., Canagarajah, B. and Friedberg, E.C. (1996) Interactions involving the human RNA polymerase II transcription/nucleotide excision repair complex TFIIH, the nucleotide excision repair protein XPG, and Cockayne syndrome group B (CSB) protein. *Biochemistry*, 35, 2157-2167.
- Jackson, D.A. and Cook, P.R. (1985) Transcription occurs at a nucleoskeleton. *Embo J*, 4, 919-925.
- Johnson, B.R., Nitta, R.T., Frock, R.L., Mounkes, L., Barbie, D.A., Stewart, C.L., Harlow, E. and Kennedy, B.K. (2004) A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation. *Proc Natl Acad Sci U S A*, 101, 9677-9682.
- Johnson, J.E., Cao, K., Ryvkin, P., Wang, L.S. and Johnson, F.B. Altered gene expression in the Werner and Bloom syndromes is associated with sequences having G-quadruplex forming potential. *Nucleic Acids Res*, 38, 1114-1122.
- Kim, T.K., Ebright, R.H. and Reinberg, D. (2000) Mechanism of ATP-dependent promoter melting by transcription factor IIIH. *Science*, 288, 1418-1422.
- Kirkwood, T.B. Global aging and the brain. *Nutr Rev*, 68 Suppl 2, S65-69.
- Kyng, K.J., May, A., Brosh, R.M., Jr., Cheng, W.H., Chen, C., Becker, K.G. and Bohr, V.A. (2003) The transcriptional response after oxidative stress is defective in Cockayne syndrome group B cells. *Oncogene*, 22, 1135-1149.
- Lammerding, J., Hsiao, J., Schulze, P.C., Kozlov, S., Stewart, C.L. and Lee, R.T. (2005) Abnormal nuclear shape and impaired mechanotransduction in emerin-deficient cells. *J Cell Biol*, 170, 781-791.

- Laugel, V., et al., (2009) Mutation update for the CSB/ERCC6 and CSA/ERCC8 genes involved in Cockayne syndrome. *Hum Mutat*, 31, 113-126.
- Le May, N., Mota-Fernandes, D., Velez-Cruz, R., Iltis, I., Biard, D. and Egly, J.M. NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. *Mol Cell*, 38, 54-66.
- Le May, N., Mota-Fernandes, D., Velez-Cruz, R., Iltis, I., Biard, D. and Egly, J.M. NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. *Mol Cell*, 38, 54-66.
- Lebedev, A., Scharffetter-Kochanek, K. and Iben, S. (2008) Truncated Cockayne syndrome B protein represses elongation by RNA polymerase I. *J Mol Biol*, 382, 266-274.
- Lee, S.K., Johnson, R.E., Yu, S.L., Prakash, L. and Prakash, S. (1999) Requirement of yeast SGS1 and SRS2 genes for replication and transcription. *Science*, 286, 2339-2342.
- Lee, S.K., Yu, S.L., Prakash, L. and Prakash, S. (2001) Requirement for yeast RAD26, a homolog of the human CSB gene, in elongation by RNA polymerase II. *Mol Cell Biol*, 21, 8651-8656.
- Lee, S.K., Yu, S.L., Prakash, L. and Prakash, S. (2002) Requirement of yeast RAD2, a homolog of human XPG gene, for efficient RNA polymerase II transcription. implications for Cockayne syndrome. *Cell*, 109, 823-834.
- Lee, S.K., Yu, S.L., Prakash, L. and Prakash, S. (2002) Yeast RAD26, a homolog of the human CSB gene, functions independently of nucleotide excision repair and base excision repair in promoting transcription through damaged bases. *Mol Cell Biol*, 22, 4383-4389.
- Liu, B., Wang, J., Chan, K.M., Tjia, W.M., Deng, W., Guan, X., Huang, J.D., Li, K.M., Chau, P.Y., Chen, D.J., Pei, D., Pendas, A.M., Cadinanos, J., Lopez-Otin, C., Tse, H.F., Hutchison, C., Chen, J., Cao, Y., Cheah, K.S., Tryggvason, K. and Zhou, Z. (2005) Genomic instability in laminopathy-based premature aging. *Nat Med*, 11, 780-785.
- Lutomska, A., Lebedev, A., Scharffetter-Kochanek, K. and Iben, S. (2008) The transcriptional response to distinct growth factors is impaired in Werner syndrome cells. *Exp Gerontol*, 43, 820-826.
- Mancini, M.A., Shan, B., Nickerson, J.A., Penman, S. and Lee, W.H. (1994) The retinoblastoma gene product is a cell cycle-dependent, nuclear matrix-associated protein. *Proc Natl Acad Sci U S A*, 91, 418-422.
- Marji, J., O'Donoghue, S.I., McClintock, D., Satagopam, V.P., Schneider, R., Ratner, D., Worman, H.J., Gordon, L.B. and Djabali, K. Defective lamin A-Rb signaling in Hutchinson-Gilford Progeria Syndrome and reversal by farnesyltransferase inhibition. *PLoS One*, 5, e11132.
- Moir, R.D., Spann, T.P. and Goldman, R.D. (1995) The dynamic properties and possible functions of nuclear lamins. *Int Rev Cytol*, 162B, 141-182.
- Moir, R.D., Spann, T.P., Herrmann, H. and Goldman, R.D. (2000) Disruption of nuclear lamin organization blocks the elongation phase of DNA replication. *J Cell Biol*, 149, 1179-1192.
- Nance, M.A. and Berry, S.A. (1992) Cockayne syndrome: review of 140 cases. *Am J Med Genet*, 42, 68-84.
- Newman, J.C., Bailey, A.D. and Weiner, A.M. (2006) Cockayne syndrome group B protein (CSB) plays a general role in chromatin maintenance and remodeling. *Proc Natl Acad Sci U S A*, 103, 9613-9618.

- Newport, J.W., Wilson, K.L. and Dunphy, W.G. (1990) A lamin-independent pathway for nuclear envelope assembly. *J Cell Biol*, 111, 2247-2259.
- Nouspikel, T., Lalle, P., Leadon, S.A., Cooper, P.K. and Clarkson, S.G. (1997) A common mutational pattern in Cockayne syndrome patients from xeroderma pigmentosum group G: implications for a second XPG function. *Proc Natl Acad Sci U S A*, 94, 3116-3121.
- Osorio, F.G., Varela, I., Lara, E., Puente, X.S., Espada, J., Santoro, R., Freije, J.M., Fraga, M.F. and Lopez-Otin, C. Nuclear envelope alterations generate an aging-like epigenetic pattern in mice deficient in Zmpste24 metalloprotease. *Aging Cell*, 9, 947-957.
- Pollex, R.L. and Hegele, R.A. (2004) Hutchinson-Gilford progeria syndrome. *Clin Genet*, 66, 375-381.
- Proietti-De-Santis, L., Drane, P. and Egly, J.M. (2006) Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. *Embo J*, 25, 1915-1923.
- Rossi, M.L., Ghosh, A.K. and Bohr, V.A. Roles of Werner syndrome protein in protection of genome integrity. *DNA Repair (Amst)*, 9, 331-344.
- Satoh, M.S. and Hanawalt, P.C. (1997) Competent transcription initiation by RNA polymerase II in cell-free extracts from xeroderma pigmentosum groups B and D in an optimized RNA transcription assay. *Biochim Biophys Acta*, 1354, 241-251.
- Scaffidi, P., Gordon, L. and Misteli, T. (2005) The cell nucleus and aging: tantalizing clues and hopeful promises. *PLoS Biol*, 3, e395.
- Schmitz, K.M., Schmitt, N., Hoffmann-Rohrer, U., Schafer, A., Grummt, I. and Mayer, C. (2009) TAF12 recruits Gadd45a and the nucleotide excision repair complex to the promoter of rRNA genes leading to active DNA demethylation. *Mol Cell*, 33, 344-353.
- Schmitz, K.M., Schmitt, N., Hoffmann-Rohrer, U., Schafer, A., Grummt, I. and Mayer, C. (2009) TAF12 recruits Gadd45a and the nucleotide excision repair complex to the promoter of rRNA genes leading to active DNA demethylation. *Mol Cell*, 33, 344-353.
- Selby, C.P. and Sancar, A. (1997) Cockayne syndrome group B protein enhances elongation by RNA polymerase II. *Proc Natl Acad Sci U S A*, 94, 11205-11209.
- Shiratori, M., Suzuki, T., Itoh, C., Goto, M., Furuichi, Y. and Matsumoto, T. (2002) WRN helicase accelerates the transcription of ribosomal RNA as a component of an RNA polymerase I-associated complex. *Oncogene*, 21, 2447-2454.
- Shumaker, D.K., Dechat, T., Kohlmaier, A., Adam, S.A., Bozovsky, M.R., Erdos, M.R., Eriksson, M., Goldman, A.E., Khoun, S., Collins, F.S., Jenuwein, T. and Goldman, R.D. (2006) Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. *Proc Natl Acad Sci U S A*, 103, 8703-8708.
- Sinclair, D.A., Mills, K. and Guarente, L. (1997) Accelerated aging and nucleolar fragmentation in yeast *sgs1* mutants. *Science*, 277, 1313-1316.
- Spann, T.P., Goldman, A.E., Wang, C., Huang, S. and Goldman, R.D. (2002) Alteration of nuclear lamin organization inhibits RNA polymerase II-dependent transcription. *J Cell Biol*, 156, 603-608.
- Stein, G.S., van Wijnen, A.J., Stein, J., Lian, J.B. and Montecino, M. (1995) Contributions of nuclear architecture to transcriptional control. *Int Rev Cytol*, 162A, 251-278.

- Tantin, D., Kansal, A. and Carey, M. (1997) Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes. *Mol Cell Biol*, 17, 6803-6814.
- Thorel, F., Constantinou, A., Dunand-Sauthier, I., Nospikel, T., Lalle, P., Raams, A., Jaspers, N.G., Vermeulen, W., Shivji, M.K., Wood, R.D. and Clarkson, S.G. (2004) Definition of a short region of XPG necessary for TFIIH interaction and stable recruitment to sites of UV damage. *Mol Cell Biol*, 24, 10670-10680.
- Troelstra, C., van Gool, A., de Wit, J., Vermeulen, W., Bootsma, D. and Hoeijmakers, J.H. (1992) ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. *Cell*, 71, 939-953.
- Turaga, R.V., Paquet, E.R., Sild, M., Vignard, J., Garand, C., Johnson, F.B., Masson, J.Y. and Lebel, M. (2009) The Werner syndrome protein affects the expression of genes involved in adipogenesis and inflammation in addition to cell cycle and DNA damage responses. *Cell Cycle*, 8, 2080-2092.
- van Gool, A.J., Citterio, E., Rademakers, S., van Os, R., Vermeulen, W., Constantinou, A., Egly, J.M., Bootsma, D. and Hoeijmakers, J.H. (1997) The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex. *Embo J*, 16, 5955-5965.
- Winkler, G.S. and Hoeijmakers, J.H. (1998) From a DNA helicase to brittle hair. *Nat Genet*, 20, 106-107.
- Yu, A., Fan, H.Y., Liao, D., Bailey, A.D. and Weiner, A.M. (2000) Activation of p53 or loss of the Cockayne syndrome group B repair protein causes metaphase fragility of human U1, U2, and 5S genes. *Mol Cell*, 5, 801-810.
- Yuan, X., Feng, W., Imhof, A., Grummt, I. and Zhou, Y. (2007) Activation of RNA polymerase I transcription by cockayne syndrome group B protein and histone methyltransferase G9a. *Mol Cell*, 27, 585-595.
- Zurita, M. and Merino, C. (2003) The transcriptional complexity of the TFIIH complex. *Trends Genet*, 19, 578-584.

Part 4

DNA Damage and Disease

DNA Damage and mRNA Levels of DNA Base Excision Repair Enzymes Following H₂O₂ Challenge at Different Temperatures

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1. Introduction

Our cells are continuously exposed to endogenous and exogenous oxidizing agents that can damage DNA leading to disruption of transcription, translation, and DNA replication (Davies, 2000). Accordingly, protective mechanisms, including the presence of cellular antioxidants and induction of enzymatic repair of damaged lesions, are necessary in order to survive in this oxidizing environment. When damaged DNA lesions are not prevented or properly repaired, they can cause mutations that increase the risk of degenerative diseases.

The baseline level of oxidative damage associated with normal cellular processes has been estimated as high as 1 base modification per 130,000 bases in nuclear DNA (Davies, 2000). However, high levels of reactive oxygen species (ROS) result in an increase in modified DNA levels. Overall more than 20 modified DNA base lesions have been identified, including 8-oxoguanine which is the most abundant DNA adduct (Cooke et al., 2003). In addition, ROS can attack DNA, generating strand breaks, sugar damage and DNA-protein cross-links. A single strand break (SSB) is a discontinuity in the sugar-phosphate backbone of one strand of a DNA duplex leaving modified ends which inhibit or block DNA polymerases and DNA ligases (Caldecott, 2001). SSBs can be produced directly from ROS attack, indirectly from DNA repair processes, by direct disintegration of deoxyribose, or by abortive DNA topoisomerase 1 activity (Dianov & Parsons, 2007; Leppard & Campoux, 2005).

DNA repair mechanisms to maintain the genomic integrity have been described. DNA base lesions and single strand breaks resulting from ROS-induced oxidative attack are mainly repaired through the base excision repair (BER) pathway (reviewed in (Caldecott, 2003; de Murcia & Menissier de Murcia, 1994; Dianov & Parsons, 2007; Wilson, 2007)). In order to repair DNA base lesions, BER is initiated by specific DNA glycosylases. For example, human 8-oxoguanine DNA-glycosylase 1 (hOGG1), a bifunctional glycosylase, recognizes and cleaves 8-oxoguanine and also catalyzes 3' of the abasic site (AP site). Following this initiation step, AP-endonuclease I (APE1) cleaves the AP site making a gap between the DNA 3'-OH and the 5'-phosphate. The SSBs, produced either directly from ROS attack or indirectly from DNA repair processes, can then be recognized by the enzyme poly (ADP-ribose) polymerase-1 (PARP1). Binding of PARP-1 to the AP sites stimulates the formation of poly (ADP-ribose) polymers and dissociation of PARP-1 from the DNA-recruiting BER

proteins at the damage site. DNA polymerase β fills the gap by DNA synthesis. Finally the resulting nick is sealed by DNA ligase, completing the short-patch repair pathway. In this pathway X-ray cross-complementing 1 (XRCC1) plays a major role in facilitating the interaction among the proteins involved in the BER pathway such as APE1, DNA polymerase β and DNA ligase III (Caldecott, 2003). Depending on the nature of AP sites, some AP sites are repaired by the long-patch repair pathway requiring different enzymes including flap endonuclease and DNA ligase I. Even though the basic DNA repair mechanisms are well described, recent evidence suggests that DNA repair mechanisms are quite complicated with more than 100 proteins involved in the repair of various lesions (Wood et al., 2001).

Deficiencies in DNA repair systems have been shown in several types of cancer (Langland et al., 2002; Lynch & Smyrk, 1996; Marchetto et al., 2004). However, whether such deficiencies in DNA repair enzymes are associated with single nucleotide polymorphisms (SNPs) is still arguable.

Several studies have examined the DNA repair capacity of different cells upon exposure to environmental agents such as oxidants or antioxidants (Astley et al., 2002; Collins et al., 1995, 2003; Torbergson & Collins, 2000). Most studies have either only monitored DNA damage or determined the mRNA expression levels of DNA repair enzymes, mostly hOGG1, in order to elucidate the role of oxidants or antioxidants on DNA repair activity. However, monitoring only DNA damage for DNA repair kinetics reflects more global effects instead of specific aspects of repair (Berwick & Vineis, 2000). In addition, the exact relationships between oxidative stress, DNA damage and induction of the mRNA expression levels of repair enzymes including hOGG1 is unclear (Hodges & Chipman, 2002; Kim et al., 2001) although hOGG1 has been shown to be inducible responding to various oxidative conditions (Kim et al., 2001; Lan et al., 2003).

As these previous studies indicate, multiple methods and markers of DNA damage and repair may be needed in order to explain molecular responses to DNA damaging agents. Furthermore, information is still lacking about the rate of DNA repair immediately following treatment with oxidants or antioxidants; this information may be important in determining steady-state damage levels following induction of oxidative stress (Collins & Harrington, 2002). Therefore the current study was undertaken to better understand the relationship between cellular DNA damage and induction of mRNA expression of repair enzymes following acute oxidant treatment using Caco-2 cells (human colon cancer cells). Oxidant (H_2O_2) treated cells were monitored over an extended recovery period, and both DNA damage levels and mRNA levels of several DNA BER enzymes (hOGG1, APE1, PARP1, XRCC1) were quantified over time in order to better understand DNA repair kinetics and the molecular responses to an oxidative DNA damaging agent.

2. Materials and methods

2.1 Chemicals and reagents

Caco-2 cells were generously donated by Dr. Bo Lonnerdal (University of California, Davis). Hydrogen peroxide, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Minimum essential medium (MEM) with Earl's salts, including 1-glutamine and 0.25% trypsin-EDTA solution, were from Gibco (Invitrogen, Carlsbad, CA). Fetal bovine

serum was obtained from Gemini (West Sacramento, CA). For the single cell gel electrophoresis assay, a commercial kit was purchased from Trevigen Co. (Gaithersburg, MD). For real time PCR, reverse transcription kits and SYBR Green PCR master mix were purchased from Applied Biosystems (Foster City, CA) and Roche (Mannheim, Germany), respectively.

2.2 Cell culture and treatments

Caco-2 cells were grown in MEM, supplemented with 10% (v/v) fetal bovine serum, L-glutamine, 1% penicillin and streptomycin (10 units/mL and 1 mg/mL respectively) at 37°C in a humidified environment composed of 5% CO₂ and 95% air; the growing medium was changed every two days. Cells were subcultured at 80-90% confluency. After seeding onto a 100 mm cell culture plate with a density of 5×10⁶ cells/plate, cells were grown for one day and treated with fresh hydrogen peroxide (100 μM) for 30 min at 37°C, a physiologically relevant temperature, or for 10 min at 4°C, a condition where DNA repair should be minimized. After washing with phosphate buffered saline (PBS), cells were incubated in growing medium (including serum) at 37°C for up to 5 h. For the cells treated with oxidant at 37°C, they were further incubated for 8 hours in order to confirm the mRNA expression pattern of some DNA repair genes. Some cells were collected for comet assay and the others were collected for RNA extraction. Each experimental treatment was performed in duplicate on 3 different days.

2.3 Measurement of DNA damage

After incubation in growing medium for 0-5 h, cells were harvested with trypsin-EDTA solution, washed twice with ice-cold PBS, and cell viability was determined with the trypan blue exclusion test. The single cell gel electrophoresis (comet assay) procedure was based on methods of Singh et al. with slight modifications (Singh et al., 1988). A commercial comet assay kit was used to measure strand breaks following the manufacturer's protocols. Briefly, cells were diluted with PBS in order to have a cell density of 1×10⁵ cells/mL and embedded into low melting point agarose on comet slides. Embedded cells were lysed in lysis solution (including 1% sodium lauryl sarcosinate) for 1 hour and unwound in alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13). Subsequently, electrophoresis was performed for 30 min at 300 mA. Cells were neutralized by washing with water, dried following immersion in ethanol, and kept at room temperature in the dark until silver staining. Silver stained cells were imaged using a Nikon E600 with a Leica LEI-750 camera.

Images were analyzed by measuring % tail DNA of each cell using CometScore software (version 1.5, www.autocomet.com). Cells (75 total) were collected from 2 slides per treatment and the whole procedure for DNA damage measurement was repeated three times independently. Slides were coded and counted blindly; after imaging and counting, slides were decoded in order to quantify differences among samples.

2.4 Measurement of expression of DNA repair enzymes

2.4.1 Total RNA extraction

Total RNA was extracted from Caco-2 cells using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The concentrations of extracted RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington,

DE) with the quality of RNA determined from the absorbance ratio of $A_{260}/A_{280} > 1.8$ and confirmed by gel electrophoresis. Extracted RNA was preserved at -80°C until used. cDNA was synthesized using 5 μg total RNA, oligo d(T)₁₆ primers, and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). Reverse transcription was performed by following the manufacturer's protocol.

2.4.2 Real-time quantitative RT-PCR (qRT-PCR)

In order to detect DNA repair enzyme genes (hOGG1, APE1, PARP1 and XRCC1) and β -actin (used as a reference gene), qRT-PCR was performed using SYBR Green PCR Master Mix reagents (Roche, Mannheim, Germany) on a PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers for each gene are shown in Table 1. Real time quantitative RT-PCR (real time qRT-PCR) was performed through the amplification for 40 cycles of 95°C (30 sec), 58°C (30 sec) and 60°C (1 min) after activation of enzyme at 95°C (10 min). The data were normalized using β -actin as an internal standard. Relative fold changes were calculated using the formula of $2^{-\Delta\Delta\text{Ct}}$ by comparing mRNA levels to the control. Triplicate qRT-PCR analyses were run for each sample.

Primers		Sequences (5' → 3')	Size (bp)	References
β -actin	Forward	TCACCCAACACTGTGCCCATCTACGA	180	Mambo et al., 2005
	Reverse	TCGGTGAGGATCTTCATGAGGTA		
hOGG1	Forward	GCGACTGCTGCGACAAGAC	250	Chevallard et al., 1998
	Reverse	TCGGGCACTGGCACTCAC		
APE1	Forward	GAG TAA GAC GGC CGC AAA GAA AAA	296	Collins et al., 2003
	Reverse	CCG AAG GAG CTG ACC AGT ATT GAT		
PARP1	Forward	CAA CTT TGC TGG GAT CCT GT	185	Mayer et al., 2002
	Reverse	TGT TTC CAA GGG CAA CTT CT		
XRCC1	Forward	CGC TGG GGA GCA AGA CTA TG	517	Noe et al., 2004
	Reverse	CAA ATC CAA CTT CCT CTT CC'		

Table 1. Primers of DNA repair enzyme genes and reference gene for RT-PCR analysis.

2.5 Statistical analysis

Each experiment was performed three times independently. Statistical evaluations were performed with GraphPad Prism (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) was used to determine the significance of the experimental variables.

Mean values for each treatment were compared with the Dunnett's multiple comparison post-test at a 95% confidence interval. Student's t-test was used to compare effects of the temperature.

3. Results

Caco-2 cells were treated with sublethal concentrations (100 μ M) of hydrogen peroxide at two different temperatures. This concentration of hydrogen peroxide showed no significant effects on the viability of Caco-2 cells under these experimental conditions and was confirmed to generate significant DNA damage including DNA SSBs and oxidative DNA adducts (Min & Ebeler, 2009). Oxidant treatment at 4°C for 10 min has been adopted in several previous studies to reduce DNA repair and so was adopted in this study as a comparison treatment where DNA repair should be minimized (Astley et al., 2002; Collins et al., 1995). The higher temperature (37°C) was used to represent physiological temperature conditions. Neither condition affected cell viability in the present study (data not shown). Levels of DNA damage (SSBs) was monitored over time by comet assay and mRNA levels of several BER enzymes were correspondingly determined by real time qRT-PCR.

Fig. 1 shows DNA damage levels during the recovery time following oxidant treatment at 4°C. Immediately after hydrogen peroxide treatment (time 0), DNA damage increased significantly. Levels of damage then decreased consistently throughout the recovery period. Fig. 2 shows corresponding levels of mRNA for several DNA BER enzymes following oxidant challenge at 4°C. Except for XRCC1, mRNA levels for all DNA repair enzymes varied significantly during the recovery time following oxidant treatment ($p < 0.05$). mRNA levels of PARP1 increased immediately after oxidant treatment at time 0. In contrast, expression levels of hOGG1, which cleaves the 8-oxoguanine lesion, decreased initially (0 h) and remained lower than control throughout the recovery period. APE1 expression also decreased initially (0 h) but then increased again over the recovery period following treatment at 4°C.

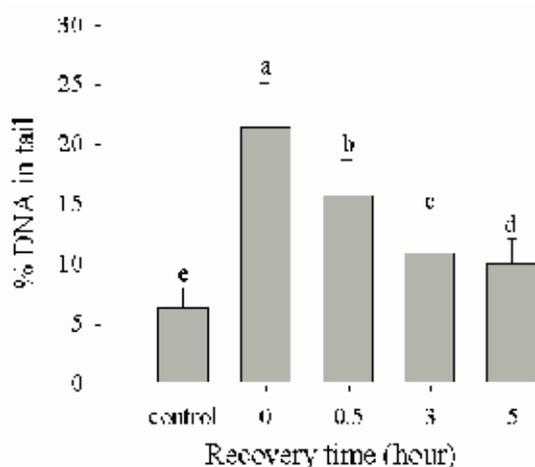


Fig. 1. DNA damage under oxidant challenge at 4°C. Bars represent mean \pm SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, $n=6$ independent replications \times 2 slides per replication).

Fig. 3 and 4 show DNA damage change and corresponding mRNA levels of DNA repair enzymes following oxidant challenge at 37°C. Levels of DNA damage increased significantly immediately following hydrogen peroxide treatment although the amount of damage was less than that of cells treated with oxidant at 4°C (t-test; $p < 0.001$) (Fig.3). The level of DNA damage decreased by 39% during the first 0.5 h, a rate of decrease that was faster than that observed following oxidant treatment at 4°C. However, the level of DNA damage did not maintain this rapid decrease over an extended period and DNA damage actually increased slightly after 3 h. Nonetheless, the level of damage was still lower than that at 0 h and levels of damaged DNA again decreased at 5 h.

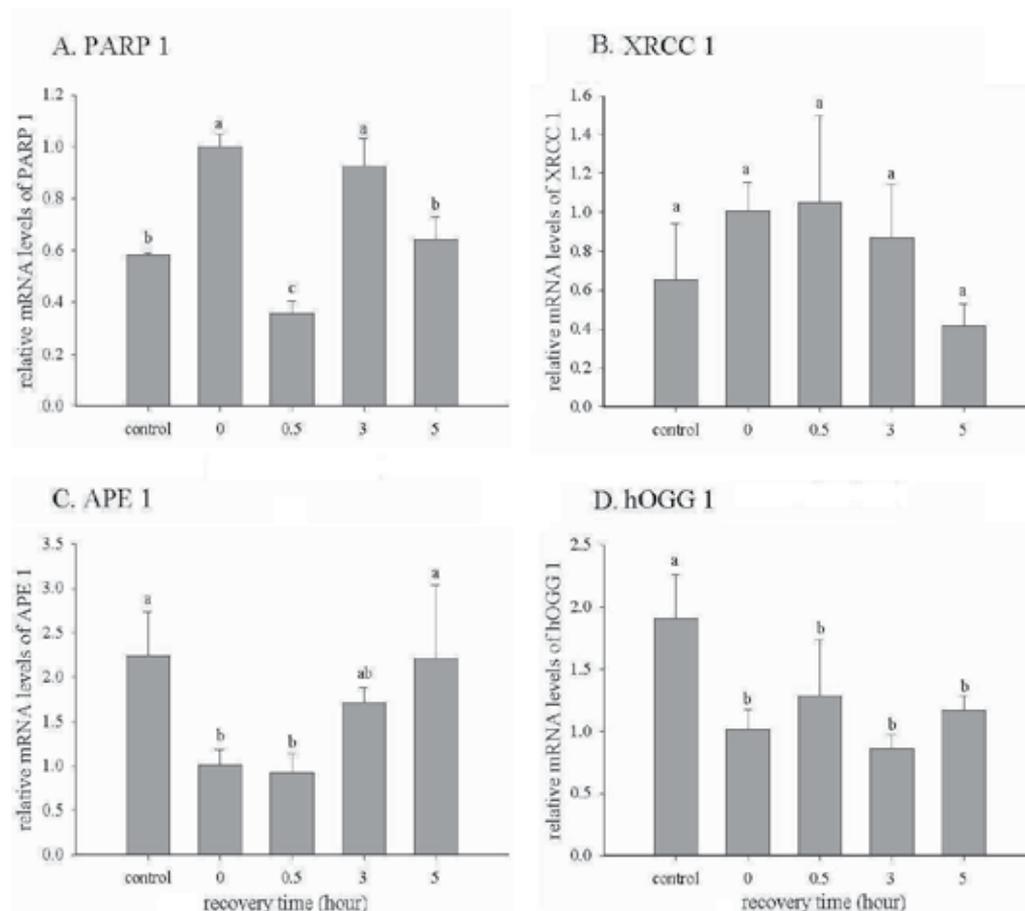


Fig. 2. Relative mRNA levels of DNA repair enzymes under oxidant challenge at 4°C. Levels are reported relative to 0 time. Bars represent mean \pm SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, $n=3$ independent replications and qRT-PCR was analyzed in triplicate for each sample).

Unlike at 4°C mRNA levels did not change significantly for any of the repair enzymes immediately (0 h) after hydrogen peroxide treatment at 37°C (Fig. 4). However, hOGG1 levels did increase at 0.5 h and then gradually decreased during the recovery. XRCC1 levels

also decreased late in the recovery period. On the other hand, APE1 expression increased late in the recovery time (3-5 h) and showed an approximately inverse relationship with hOGG1 levels. mRNA expression levels of PARP1, a SSB recognizing enzyme, changed dynamically at 4°C however, at 37°C PARP1 levels generally were maintained at the basal levels except for decreases which occurred at 3 and 5 h (Fig. 4).

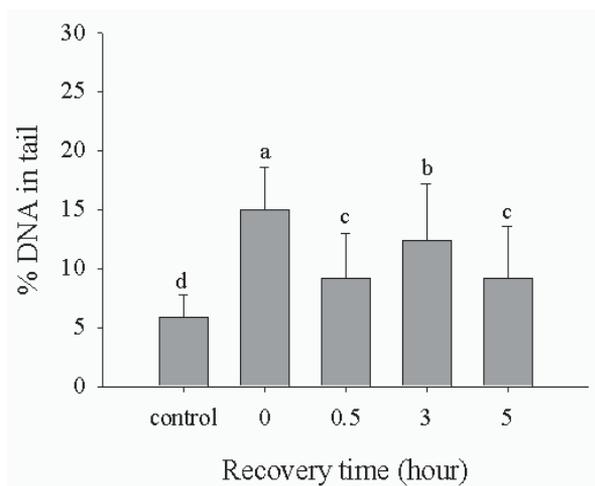


Fig. 3. DNA damage under oxidant challenge at 37°C. Bars represent mean \pm SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, $n=6$ independent replications \times 2 slides per replication).

4. Discussion

SSBs are a common type of DNA damage produced not only by ROS directly, but also indirectly during DNA repair processes. Moreover, no matter what the origins of SSBs are, SSBs are mostly repaired by the BER pathway. In this study, acute oxidative stress was induced in the Caco-2 cells by H₂O₂ treatment. Hydrogen peroxide has been shown previously to generate significant DNA damage including DNA base lesions and SSBs (Cantoni et al., 1987; Dizdaroglu, 1994; Min & Ebeler, 2009). In order to obtain a more complete picture of the cellular response to DNA damaging agents, DNA damage as a function of SSBs and corresponding mRNA expression levels of DNA repair enzymes were monitored. The measured DNA damage is the result of a balance between production of breaks by specific DNA base lesion glycosylases and the sealing of gaps by polymerases and ligases (Cantoni et al., 1987). Among DNA BER enzymes, PARP1, which recognizes and binds to AP sites, and XRCC1, a coordinating protein of the DNA BER pathway, were evaluated here. In addition, hOGG1, a glycosylase which cleaves 8-oxoguanine, and APE1, an endonuclease which cleaves AP sites, were also monitored. We examined DNA damage and repair at two different temperatures. Several studies have induced DNA damage at low temperature in order to minimize the possibilities of DNA repair (Astley et al., 2002; Collins et al., 1995). Accordingly our study adopted low temperature as a reference condition and also monitored responses at 37°C in order to elucidate the biochemical responses to DNA damaging agents at physiological temperature.

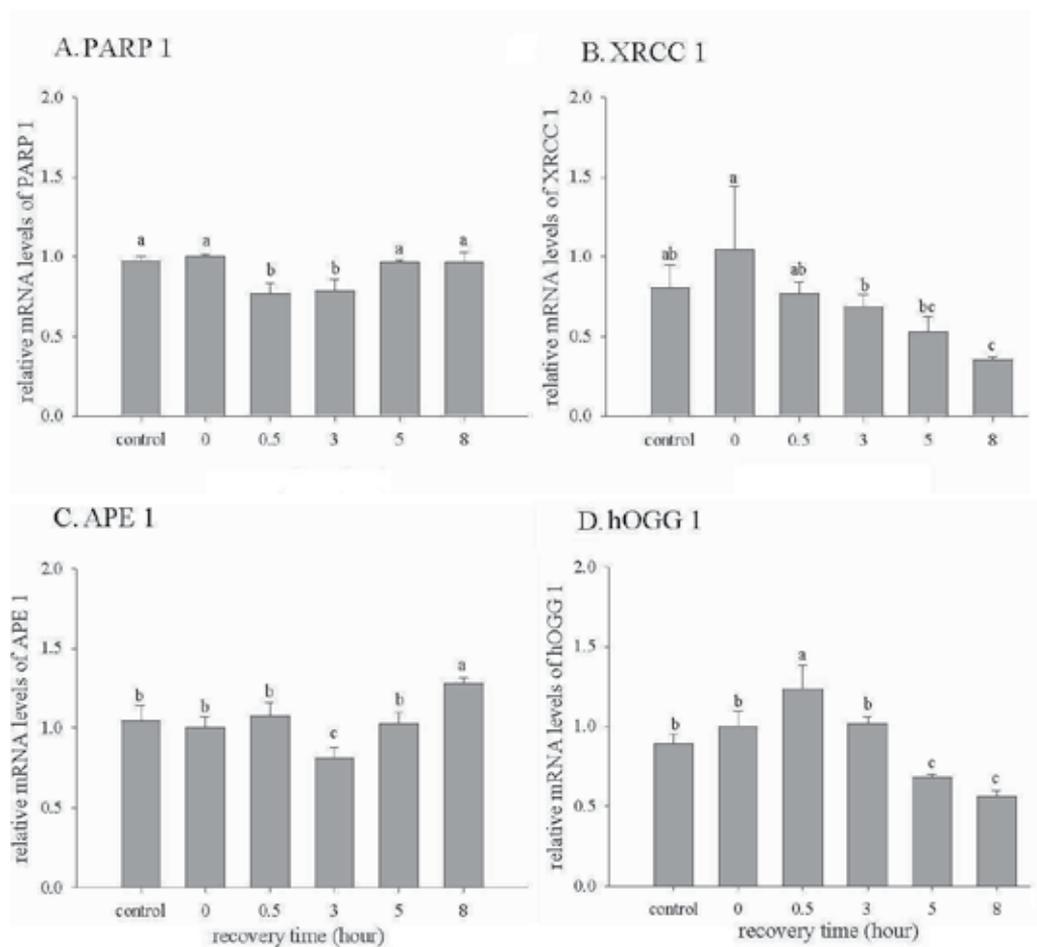


Fig. 4. Relative mRNA levels of DNA repair enzymes under oxidant challenge at 37°C. Levels are reported relative to the 0 time. Bars represent mean \pm SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, $n=3$ independent treatments and qRT-PCR was analyzed in triplicate for each sample).

Higher levels of DNA damage followed by steady repair over time were observed following oxidant treatment at 4°C compared to treatment at 37°C. Foray et al. (1995) have shown more DNA double strand breaks at 4°C compared to 37°C following ionizing radiation, consistent with our study using a chemical oxidant. In our study, mRNA levels of PARP1 responded quickly to increased DNA damage at 4°C while the expression levels of hOGG1 and APE1 decreased immediately following oxidant treatment. Early decreases of APE 1 have been shown by Morita-Fujimura et al. after cold-injury induced brain trauma in mice (Morita-Fujimura et al., 1999). The increase of PARP1 immediately after oxidant treatment and the late gradual increase of APE1 may be factors contributing to the decrease in DNA damage (i.e., increase in repair) over time following the low temperature oxidant challenge. Therefore, although low temperature is typically associated with reduced metabolic and

enzymatic activity, the effects of cold temperature on DNA damage appear to be complex and more investigations are needed to understand the effects of cold temperature on DNA damage and subsequent repair activity.

Levels of SSBs initially and over time, were different following oxidant challenge at 37°C compared to oxidant challenge at 4°C. It is possible that DNA repair was actually initiated during the hydrogen peroxide treatment at the higher temperature resulting in lower DNA damage levels immediately after oxidant treatment. This could at least partially account for the lower level of damage observed at 0 h compared to the treatment at 4°C. In addition, mRNA expression patterns of the repair genes were different from those at 4°C. For example, increased levels of hOGG1 within the first 0.5 h following oxidant challenge were consistent with greater DNA repair (i.e., decreased levels of DNA damage) that was observed following oxidant treatment at 37°C. The inverse relationship between APE1 and hOGG1 mRNA levels from 3 to 8 h at 37°C is consistent with hOGG1 producing AP sites which then induce mRNA production of APE1 (Hill et al., 2001).

Measuring the mRNA expression levels of DNA BER genes has been indicated to be a sensitive end point for determining the effects of chronic oxidative stress to DNA (Rusyn et al., 2004). In addition, consistent with our results following oxidant challenge at 37°C expression of hOGG1 mRNA, has been shown to be inducible responding to various conditions and reflecting DNA repair, at least initially. However, our results indicate that no single gene reflects the overall DNA repair response at any one point in time following oxidant treatment and it is difficult to fully relate changes in mRNA expression levels to the observed DNA damage repair kinetics.

5. Conclusions

Our study indicates that DNA damage induced by oxidant at physiological temperature (37°C) is lower as compared to damage at low temperatures. In addition, the pattern of mRNA expression of DNA repair processing enzymes is different over time following treatment. Some of the changes in DNA damage levels over the extended recovery period could be associated with the overall pattern of mRNA expression of several DNA repair enzymes, however, our results indicate that an individual gene alone may not accurately reflect the overall DNA repair capacity. Our study also indicates that protocols using low temperatures to minimize DNA repair may actually result in conditions that enhance DNA damage and result in very different repair kinetics than those that occur at a physiologic temperature. Experimental protocols should be carefully evaluated and interpreted if nonphysiologic conditions are used. Further studies comparing oxidative damage, mRNA expression and protein/enzyme levels and their activity are needed in order to fully understand the molecular responses to DNA damaging agents in the DNA damage/repair processes under variety of conditions.

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7. References

- Astley, S. B, Elliott, R. M., Archer, D. B, & Southon, S. (2002). Increased cellular carotenoid levels reduce the persistence of DNA single-strand breaks after oxidative challenge. *Nutr. Cancer*, 43, 2, pp. 202-213.
- Berwick, M., & Vineis, P. (2000). Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. *J. Natl. Cancer Inst.*, 92, 11, pp. 874-897.
- Caldecott, K. W. (2001). Mammalian DNA single-strand break repair: an X-ra(y)ted affair. *Bioessays*, 23, 5, pp. 447-455.
- Caldecott, K. W. (2003). XRCC1 and DNA strand break repair. *DNA Repair (Amst)*, 2, 9, pp. 955-969.
- Cantoni, O., Murray, D., & Meyn, R. E. (1987). Induction and repair of DNA single-strand breaks in EM9 mutant CHO cells treated with hydrogen peroxide. *Chem. Biol. Interact.*, 63, 1, pp. 29-38.
- Chevillard, S., Radicella, J. P., Levalois, C., Lebeau, J., Poupon, M. F., Oudard, S., Dutrillaux, B., & Boiteux, S. (1998). Mutations in OGG1, a gene involved in the repair of oxidative DNA damage, are found in human lung and kidney tumours. *Oncogene*, 16, 23, pp. 3083-3086.
- Collins, A. R., Harrington, V., Drew, J., & Melvin, R. (2003). Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis*, 24, 3, pp. 511-515.
- Collins, A. R., Ma, A. G., & Duthie, S. J. (1995). The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. *Mutat. Res.*, 336, 1, pp. 69-77.
- Collins, A., & Harrington, V. (2002). Repair of oxidative DNA damage: assessing its contribution to cancer prevention. *Mutagenesis*, 17, 6, pp. 489-493.
- Cooke, M. S., Evans, M. D., Dizdaroglu, M., & Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.*, 17, 10, pp. 1195-1214.
- Davies, K. J. (2000). Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life*, 50, 4-5, pp. 279-289.
- de Murcia, G., & Menissier de Murcia, J. (1994). Poly(ADP-ribose) polymerase: a molecular nick-sensor. *Trends Biochem. Sci.*, 19, 4, pp. 172-176.
- Dianov, G. L. & Parsons, J. L. (2007). Co-ordination of DNA single strand break repair. *DNA Repair (Amst)*, 6, 4, pp. 454-460.
- Dizdaroglu, M. (1994). Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry. *Methods Enzymol.*, 234, pp. 3-16.
- Foray, N., Arlett, C. F., & Malaise, E. P. (1995). Dose-rate effect on induction and repair rate of radiation-induced DNA double-strand breaks in a normal and an ataxia telangiectasia human fibroblast cell line. *Biochimie*, 77, 11, pp. 900-905.
- Hill, J. W., Hazra, T. K., Izumi, T., & Mitra, S. (2001). Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. *Nucleic Acids Res.* 29, 2, pp. 430-438.
- Hodges, N. J., & Chipman, J. K. (2002). Down-regulation of the DNA-repair endonuclease 8-oxo-guanine DNA glycosylase 1 (hOGG1) by sodium dichromate in cultured human A549 lung carcinoma cells. *Carcinogenesis*, 23, 1, pp. 55-60.
- Kim, H. N., Morimoto, Y., Tsuda, T., Ootsuyama, Y., Hirohashi, M., Hirano, T., Tanaka, I., Lim, Y., Yun, I. G., & Kasai, H. (2001). Changes in DNA 8-hydroxyguanine levels, 8-

- hydroxyguanine repair activity, and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos. *Carcinogenesis*, 22, 2, pp. 265-269.
- Lan, J., Li, W., Zhang, F., Sun, F. Y., Nagayama, T., O'Horo, C., & Chen, J. (2003). Inducible repair of oxidative DNA lesions in the rat brain after transient focal ischemia and reperfusion. *J. Cereb. Blood Flow Metab.*, 23, 11, pp. 1324-1339.
- Langland, G., Elliott, J., Li, Y., Creaney, J., Dixon, K., & Groden, J. (2002). The BLM helicase is necessary for normal DNA double-strand break repair. *Cancer Res.*, 62, 10, pp. 2766-2770.
- Leppard, J. B., & Champoux, J. J. (2005). Human DNA topoisomerase I: relaxation, roles, and damage control. *Chromosoma*, 114, 2, pp. 75-85.
- Lynch, H. T., & Smyrk, T. (1996). Hereditary nonpolyposis colorectal cancer (Lynch syndrome). An updated review. *Cancer*, 78, 6, pp. 1149-1167.
- Mambo, E., Chatterjee, A., de Souza-Pinto, N. C., Mayard, S., Hogue, B. A. Hoque, M. O., Dizdaroglu, M., Bohr, V. A., & Sidransky, D. (2005). Oxidized guanine lesions and hOgg1 activity in lung cancer. *Oncogene*, 24, 28, pp. 4496-4508.
- Marchetto, M. C., Muotri, A. R., Burns, D. K., Friedberg, E. C., & Menck, C. F. (2004). Gene transduction in skin cells: preventing cancer in xeroderma pigmentosum mice. *Proc. Natl. Acad. Sci. U S A*, 101, 51, pp. 17759-17764.
- Mayer, C., Popanda, O., Zelezny, O., von Brevern, M. C., Bach, A., Bartsch, H., & Schmezer, P. (2002). DNA repair capacity after gamma-irradiation and expression profiles of DNA repair genes in resting and proliferating human peripheral blood lymphocytes. *DNA Repair (Amst)*, 1, 3, pp. 237-250.
- Min, K. & Ebeler, S. E. (2009). Quercetin inhibits hydrogen peroxide-induced DNA damage and enhances DNA repair in Caco-2 cells. *Food Chem. Tox.*, 47, pp. 2716-2722.
- Morita-Fujimura, Y., Fujimura, M., Kawase, M., & Chan, P. H. (1999). Early decrease in apurinic/aprimidinic endonuclease is followed by DNA fragmentation after cold injury-induced brain trauma in mice. *Neuroscience*, 93, 4, pp. 1465-1473.
- Noe, V., Penuelas, S., Lamuela-Raventos, R. M., Permanyer, J., Ciudad, C. J., Izquierdo-Pulido, M. (2004). Epicatechin and a cocoa polyphenolic extract modulate gene expression in human Caco-2 cells. *J. Nutr.*, 134, 10, pp. 2509-2516.
- Rusyn, I., Asakura, S., Pachkowski, B., Bradford, B. U., Denissenko, M. F., Peters, J. M., Holland, S. M., Reddy, J. K., Cunningham, M. L., & Swenberg, J. A. (2004). Expression of base excision DNA repair genes is a sensitive biomarker for in vivo detection of chemical-induced chronic oxidative stress: identification of the molecular source of radicals responsible for DNA damage by peroxisome proliferators. *Cancer Res.* 64, 3, pp. 1050-1057.
- Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, 175, 1, pp. 184-191.
- Torbergson, A. C., & Collins, A. R. (2000). Recovery of human lymphocytes from oxidative DNA damage; the apparent enhancement of DNA repair by carotenoids is probably simply an antioxidant effect. *Eur. J. Nutr.*, 39, 2, pp. 80-85.
- Wilson, D. M & Bohr, V. A. (2007). The mechanics of base excision repair, and its relationship to aging and disease. *DNA Repair (Amst)*, 6, 4, pp. 544-559.

Wood, R. D., Mitchell, M., Sgouros, J., & Lindahl, T. (2001). Human DNA repair genes, *Science*, 291, 5507, pp. 1284-1289.

Relationship Between DNA Damage and Energy Metabolism: Evidence from DNA Repair Deficiency Syndromes

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1. Introduction

It is estimated that the human genome incurs on the order of 1,000-1,000,000 DNA lesions/cell/day (Lodish 2000). A majority of these are thought to be due to endogenous sources, including reactive oxygen and nitrogen species (ROS, RNS) that can oxidize cellular macromolecules including lipid, protein and nucleic acid. These free radicals can be generated for specific purposes by cellular enzymes; for example, nitric oxide synthases generate NO in endothelial cells for signaling purposes, while NADPH oxidase and myeloperoxidase generate ROS in granulocytes to kill invading pathogens. However, ROS can also occur as a byproduct of cellular energy metabolism (Beckman and Ames 1998). Most cells use mitochondrial respiration as a means of energy production. The process of moving electrons across the mitochondrial membrane can at some frequency result in their transfer to molecular oxygen and the generation of superoxide and hydrogen peroxide, which together can generate the highly reactive hydroxyl radical and damage nearby molecules, including DNA.

The frequency of superoxide formation is influenced by many factors, including the rate of electron transport and the capacity of the mitochondria to couple the proton gradient created across the inner membrane to ATP production, or to dissipate it in the form of heat through the use of uncoupling proteins. High rates of electron transport and efficient ATP production or uncoupling are consistent with reduced ROS generation, while low rates of transport or inefficient ATP production (for example when ATP/ADP ratios are already high) are consistent with increased ROS generation. Different substrates may also influence mitochondrial ROS production. Oxidation of ketone bodies, for example, may generate more ROS than oxidation of acetyl CoA from glucose (Tieu et al. 2003). Substrate utilization further depends on cell type and nutritional status. For example, neurons prefer to oxidize glucose, but can also use ketone bodies derived from fat oxidation in the liver. Skeletal muscle can use either fat or glucose, depending on availability, but tends to use one at the exclusion of the other (Randle et al. 1963). In cooperation with mitochondria, peroxisomes also play a major role in energy metabolism by oxidizing long-chain fatty acids. Peroxisomes can further participate in ketogenesis, amino acid oxidation and the oxidative phase of the pentose phosphate pathway depending on substrate availability. Like mitochondria, they are a major

source of ROS and RNS generating enzymes, and thus a potential source of oxidative macromolecular damage by leakage of ROS/RNS across the organelle membrane. Mitochondria and peroxisomes also produce abundant amounts of antioxidant enzymes such as superoxide dismutase and catalase to neutralize ROS. Thus, mitochondria and peroxisomes can both contribute to ROS as well as play a role in ROS detoxification.

In order to generate energy efficiently, cells oxidize carbon units derived from glucose, amino acids or fatty acids in mitochondria. The use of these substrates is governed by nutritional status and is further subject to hormonal control. Insulin and glucagon are major regulators of organismal substrate utilization. In the fed state, elevated blood glucose promotes insulin secretion by the pancreas and transport of glucose into insulin-responsive tissues including liver, muscle and fat. In the liver, glucose is stored as glycogen or used as a substrate for *de novo* lipogenesis. Insulin signaling also inhibits release of free fatty acids from white adipose tissue and promotes *de novo* lipogenesis and dietary lipid repackaging in the liver for storage in white adipose tissue. After a meal, glucose levels fall and counter-regulatory hormones such as glucagon reverse the actions of insulin and glucose by promoting hepatic glycogenolysis and gluconeogenesis for glucose-dependent tissues such as red blood cells and neurons, and release of fatty acids from the white adipose tissue for oxidation in other organs. Defects in insulin signaling caused by overeating are associated with a spectrum of pathologies including diabetes, obesity and atherosclerosis collectively known as the metabolic syndrome. Although the underlying mechanisms are not entirely clear, it is associated with chronic inflammation and oxidative stress (Hotamisligil 2006). In stark contrast to metabolic syndrome is the spectrum of phenotypes associated with dietary restriction (DR, also known as calorie restriction), defined as reduced food intake without malnutrition. Originally described in rodents to reduce the incidence of cancer and extend lifespan (McCay, Crowel, and Maynard 1935), DR has proven efficacy at increasing lifespan, stress resistance and metabolic fitness in a wide range of experimental organisms. In mammals, the DR state is characterized by reduced serum glucose, reduced growth factors and growth factor signaling, improved insulin sensitivity, increased resistance to oxidative stress and reduced adiposity (Fontana and Klein 2007). While the molecular mechanisms underlying the benefits of DR remain unclear, reduced steady state levels of macromolecular oxidative damage suggest reduced ROS production and/or increased antioxidant defenses play a role.

When DNA damage occurs, there are a number of overlapping repair pathways that recognize and remove the damage, as well as a battery of signaling pathways that influence immediate decisions on cell fate and longer-term adaptations to stress. DNA damage repair pathways are distinguished in large part by the lesions that they recognize. Oxidative base lesions are typically recognized by the base excision repair (BER) pathway, while bulky helix distorting lesions are typically removed by the nucleotide excision repair (NER) pathway. Although the latter is chiefly responsible for removal of UV lesions from sunlight, endogenous oxidative lesions are also partially dependent on NER pathways (Brooks et al. 2000). Oxidative stress can also cause breaks in the sugar-phosphate backbone, resulting in single strand breaks that can interrupt transcription or replication. When the density of such breaks is high, they can occur nearby on opposite strands and result in double strand breaks. Such lesions can be repaired by homologous recombination in the presence of a sister chromatid (for example during S phase of the cell cycle) or by non-homologous recombination during other phases of the cell cycle when the sister chromatid is not readily available to serve as a template for repair. The so-called DNA damage response (DDR) is

not a single response but a network of signaling and repair pathways activated by genotoxic stress. Upon DNA damage such as a double strand break or a collapsed replication fork, the serine/threonine kinases ATM or ATR, respectively, initiate a cascade of cellular responses resulting in cell cycle arrest and recruitment of repair factors. One of the targets of ATM and ATR is the tumor suppressor p53, which is stabilized by phosphorylation and activates transcription of genes involved in cell fate, including apoptosis or senescence. Other proteins such as poly ADP ribose polymerase (PARP) are activated by DNA damage and can have indirect effects on cell fate decisions by depleting ATP and NAD⁺.

Given the connection between production of ROS by cellular metabolism and DNA damage, one might predict coordinate regulation of the cellular response to DNA damage and growth and metabolism on the cellular and organismal levels. In this chapter, we will discuss existing evidence of such a connection. First, we will consider metabolic changes associated with defects in NER disorders and their resemblance to the adaptive response to DR, particularly in mouse models of these diseases. Next, we will consider metabolic changes in a variety of other DNA damage repair and signaling disorders, ranging from DR-like phenotypes to metabolic disorder. We will conclude by reviewing the evidence linking DNA damage repair and signaling pathways directly and indirectly to changes in cellular growth and energy metabolism.

2. Metabolic defects in nucleotide excision repair deficiency syndromes

Although the mutations causing the segmental progerias Cockayne syndrome (CS) and trichothiodystrophy (TTD) are known, how alterations in the associated nucleotide excision DNA repair proteins cause pleiotropic disease symptoms including dwarfism and cachexia are not yet clear. In mouse models of these disorders, unrepaired endogenous DNA damage is linked to perturbations in energy metabolism and alterations in insulin/insulin-like growth factor-1 (IGF-1) signaling. Paradoxically, these changes resemble beneficial adaptive responses to DR associated with improved metabolic fitness and extended longevity. In this section, we will discuss perturbations in growth and energy metabolism associated with defects in NER proteins in human disease and mouse models, and the potential role of DNA damage in eliciting these changes.

2.1 Cockayne syndrome and trichothiodystrophy

NER is an evolutionarily conserved pathway required for the removal of UV-induced DNA damage. It is divided into two branches based on how the lesion is initially recognized. Global genome (GG)-NER can occur anywhere in the genome upon recognition of DNA helical distortions by the XPC/HR23B/CEN2 complex. Transcription-coupled (TC)-NER occurs only on the transcribed DNA strand and is initiated by the stalling of an elongating RNA polymerase, for example by steric hindrance at the site of a bulky adduct. TC-NER specific proteins CSA and CSB participate in the upstream events surrounding the stalling of an RNA polymerase at the site of DNA damage. Although both CSA and CSB are involved in ubiquitination and protein turnover - CSA is a component of a ubiquitin ligase complex (Groisman et al. 2003), and CSB contains a ubiquitin-binding domain required for UV damage repair (Anindya et al. 2010) - their exact roles in TC-NER remain unclear. Once the TC-NER or GG-NER machinery recognizes the lesion, the helicases XPB and XPD unwind the damaged DNA, allowing validation of the lesion by XPA, endonucleolytic cleavage of

the phosphodiester backbone by XPG and XPF-ERCC1 and removal of the damaged oligonucleotide in preparation for repair DNA synthesis.

Mutations in CSA, CSB, XPG, XPD and XPB are associated with CS, a rare progressive disease characterized by photosensitivity, dwarfism, loss of subcutaneous fat and neurodegeneration. CS was first described by Edward Cockayne in 1936 (Cockayne 1936). To date, approximately 200 cases have been reported in the literature (Nance and Berry 1992; Ozdirim et al. 1996; Pasquier et al. 2006; Rapin et al. 2006). Despite UV sensitivity, CS is not associated with an elevated risk of skin cancer as observed in the related NER deficiency syndrome xeroderma pigmentosum (XP). Although there is a range in the onset and severity of CS, typical presentation involves normal in utero growth and birth weight followed by a profound postnatal growth failure within the first two years. This growth failure is also observed in the postnatal brain, resulting in developmental microcephaly and cognitive impairment. Neuropathological defects include demyelination and atrophy of white matter, calcification of the basal ganglia, cerebellar atrophy, demyelination of peripheral nerves, retinopathy, and neuronal loss in the inner ear (Weidenheim, Dickson, and Rapin 2009). Based on the involvement of white matter, CS is considered a form of leukodystrophy. Interestingly, such white matter diseases can be caused by various genetic defects in lysosome or peroxisome metabolism and typically present postnatally after normal birth and early development (Kohlschutter et al. 2010). Diabetes mellitus, early hypertension and atherosclerosis are also prevalent in CS (Rapin et al. 2006). Besides the nervous system and adipose tissue, other organ systems appear proportionately smaller in size yet unimpaired (Weidenheim, Dickson, and Rapin 2009). Death occurs around 12 years of age often from cachexia or an intercurrent illness such as respiratory infection.

The fact that weight is more affected than height in CS led to the use of the term cachexia, or wasting, in association with this disease (Nance and Berry 1992). Although it is not clear that lean mass is preferentially affected as is typical with cachexia, adipose tissue is clearly affected. CS is classified as a lipodystrophy, indicating the abnormal redistribution of fat. Subcutaneous fat loss leads to sunken eyes and a wizened appearance that are further defining characteristics of the disease. Adipocytes are a major site of energy storage in the form of triglycerides, as well as a source of lipokines involved in a variety of processes including appetite control, immune function and temperature regulation. CS patients presumably still have functioning adipocytes that can produce adipokines but do not store triglycerides for reasons that remain unclear. This is distinct from generalized lipoatrophy, in which adipocytes and associated adipokines are lost, resulting in hyperlipidemia and insulin resistance despite the paucity of fat. Interestingly, lipids are also a major component of myelin sheaths that are lost in CS, although no connection between altered lipid metabolism and demyelination has been reported.

The cause of dwarfism in CS is not known; however there are no consistent data to suggest alteration of endocrine function (Rapin et al. 2006; Nance and Berry 1992). For example, growth hormone (GH) levels are normal to elevated. Whether or not transient perturbation of GH/IGF-1 signaling, which has been reported in mouse models (see below), is relevant in the human disease remains unknown.

Mutations in XPD and XPB, as well as another TFIIH component, p8, can cause another photosensitivity disorder with characteristic growth failure and neurological involvement known as TTD (Morice-Picard et al. 2009). Unlike CS, TTD patients present with characteristic brittle hair and nails caused by a transcriptional defect in terminally differentiating keratinocytes. Despite this difference, there are many similarities between

the diseases as would be expected if they share a common basis in defective transcription-coupled DNA repair. As with CS, TTD shows no increase skin cancer risk despite photosensitivity. TTD patients also demonstrate dysmyelination and lipodystrophy. Despite the hypothesis that CS and TTD share a common basis in defect transcription-coupled DNA repair (Andressoo, Hoeijmakers, and Mitchell 2006), this is by no means the only hypothesis regarding the etiology of these diseases. For example, the requirement for the CAK complex of TFIIH in the phosphorylation and regulation of nuclear hormone receptors, which play a major role in cellular and organismal metabolism, has led to the competing hypothesis that disease symptoms are caused by defects in gene expression regulated by these transcriptional activators (Compe et al. 2007; Keriél et al. 2002; Brooks, Cheng, and Cooper 2008).

2.2 Mouse models of NER progeria

Mouse models of CS engineered by disabling the CSA or CSB genes share some characteristics of human CS but in a milder form. Knockout mice are born normally, but are photosensitive and display an age-dependent loss of photoreceptor cells (van der Horst et al. 2002; van der Horst et al. 1997). Although they develop normally, CSB mice remain lean in adulthood and have normal lifespans (Dolle et al. 2006). Interestingly, CSA and CSB mice are resistant to renal ischemia reperfusion injury, a form of acute oxidative and inflammatory stress, and display improved glucose tolerance and insulin sensitivity (Susa et al. 2009). Both of these phenotypes are typical of DR mice (Mitchell et al. 2009). TTD mice created by mutating residue 722 from an R to a W in the XPD C terminus faithfully recapitulate the brittle hair phenotype of the human TTD, but like the CS mice have an overall milder phenotype than the corresponding human disease (de Boer et al. 1998). TTD mice display end of life pathologies consistent with both accelerated aging (osteoporosis, aortic sarcopenia, lymphoid depletion) as well as DR (reduced inflammatory dermatitis, reduced pituitary adenoma, reduced subcutaneous fat) (Wijnhoven et al. 2005). Duodenal epithelial hyperplasia has been proposed to play a role in reduced food absorbance leading to the overall DR-like phenotype (Wijnhoven et al. 2005). XP-CS mice, engineered with a different point mutation in the XPD gene (G602D) associated in patients with the symptoms of both CS and XP also recapitulate the mild features of mouse CS as well as the severe skin cancer susceptibility of XP (Andressoo et al. 2006).

Another group of mouse models of NER deficiency share a more severe core phenotype of dwarfism, ataxia, failure to thrive and death before weaning at 3-4 weeks of age. This so-called "NER progeria" was first described in mice lacking the endonuclease *Erc1* (Melton et al. 1998; Weeda et al. 1997) and subsequently in a number of genetic models of NER deficiency, including CSB/XPA (Murai et al. 2001), XPA/TTD (de Boer et al. 1998), XPA/XPCS (Andressoo et al. 2006), CSB/XPC (Laposa, Huang, and Cleaver 2007) double homozygous mutants as well as XPG (Sun et al. 2003) and XPF (Tian et al. 2004) single homozygous mutants.

Common metabolic features of NER progeria in mice include reduced blood glucose and reduced insulin, disproportionate reduction of white adipose tissue weight, and accumulation of triglycerides in the liver (van de Ven et al. 2007). Although the cause of death is not clear, animals may inevitably succumb to hypoglycemia. Reduced food intake does not seem to be the cause of metabolic perturbations. Liver transcriptome analysis of *Erc1*^{-/-} and CSB/XPA mice revealed altered expression of genes involved in carbohydrate and oxidative metabolism and peroxisome biogenesis suggestive of increased glycogen

synthesis, decreased glycolysis, and decreased oxidative metabolism (Niedernhofer et al. 2006). Global metabolic profiling by NMR revealed that *Ercc1*^{-/-} mice have altered lipid and energy metabolism and a shift toward ketosis when compared to age-matched controls (Nevedomskaya et al. 2010). *Ercc1*^{-/-} mice show several metabolic adaptations that resemble those seen in DR, including decreased LDL and VLDL, increased HDL, and decreased serum glucose (Nevedomskaya et al. 2010).

Another common feature of NER progeria that may underlie the growth retardation is reduced mRNA and serum protein expression of IGF-1 (Niedernhofer et al. 2006; van der Pluijm et al. 2007; van de Ven et al. 2006). Unlike long-lived endocrine deficient dwarfs (Ames, Snell) with reduced GH secretion due to defective anterior pituitary development (Bartke and Brown-Borg 2004), NER-deficient mice have an intact pituitary and normal to elevated GH levels, consistent with normal hypothalamic and pituitary function. Instead, GH receptor mRNA levels are reduced in multiple tissues, resulting in reduced GH-dependent IGF-1 production. Liver transcriptome analysis confirmed a general downregulation of multiple components of the postnatal GH/IGF-1 axis. Nonetheless, in select mouse models of severe NER progeria, effects on serum IGF-1 and glycemic index can be transient, occurring prior to weaning but normalizing in animals that survive this apparent developmental bottleneck (van de Ven et al. 2006).

Global profiling of gene expression in liver confirmed a significant overlap between NER progeria and long-lived dwarfism (Schumacher et al. 2008) consistent with the physiologic data. Paradoxically, the core feature of IGF-1 signaling attenuation increases lifespan in several species (Rincon et al. 2004; Longo and Finch 2003) but is associated with decreased longevity in NER progeria. Altered growth and energy metabolism has thus been interpreted as an adaptive response to endogenous genotoxic stress. Whether or not this response is maladaptive in this model is not known, but does appear to be so in mouse models of models of Hutchinson-Gilford progeria syndrome (HGPS) (Marino et al. 2010) as discussed below.

2.3 Evidence for a role of DNA repair in CD and TTD phenotypes

What is the evidence that defects in DNA repair, and in particular TC-NER, are causative of the pleiotropic disease symptoms including disturbances in growth and energy metabolism? Most NER proteins associated with disorders in man and mouse are multifunctional with distinct roles in several different cellular processes. For example, CSB was originally cloned as a TC-NER factor, but can also function in chromatin remodeling, transcriptional initiation (Le May, Mota-Fernandes et al. 2010), transcriptional elongation (Le May, Egly, and Coin 2010), BER via interactions with the BER glycosylase Ogg1 and stimulation of APE1 incision (Wong et al. 2007) and rRNA synthesis (Bradsher et al. 2002). Similarly, the TFIIH complex containing the XPB and XPD helicases plays a role in transcriptional initiation and activated transcription by a subset of nuclear hormone receptors (Keriel et al. 2002; Compe et al. 2007). Roles for many of these functions have been proposed to be causative of one or more symptoms of CS, TTD or XP. However, due in large part to the high degree of overlap between symptoms in multiple different mouse models, where the effects of homozygous mutations can be interrogated against a standardized genetic and environmental background, it has been hypothesized that these conditions have a common underlying cause (Andressoo, Hoeijmakers, and Mitchell 2006; van de Ven et al. 2007). Currently, the only known common function of each of the proteins, including CSA, CSB, XPD, XPB and XPG, is the transcription-coupled arm of NER. Based on current data, this makes a defect in

TC-NER a plausible cause of overlapping disease symptoms, but does not rule out common pathways that are currently unknown or untested for all relevant disease loci.

If indeed defects in the TC-NER pathway are causative of disease symptoms, what are the relevant endogenous DNA lesions? Most of what is known about the function of TC-NER proteins is derived from cell-based experiments with UV as the source of DNA damage. UV irradiation produces two types of bulky lesions which are substrates for NER; cyclobutane pyrimidine dimers, mainly removed by TC-NER and pyrimidine 6-4 pyrimidone photoproducts, typically processed by GG-NER. Indeed, cells deficient in CSB fail to repair cyclobutane dimers but repair 6-4 photoproducts efficiently (Barrett et al. 1991). UV light may possibly damage keratinocytes of CS patients, but has little capacity to induce lesions in other relevant tissues. Most bulky lesions are induced by exogenous sources, but some bulky lesions are created by endogenous sources (De Bont and van Larebeke 2004).

8,5'-cyclopurine-2'-deoxynucleosides (cyPudNs) are endogenous lesions formed in DNA by the hydroxyl radical (Jaruga, Theruvathu, et al. 2004; Dizdaroglu et al. 1987). cyPudNs are chemically stable lesions which are expected to accumulate slowly and are candidates for lesions which could cause neurodegeneration (Kuraoka et al. 2000; Brooks 2008). cyPudNs block transcription and, unlike most oxidative DNA lesions, cyPudNs are repaired by NER rather than BER (Brooks et al. 2000; Kuraoka et al. 2000). *In vitro* assays using CHO and NER-deficient CHO cells show that ERCC1 and XPG are required for excision of the 8,5'-(S)-cyclo-2'-deoxyadenosine (cyclo-dA) lesion, while BER glycosylases are not active on the cyclo-dA lesion (Brooks et al. 2000). Using a host cell reactivation assay, Brooks et al. found that cyclo-dA is a substrate for NER *in vivo* (Brooks et al. 2000). Oxidative lesions are elevated in and may contribute to neurodegeneration (Kruman 2004). TC-NER may protect neurons from oxidative lesions, the accumulation of which may lead to the neuronal death observed in CS. Nonetheless, it remains controversial whether or not cells from CS patients or mice are hypersensitive to oxidative stress (van de Ven et al. 2006). Thus despite the growing literature on molecular functions of CSA, CSB and other TC-NER proteins, proof that unrepaired endogenous oxidative lesions cause CS and/or TTD is lacking. Identification of such a lesion and its target tissue awaits.

3. Metabolic defects in other DNA repair and maintenance disorders

A number of human syndromes and mouse models with defects in DNA damage repair and signaling also display perturbations in growth and energy metabolism. Some of these show characteristics of adaptive changes reminiscent of DR as described above in TC-NER syndromes, while others display insulin resistance and atherosclerosis resembling metabolic syndrome on the opposite end of the energy spectrum. In this section, we will describe a number of syndromes and/or mouse models with known defects in DNA repair, genome maintenance or DNA damage-related signal transduction with an emphasis on changes in growth, energy metabolism, adiposity and glucose homeostasis.

3.1 Hutchinson-Gilford Progeria Syndrome

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare autosomal dominant progeria characterized by failure to thrive, growth retardation unrelated to GH deficiency, baldness and decreased body fat without insulin resistance (Merideth et al. 2008). Atherosclerosis is thought to result from the general accelerated aging observed in all tissues and organs rather than from elevated serum lipoproteins (Al-Shali and Hegele 2004). Vascular disease

and stroke are the main causes of death in HGPS, with a life expectancy of about 13 years of age (Merideth et al. 2008). HGPS is caused by a mutation in Lamin A, a component of the nuclear envelope, which leads to a truncated protein missing the Zmpste24 cleavage site (Merideth et al. 2008).

Mouse models deficient in *Zmpste24* exhibit nuclear architecture abnormalities, severe growth retardation, loss of subcutaneous adipose tissue, accumulation of lipid in ectopic sites such as liver, and premature death (Pendas et al. 2002; Varela et al. 2005). Aberrations in cardiac muscle of *Zmpste24* mice include thinning of the ventricular wall, muscle degeneration, increased inflammation and interstitial fibrosis, suggesting that cardiomyopathy and heart failure contribute to death in this model (Pendas et al. 2002). *Zmpste24* mice exhibit increased autophagy in skeletal muscle, possibly due to decreased circulating glucose and insulin and increased adiponectin, resulting in AMPK activation and suppression of the mTOR pathway (Marino et al. 2008). Gene expression data of livers of *Zmpste24* mice indicate a shift from glucose to lipid metabolism, a response also seen in starvation (Marino et al. 2008). *Zmpste24* mice show decreased IGF-1 and GHR expression in the liver, along with suppressed levels of IGF-1 and increased levels of GH in the serum (Marino et al. 2010). Treatment of *Zmpste24* mice with recombinant IGF-1 using a subcutaneous minipump rescues some of the progeroid phenotypes, resulting in improved body weight, increased subcutaneous fat, reduced kyphosis and reduced alopecia (Marino et al. 2010). Serum GH levels were restored (reduced to normal) and lifespan of *Zmpste24* mice was extended by 18% by IGF-1 treatment (Marino et al. 2010). Thus, chronic perturbation of the somatotroph axis in response to defects in nuclear architecture has been interpreted as a maladaptive response that actually accelerates disease symptoms.

Does nuclear architecture have any impact on DNA damage repair or signaling? Evidence for a connection between nuclear architecture and DNA damage comes from cells of *Zmpste24* deficient mice. These cells display increased chromosomal abnormalities and γ -H2AX phosphorylation indicative of greater DNA damage and reduced genomic stability (Liu et al. 2005). Fibroblasts from HGPS patients senesce prematurely, indicating a cell-autonomous alteration in proliferative capacity (Bridger and Kill 2004; Allsopp et al. 1992). Thus, alterations in nuclear architecture leading to chromosome instability can indirectly activate the DNA damage response (Liu et al. 2005; Verstraeten et al. 2007).

3.2 Werner syndrome

Werner syndrome is a segmental progeria characterized by short stature, early graying and loss of hair. There is no evidence for endocrine deficiency as an explanation for growth deficiency (Monnat 2010). Type 2 diabetes mellitus and dyslipidemia leading to atherosclerosis are also common features of Werner syndrome. Patients with Werner syndrome typically live into their mid-50s and die from premature cardiovascular disease or cancer (Huang et al. 2006; Martin 1985). Werner syndrome patients show accelerated brain accumulation of amyloid β peptide and hyperphosphorylated tau, both common in age-associated disorders (Leverenz, Yu, and Schellenberg 1998). Werner syndrome is also associated with loss of myelin fibers in both the central and peripheral nervous system (Umehara et al. 1993) although without the significant delays in neuronal development observed in CS. Werner syndrome is caused by a defect in Werner (WRN), a Rec-Q helicase which hydrolyzes ATP to separate double-stranded DNA for replication, recombination, transcription and repair (Monnat 2010). The WRN protein is also essential for maintaining chromosomal integrity through intact recombination or DNA replication (Monnat 2010).

Many features of Werner syndrome are faithfully recapitulated in a mouse model in which the WRN gene is lacking the helicase domain (WRN^{Δhel/Δhel}) (Huang et al. 2006; Lachapelle, Oesterreich, and Lebel 2011). WRN^{Δhel/Δhel} mice have elevated levels of ROS and oxidative DNA damage in liver and heart, and elevated serum triglycerides, glucose and insulin; all of which return to wildtype level upon long-term vitamin C treatment (Lebel et al. 2010). WRN appears to play a role in protecting cells from oxidative damage, and the loss of WRN leads to changes resembling the metabolic syndrome.

3.3 Ataxia Telangiectasia

Deficiency in the DNA damage sensor ATM (Ataxia Telangiectasia Mutated) leads to ataxia telangiectasia (A-T). A-T is a rare autosomal disorder characterized by cerebellar ataxia, elevated cancer incidence, immune dysfunction and elevated sensitivity to ionizing radiation (Shackelford 2005). The life expectancy for patients with A-T is roughly 20 years (Chun and Gatti 2004). A-T patients display signs of premature aging as well as insulin resistance and lowered insulin receptor affinity (Lavin 2000). Mouse models of ATM deficiency show an age-dependent increase in blood glucose and decrease in insulin sensitivity, consistent with a conserved role of this protein in metabolic function (Miles et al. 2007). In an *ApoE*^{-/-} mouse model, haploinsufficiency of ATM leads to accelerated atherosclerosis and multiple features of metabolic syndrome relative to the *ApoE*^{-/-} mouse (Mercer et al. 2010).

3.4 Seckel syndrome

Stalled replication forks activate A-T and Rad-related protein (ATR), which leads to cell cycle checkpoint activation. Defective ATR signaling in humans causes Seckel syndrome, characterized by growth retardation and severe microcephaly (O'Driscoll and Jeggo 2008). While growth hormone secretion is normal, Seckel syndrome features high circulating IGF-1 levels and slightly decreased binding affinity for the IGF-1 receptor (Ducos et al. 2001; Schmidt et al. 2002). Whether this represents a constitutive defect in IGF-1 signalling or an adaptive response to a defective DNA damage response remains unknown.

3.5 TP53

Activation of the tumor suppressor p53 by genotoxic or other forms of stress can trigger various outcomes that reduce the chance of a damaged cell progressing into a tumor, ranging from cellular senescence to apoptosis. Besides its role in the response to genotoxic stress, p53 plays a central role in cellular energy metabolism through regulation of oxidative phosphorylation, glucose transporter expression and fatty acid synthase (Zhang, Qin, and Wang 2010). Mice engineered with defects in p53 are highly cancer prone, but also display a number of metabolic phenotypes. For example, phosphorylation of p53 at Ser18 by ATM is an important regulator of glucose homeostasis, as a S18A mutation renders mice insulin resistant (Armata et al. 2010). p53 is activated in adipose tissue upon high fat diet-induced obesity and insulin resistance in mice; inhibition of p53 in adipose tissue rescues senescence and insulin resistance in diabetic mice (Minamino et al. 2009). Taken together, these data suggest that p53 activation, for example by oxidative stress derived from over-nutrition and potentially by isolated DNA damage, can promote the onset of metabolic syndrome.

3.6 DNA-PK, KU

DNA-PK, Ku70 and Ku80 form a complex at double strand breaks to facilitate non-homologous end joining (NHEJ). Mice deficient in the catalytic subunit of DNA-PK (DNA-PKcs) exhibit accelerated aging, growth defects and decreased lifespan (Espejel et al. 2004). Mice deficient in Ku80 display premature aging symptoms including osteopenia, atrophic skin, hepatocellular degeneration and age-specific mortality; *Ku70*^{-/-} mice display growth retardation (Gu et al. 1997; Vogel et al. 1999). Increased lymphoma and defects in B and T cells of DNA-PK, Ku70 and Ku80 mice are attributed to their lack of NHEJ resulting in deficient V(D)J recombination required for adaptive immunity; however, premature aging phenotypes are not seen in Rag-1 (V(D)J deficient) mice and thus not related to the lack of adaptive immunity and associated inflammation (Holcomb, Vogel, and Hasty 2007). Reduced size in Ku80 mice is not due to reduced IGF-1, but may instead be related to defects in cell-autonomous proliferation (van de Ven et al. 2006).

3.7 NEIL1 DNA glycosylase deficiency

NEIL1 glycosylase is the homologue of the bacterial formamidopyrimidine DNA glycosylase and initiates repair of oxidative lesions in BER, acting specifically on 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine lesions (Jaruga, Birincioglu, et al. 2004). A mouse model deficient in NEIL-1 glycosylase develops severe obesity, dyslipidemia, fatty liver disease and hyperinsulinemia in the absence of exogenous oxidative stress (Vartanian et al. 2006). The development of metabolic syndrome in *NEIL1*^{-/-} mice is accelerated on a high-fat diet, indicating NEIL1 absence renders mice more susceptible to oxidative stress-induced metabolic syndrome (Sampath et al. 2011). Although no human diseases are associated with deficiency of NEIL1, several polymorphisms of NEIL1 with different activities on oxidized bases are found in humans (Roy et al. 2007). Whether or not these polymorphisms may lead to susceptibility to metabolic disease is not understood.

3.8 SIRT6

SIRT6 is the mammalian homologue of yeast Sir2 and is an NAD-dependent histone deacetylase. SIRT6 was originally described as a component of the BER system, as SIRT6 deficient MEFs are hypersensitive to BER-lesion inducing agents such as methyl methanesulfonate and hydrogen peroxide; sensitivity was restored to that of wildtype by introducing the dRP lyase domain of Pol β (Mostoslavsky et al. 2006). Although no direct interaction between SIRT6 and BER factors has been reported to date, SIRT6 appears to impact DNA repair by stabilizing chromatin and facilitating DNA-PK dependent damage signaling (Lombard 2009; McCord et al. 2009). SIRT6 also influences metabolism both by deacetylating histone H3 lysine 9 and by repressing HIF1 α to control the expression of glycolytic genes, which explains the glucose imbalance seen in SIRT6 knockout mice (Zhong et al. 2010). The SIRT6 phenotype is predominantly a metabolic one, with mice developing normally, although smaller than wildtypes, until 2 weeks of age, when they suffer from a degenerative wasting and severe hypoglycemia resulting in death (Mostoslavsky et al. 2006). Like NER progeria, SIRT6 knockout mice also have reduced serum IGF-1, likely contributing to their small size. Neural-specific deletion of SIRT6 does not rescue the postnatal growth failure, but does rescue the severe hypoglycemia that leads to death in full-body knockouts of SIRT 6 (Schwer et al. 2010; Mostoslavsky et al. 2006). Neural-specific

SIRT6 mice survive much longer than the whole-body knockouts, and by one year of age become obese (Schwer et al. 2010).

Syndrome	Affected gene	Metabolic feature
Cockayne Syndrome	CSA/CSB	Loss of subcutaneous fat
Trichothiodystrophy	XPB/XPD/TTDA	Loss of subcutaneous fat
Hutchinson-Gilford Progeria Syndrome	Lamin A	Loss of subcutaneous fat, atherosclerosis
Werner Syndrome	WRN	Lipid accumulation in blood, insulin resistance
Ataxia telangiectasia	ATM	Insulin resistance
Seckel syndrome	ATR	Increased IGF-1

Table 1. Human DNA repair diseases resulting in aberrant metabolism.

4. Cell culture models

In cultured cells, a number of recent studies on gene expression, signal transduction and protein interaction networks upon genotoxic stress point to both direct and indirect links between various forms of DNA damage and pathways regulating growth and energy metabolism.

In mammals, organismal growth and metabolism are controlled by availability of nutrients and energy, which in turn influence secretion of circulating regulatory hormones including insulin from the pancreas, growth hormone from the pituitary, and growth-hormone dependent IGF-1 from the liver. Cellular responses to nutrients, energy and growth factor availability are controlled at the cell surface by receptor tyrosine kinases including the insulin receptor (IR) and IGF-1 receptor (IGF-1R). Binding of peptide hormones to their cognate receptors activates a signal transduction cascade resulting in the phosphorylation and activation of downstream kinases including AKT and mTOR. AKT exerts control over energy metabolism by phosphorylating and inactivating FOXO transcription factors as well as TSC1, a major negative regulator of mTOR. mTOR activation, which requires growth factors as well as nutrients (amino acids) and energy, results in phosphorylation and activation of ribosomal protein S6 kinase, promoting protein translation and increased cell size and growth. mTOR also phosphorylates and inactivates the translational repressor eIF-4E-binding protein 1 (4EBP1), further promoting protein synthesis.

The role of protein kinases in DNA repair and the DDR is firmly established. In response to ionizing radiation, DNA damage response proteins ATM and ATR phosphorylate checkpoint kinases Chk1 and Chk2, as well as p53 to block cell cycle progression (Stokes et al. 2007). The kinase DNA-PK, a PI3-type protein kinase in the same family as ATM and ATR, is directly involved in the repair of DNA double strand breaks through the process of NHEJ. However, each of these kinases can target additional substrates involved in growth and energy metabolism outside of canonical DNA repair and damage signaling pathways in response to growth factor stimulation. For example, DNA-PK is recruited by the upstream-stimulatory factor to the promoter of fatty acid synthase (FAS), the master regulator of fatty acid synthesis, upon insulin stimulation (Wong et al. 2009). DNA-PK is required for transient DNA breaks at the FAS promoter and transcription of FAS (Wong et al. 2009). ATM is required for phosphorylation of 4EBP1 on Ser 111 to promote protein anabolism

upon insulin stimulation (Yang and Kastan 2000). This may be due indirectly to phosphorylation and inactivation of the mTOR repressor TSC2 by ATM (Alexander et al. 2010; Yang and Kastan 2000). ATM is also required for IGF-1 stimulated phosphorylation (activation) of AMPK, a cellular sensor of energy activated by low ATP/AMP ratios, to suppress energy-demanding processes such as cell growth in PANC and HeLa cells (Suzuki et al. 2004). ATM is also capable of inhibiting the stress signaling kinase JNK, whose activity is linked to several features of the metabolic syndrome (Schneider et al. 2006). Recently, ATM has been shown to be involved in the activation of autophagy through inhibition of mTOR (Alexander et al. 2010). DNA damage response proteins thus appear to be important regulators of cell growth and energy metabolism in response to environmental cues such as nutrient availability.

Importantly, there is mounting evidence that DDR proteins can also control these same metabolic pathways in response to DNA damage. One of the first clues was that ATM regulates expression of the IGF-1R in response to ionizing radiation (Peretz et al. 2001). Subsequent analysis of the ATM and ATR substrate pathway following ionizing radiation revealed several connections to the insulin-IGF-1-AKT pathway, including previously unidentified substrates insulin receptor substrate 2 (IRS2), AKT3 and its regulators HSP90 (heat shock protein 90) and PP2A (protein phosphatase 2A) (Matsuoka et al. 2007). Downstream targets of AKT including the transcription factor FOXO1, TSC1, S6K and 4E-BP1 were also identified as ATM and/or ATR substrates (Matsuoka et al. 2007). Cells lacking ATM demonstrate elevated mTOR and glycerophospholipid pathways when exposed to ionizing radiation (Varghese et al. 2010). Upon UV irradiation, DNA-PK is also required for translational reprogramming by directly or indirectly targeting the amino acid deprivation sensor GCN2 (Powley et al. 2009). The net outcome of this signal transduction cascade is to reduce general translation while at the same time to increase translation of proteins involved in adaptation to DNA damage, including NER proteins.

In addition to direct effects on cellular metabolism, activation of the DDR can trigger senescence-associated inflammatory cytokine secretion including IL-6 that can have an indirect effect on metabolism (Rodier et al. 2009). In 3T3-L1 adipocytes, for example, IL-6 inhibits phosphoenolpyruvate carboxykinase, which is required for triglyceride biosynthesis, thus increasing fatty acid mobilization (Feingold et al. 2011).

Another target of DNA damage-induced signal transduction is the tumor suppressor p53. p53 is normally a short-lived protein, but stabilization by phosphorylation promotes its activity as a transcriptional activator of cell-cycle inhibitors such as p21. p53 is stabilized as a result of multiple forms of stress, including genotoxic stress from ionizing radiation, ultraviolet radiation and ROS. In addition to cell cycle targets, p53 targets include genes involved directly in glycolysis (repression of phosphoglycerate mutase, (Kondoh et al. 2007)), and indirectly in respiration (activation of synthesis of cytochrome oxidase 2, a factor involved in COX assembly (Matoba et al. 2006)). In both cases, loss of p53 results in an increased glycolytic rate and decreased oxidative metabolism as is typically seen in cancer cells. TIGAR, another p53 target, is activated by low levels of stress and functions to repress glycolysis and to increase flux through the pentose phosphate pathway, resulting in increased generation of reduced glutathione, reduced ROS levels and protection from apoptosis (Bensaad et al. 2006). In some cells, glucose utilization by glycolysis competes with the pentose phosphate pathway responsible for generating NADPH reducing equivalents. Because NADPH is required for production of one of the major cellular antioxidants, reduced glutathione, increased glycolysis can come at the expense of

increased, rather than decreased, ROS. However, it is worth pointing out that this may be cell-type specific, as in other cells reduced glycolysis results in increased apoptosis.

ATR may be the kinase responsible for phosphorylating and stabilizing p53 in response to genotoxic stress (Colman, Afshari, and Barrett 2000). ATR is recruited to stalled replication forks by the single strand DNA binding protein RPA and the ATR-interacting protein ATRIP. Single-stranded DNA regions serve as a platform for RPA-ATRIP-ATR recruitment in the context of stalled elongating replication machinery (Ljungman 2007). p53 can be also be stabilized even in the absence of DNA damage by inhibiting transcriptional elongation by RNA PolII (Bode and Dong 2004; Ljungman 2007). Thus, lesions that block an elongating RNA PolII and activate TC-NER can also signal through p53. Interestingly, the production of UV-induced DNA damage foci in quiescent fibroblasts requires ATR and is defective in primary cells from patients with Seckel syndrome (O'Driscoll et al. 2003).

In cells deficient in TC-NER, unrepaired UV-induced lesions cause downregulation of both the GHR and IGF-1R (Garinis et al. 2009). This is consistent with the finding in NER progeroid mice of reduced insulin/IGF-1 signaling (Niedernhofer et al. 2006; van der Pluijm et al. 2007; van de Ven et al. 2006), and suggests that this effect can be cell autonomous *in vivo* rather than driven primarily by neuronal or neuroendocrine control. Interestingly, downregulation of growth receptors upon UV treatment was not inhibited *in vitro* by inhibitors of AKT, MAPK or JAK, but whether it requires DDR signaling through ATM, ATR or DNA-PK is not reported (Garinis et al. 2009). The identity of the signaling pathway from the lesion to receptor downregulation is currently not known.

In addition to impacting cellular metabolism by activating signal transduction pathways, DNA damage repair pathways can also directly affect cellular energy status. PARP is activated upon oxidative base damage and consumes both ATP and NAD⁺ in the polyadenylation of various local substrates (Gagne et al. 2006), thus reducing available cellular energy currencies. Depending on the amount of damage and level of PARP activation, cellular energy stores can be depleted to pathological levels resulting in cell death. In the absence of exogenous DNA damage, PARP ablation results in increased NAD⁺ levels and increased activation of the NAD⁺-dependent deacetylase SIRT1, phenocopying aspects of SIRT1 deacetylase activation on mitochondrial metabolism (Bai et al. 2011). Thus, PARP provides a link between DNA damage and energy metabolism through direct effects on energy currencies as well as indirect effects of NAD⁺ and ATP dependent enzymes.

5. Conclusions

Cellular energy metabolism is a major source of ROS that can damage cellular components, including DNA. Oxidative DNA damage can in turn activate the DDR, a network of repair and signaling activities required for damage removal and stress adaptation. Inborn errors in DNA damage repair and signaling in human syndromes and mouse models typically display pathologies consistent with perturbation of growth and energy metabolism, including dwarfism and changes in insulin signaling and lipid accumulation. Interestingly, some disorders present with phenotypes reminiscent of the maladaptive response to nutrient/energy excess seen in metabolic syndrome. For example, in in A-T and Werner syndrome, these symptoms include dyslipidemia and insulin resistance. In other disorders, including mouse models of CS and TTD, symptoms appear on the opposite end of the nutrient/energy spectrum, with hypoglycemia, increased insulin sensitivity and reduced

adiposity reminiscent of the beneficial adaptations to DR. Over time, however, chronic activation of adaptations such as reduced IGF-1 that may be beneficial in the context of a wildtype mammal on a restricted diet may in fact become maladaptive in the context of genome instability. For example, mouse models of severe NER progeria have reduced IGF-1, but nonetheless have shortened lifespans, while restoration of IGF-1 levels in a mouse model of HGPS ameliorates disease symptoms. On the cellular level, recent data suggest that proteins involved in the DNA damage response can exert both direct and indirect control over pathways involved in energy metabolism and substrate utilization, including insulin and IGF-1 signaling. Which of these genetic defects leads to constitutive alterations in metabolic processes and which to adaptive responses to genotoxic stress remains to be fully elucidated. Furthermore, to what degree unrepaired DNA damage itself serves as the trigger for metabolic changes, and the identity of the causative lesion, is in most cases unknown. In conclusion, despite recent emerging data on a connection between the DNA damage response and energy metabolism, there is a relative dearth of studies on cellular or organismal energy metabolism in the context of genome instability disorders, with much remaining to be done in this burgeoning field.

6. References

- Al-Shali, K. Z., and R. A. Hegele. 2004. Laminopathies and atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* 24 (9):1591-5.
- Alexander, A., S. L. Cai, J. Kim, A. Nanez, M. Sahin, K. H. MacLean, K. Inoki, K. L. Guan, J. Shen, M. D. Person, D. Kusewitt, G. B. Mills, M. B. Kastan, and C. L. Walker. 2010. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proceedings of the National Academy of Sciences of the United States of America* 107 (9):4153-8.
- Allsopp, R. C., H. Vaziri, C. Patterson, S. Goldstein, E. V. Younglai, A. B. Futcher, C. W. Greider, and C. B. Harley. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* 89 (21):10114-8.
- Andressoo, J. O., J. H. Hoeijmakers, and J. R. Mitchell. 2006. Nucleotide excision repair disorders and the balance between cancer and aging. *Cell cycle* 5 (24):2886-8.
- Andressoo, J. O., J. R. Mitchell, J. de Wit, D. Hoogstraten, M. Volker, W. Toussaint, E. Speksnijder, R. B. Beems, H. van Steeg, J. Jans, C. I. de Zeeuw, N. G. Jaspers, A. Raams, A. R. Lehmann, W. Vermeulen, J. H. Hoeijmakers, and G. T. van der Horst. 2006. An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. *Cancer cell* 10 (2):121-32.
- Anindya, R., P. O. Mari, U. Kristensen, H. Kool, G. Giglia-Mari, L. H. Mullenders, M. Fousteri, W. Vermeulen, J. M. Egly, and J. Q. Svejstrup. 2010. A ubiquitin-binding domain in Cockayne syndrome B required for transcription-coupled nucleotide excision repair. *Molecular cell* 38 (5):637-48.
- Armata, H. L., D. Golebiowski, D. Y. Jung, H. J. Ko, J. K. Kim, and H. K. Sluss. 2010. Requirement of the ATM/p53 tumor suppressor pathway for glucose homeostasis. *Mol Cell Biol* 30 (24):5787-94.
- Bai, P., C. Canto, H. Oudart, A. Brunyanszki, Y. Cen, C. Thomas, H. Yamamoto, A. Huber, B. Kiss, R. H. Houtkooper, K. Schoonjans, V. Schreiber, A. A. Sauve, J. Menissier-de

- Murcia, and J. Auwerx. 2011. PARP-1 Inhibition Increases Mitochondrial Metabolism through SIRT1 Activation. *Cell metabolism* 13 (4):461-8.
- Barrett, S. F., J. H. Robbins, R. E. Tarone, and K. H. Kraemer. 1991. Evidence for defective repair of cyclobutane pyrimidine dimers with normal repair of other DNA photoproducts in a transcriptionally active gene transfected into Cockayne syndrome cells. *Mutation research* 255 (3):281-91.
- Bartke, A., and H. Brown-Borg. 2004. Life extension in the dwarf mouse. *Curr Top Dev Biol* 63:189-225.
- Beckman, K. B., and B. N. Ames. 1998. The free radical theory of aging matures. *Physiol Rev* 78 (2):547-81.
- Bensaad, K., A. Tsuruta, M. A. Selak, M. N. Vidal, K. Nakano, R. Bartrons, E. Gottlieb, and K. H. Vousden. 2006. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 126 (1):107-20.
- Bode, A. M., and Z. Dong. 2004. Post-translational modification of p53 in tumorigenesis. *Nature reviews. Cancer* 4 (10):793-805.
- Bradsher, J., J. Auriol, L. Proietti de Santis, S. Iben, J. L. Vonesch, I. Grummt, and J. M. Egly. 2002. CSB is a component of RNA pol I transcription. *Molecular cell* 10 (4):819-29.
- Bridger, J. M., and I. R. Kill. 2004. Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis. *Exp Gerontol* 39 (5):717-24.
- Brooks, P. J. 2008. The 8,5'-cyclopurine-2'-deoxynucleosides: candidate neurodegenerative DNA lesions in xeroderma pigmentosum, and unique probes of transcription and nucleotide excision repair. *DNA repair* 7 (7):1168-79.
- Brooks, P. J., T. F. Cheng, and L. Cooper. 2008. Do all of the neurologic diseases in patients with DNA repair gene mutations result from the accumulation of DNA damage? *DNA repair* 7 (6):834-48.
- Brooks, P. J., D. S. Wise, D. A. Berry, J. V. Kosmoski, M. J. Smerdon, R. L. Somers, H. Mackie, A. Y. Spoonde, E. J. Ackerman, K. Coleman, R. E. Tarone, and J. H. Robbins. 2000. The oxidative DNA lesion 8,5'-(S)-cyclo-2'-deoxyadenosine is repaired by the nucleotide excision repair pathway and blocks gene expression in mammalian cells. *The Journal of biological chemistry* 275 (29):22355-62.
- Chun, H. H., and R. A. Gatti. 2004. Ataxia-telangiectasia, an evolving phenotype. *DNA repair* 3 (8-9):1187-96.
- Cockayne, E. A. 1936. Dwarfism with retinal atrophy and deafness. *Arch Dis Child* 11 (61):1-8.
- Colman, M. S., C. A. Afshari, and J. C. Barrett. 2000. Regulation of p53 stability and activity in response to genotoxic stress. *Mutation research* 462 (2-3):179-88.
- Compe, E., M. Malerba, L. Soler, J. Marescaux, E. Borrelli, and J. M. Egly. 2007. Neurological defects in trichothiodystrophy reveal a coactivator function of TFIIH. *Nature neuroscience* 10 (11):1414-22.
- de Boer, J., J. de Wit, H. van Steeg, R. J. Berg, H. Morreau, P. Visser, A. R. Lehmann, M. Duran, J. H. Hoeijmakers, and G. Weeda. 1998. A mouse model for the basal transcription/DNA repair syndrome trichothiodystrophy. *Molecular cell* 1 (7):981-90.
- De Bont, R., and N. van Larebeke. 2004. Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis* 19 (3):169-85.

- Dizdaroglu, M., M. L. Dirksen, H. X. Jiang, and J. H. Robbins. 1987. Ionizing-radiation-induced damage in the DNA of cultured human cells. Identification of 8,5-cyclo-2-deoxyguanosine. *The Biochemical journal* 241 (3):929-32.
- Dolle, M. E., R. A. Busuttill, A. M. Garcia, S. Wijnhoven, E. van Drunen, L. J. Niedernhofer, G. van der Horst, J. H. Hoeijmakers, H. van Steeg, and J. Vijg. 2006. Increased genomic instability is not a prerequisite for shortened lifespan in DNA repair deficient mice. *Mutat Res* 596 (1-2):22-35.
- Ducos, B., S. Cabrol, M. Houang, L. Perin, M. Holzenberger, and Y. Le Bouc. 2001. IGF type 1 receptor ligand binding characteristics are altered in a subgroup of children with intrauterine growth retardation. *J Clin Endocrinol Metab* 86 (11):5516-24.
- Espejel, S., M. Martin, P. Klatt, J. Martin-Caballero, J. M. Flores, and M. A. Blasco. 2004. Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. *EMBO Rep* 5 (5):503-9.
- Feingold, K. R., A. Moser, J. K. Shigenaga, and C. Grunfeld. 2011. Inflammation inhibits the expression of phosphoenolpyruvate carboxykinase in liver and adipose tissue. *Innate Immun.*
- Fontana, L., and S. Klein. 2007. Aging, adiposity, and calorie restriction. *Jama* 297 (9):986-94.
- Gagne, J. P., M. J. Hendzel, A. Droit, and G. G. Poirier. 2006. The expanding role of poly(ADP-ribose) metabolism: current challenges and new perspectives. *Curr Opin Cell Biol* 18 (2):145-51.
- Garinis, G. A., L. M. Uittenboogaard, H. Stachelscheid, M. Fousteri, W. van Ijcken, T. M. Breit, H. van Steeg, L. H. Mullenders, G. T. van der Horst, J. C. Bruning, C. M. Niessen, J. H. Hoeijmakers, and B. Schumacher. 2009. Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. *Nature cell biology* 11 (5):604-15.
- Groisman, R., J. Polanowska, I. Kuraoka, J. Sawada, M. Saijo, R. Drapkin, A. F. Kisselev, K. Tanaka, and Y. Nakatani. 2003. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113 (3):357-67.
- Gu, Y., K. J. Seidl, G. A. Rathbun, C. Zhu, J. P. Manis, N. van der Stoep, L. Davidson, H. L. Cheng, J. M. Sekiguchi, K. Frank, P. Stanhope-Baker, M. S. Schlissel, D. B. Roth, and F. W. Alt. 1997. Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity* 7 (5):653-65.
- Holcomb, V. B., H. Vogel, and P. Hasty. 2007. Deletion of Ku80 causes early aging independent of chronic inflammation and Rag-1-induced DSBs. *Mechanisms of ageing and development* 128 (11-12):601-8.
- Hotamisligil, G. S. 2006. Inflammation and metabolic disorders. *Nature* 444 (7121):860-7.
- Huang, S., L. Lee, N. B. Hanson, C. Lenaerts, H. Hoehn, M. Poot, C. D. Rubin, D. F. Chen, C. C. Yang, H. Juch, T. Dorn, R. Spiegel, E. A. Oral, M. Abid, C. Battisti, E. Lucci-Cordisco, G. Neri, E. H. Steed, A. Kidd, W. Isley, D. Showalter, J. L. Vittone, A. Konstantinow, J. Ring, P. Meyer, S. L. Wenger, A. von Herbay, U. Wollina, M. Schuelke, C. R. Huizenga, D. F. Leistriz, G. M. Martin, I. S. Mian, and J. Oshima. 2006. The spectrum of WRN mutations in Werner syndrome patients. *Hum Mutat* 27 (6):558-67.
- Jaruga, P., M. Birincioglu, T. A. Rosenquist, and M. Dizdaroglu. 2004. Mouse NEIL1 protein is specific for excision of 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine from oxidatively damaged DNA. *Biochemistry* 43 (50):15909-14.

- Jaruga, P., J. Theruvathu, M. Dizdaroglu, and P. J. Brooks. 2004. Complete release of (5S)-8,5'-cyclo-2'-deoxyadenosine from dinucleotides, oligodeoxynucleotides and DNA, and direct comparison of its levels in cellular DNA with other oxidatively induced DNA lesions. *Nucleic acids research* 32 (11):e87.
- Keriel, A., A. Sary, A. Sarasin, C. Rochette-Egly, and J. M. Egly. 2002. XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. *Cell* 109 (1):125-35.
- Kohlschutter, A., A. Bley, K. Brockmann, J. Gartner, I. Krageloh-Mann, A. Rolfs, and L. Schols. 2010. Leukodystrophies and other genetic metabolic leukoencephalopathies in children and adults. *Brain Dev* 32 (2):82-9.
- Kondoh, H., M. E. Leonart, D. Bernard, and J. Gil. 2007. Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization. *Histol Histopathol* 22 (1):85-90.
- Kruman, II. 2004. Why do neurons enter the cell cycle? *Cell Cycle* 3 (6):769-73.
- Kuraoka, I., C. Bender, A. Romieu, J. Cadet, R. D. Wood, and T. Lindahl. 2000. Removal of oxygen free-radical-induced 5',8-purine cyclodeoxynucleosides from DNA by the nucleotide excision-repair pathway in human cells. *Proceedings of the National Academy of Sciences of the United States of America* 97 (8):3832-7.
- Lachapelle, S., S. Oesterreich, and M. Lebel. 2011. The Werner syndrome helicase protein is required for cell proliferation, immortalization, and tumorigenesis in Scaffold Attachment Factor B1 deficient mice. *Aging (Albany NY)* 3 (3):277-90.
- Laposa, R. R., E. J. Huang, and J. E. Cleaver. 2007. Increased apoptosis, p53 up-regulation, and cerebellar neuronal degeneration in repair-deficient Cockayne syndrome mice. *Proceedings of the National Academy of Sciences of the United States of America* 104 (4):1389-94.
- Lavin, M. F. 2000. An unlikely player joins the ATM signalling network. *Nature cell biology* 2 (12):E215-7.
- Le May, N., J. M. Egly, and F. Coin. 2010. True lies: the double life of the nucleotide excision repair factors in transcription and DNA repair. *Journal of nucleic acids* 2010.
- Lebel, M., L. Massip, C. Garand, and E. Thorin. 2010. Ascorbate improves metabolic abnormalities in Wrn mutant mice but not the free radical scavenger catechin. *Ann N Y Acad Sci* 1197:40-4.
- Leverenz, J. B., C. E. Yu, and G. D. Schellenberg. 1998. Aging-associated neuropathology in Werner syndrome. *Acta Neuropathol* 96 (4):421-4.
- Liu, B., J. Wang, K. M. Chan, W. M. Tjia, W. Deng, X. Guan, J. D. Huang, K. M. Li, P. Y. Chau, D. J. Chen, D. Pei, A. M. Pendas, J. Cadinanos, C. Lopez-Otin, H. F. Tse, C. Hutchison, J. Chen, Y. Cao, K. S. Cheah, K. Tryggvason, and Z. Zhou. 2005. Genomic instability in laminopathy-based premature aging. *Nature medicine* 11 (7):780-5.
- Ljungman, M. 2007. The transcription stress response. *Cell Cycle* 6 (18):2252-7.
- Lodish, H., ed. 2000. *Molecular Cell Biology*. 4 ed. New York: W.H. Freeman and Company.
- Lombard, D. B. 2009. Sirtuins at the breaking point: SIRT6 in DNA repair. *Aging (Albany NY)* 1 (1):12-6.
- Longo, V. D., and C. E. Finch. 2003. Evolutionary medicine: from dwarf model systems to healthy centenarians? *Science* 299 (5611):1342-6.
- Marino, G., A. P. Ugalde, A. F. Fernandez, F. G. Osorio, A. Fueyo, J. M. Freije, and C. Lopez-Otin. 2010. Insulin-like growth factor 1 treatment extends longevity in a mouse model of human premature aging by restoring somatotroph axis function.

- Proceedings of the National Academy of Sciences of the United States of America* 107 (37):16268-73.
- Marino, G., A. P. Ugalde, N. Salvador-Montoliu, I. Varela, P. M. Quiros, J. Cadinanos, I. van der Pluijm, J. M. Freije, and C. Lopez-Otin. 2008. Premature aging in mice activates a systemic metabolic response involving autophagy induction. *Human molecular genetics* 17 (14):2196-211.
- Martin, G. M. 1985. Genetics and aging; the Werner syndrome as a segmental progeroid syndrome. *Adv Exp Med Biol* 190:161-70.
- Matoba, S., J. G. Kang, W. D. Patino, A. Wragg, M. Boehm, O. Gavrilova, P. J. Hurley, F. Bunz, and P. M. Hwang. 2006. p53 regulates mitochondrial respiration. *Science* 312 (5780):1650-3.
- Matsuoka, S., B. A. Ballif, A. Smogorzewska, E. R. McDonald, 3rd, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi, and S. J. Elledge. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316 (5828):1160-6.
- McCay, C.M., M.F. Crowel, and L.A. Maynard. 1935. The effect of retarded growth upon the length of the life span and upon the ultimate body size. *J Nutr* 10:63-79.
- McCord, R. A., E. Michishita, T. Hong, E. Berber, L. D. Boxer, R. Kusumoto, S. Guan, X. Shi, O. Gozani, A. L. Burlingame, V. A. Bohr, and K. F. Chua. 2009. SIRT6 stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair. *Aging (Albany NY)* 1 (1):109-21.
- Melton, D. W., A. M. Ketchen, F. Nunez, S. Bonatti-Abbondandolo, A. Abbondandolo, S. Squires, and R. T. Johnson. 1998. Cells from ERCC1-deficient mice show increased genome instability and a reduced frequency of S-phase-dependent illegitimate chromosome exchange but a normal frequency of homologous recombination. *J Cell Sci* 111 (Pt 3):395-404.
- Mercer, J. R., K. K. Cheng, N. Figg, I. Gorenne, M. Mahmoudi, J. Griffin, A. Vidal-Puig, A. Logan, M. P. Murphy, and M. Bennett. 2010. DNA damage links mitochondrial dysfunction to atherosclerosis and the metabolic syndrome. *Circulation research* 107 (8):1021-31.
- Merideth, M. A., L. B. Gordon, S. Clauss, V. Sachdev, A. C. Smith, M. B. Perry, C. C. Brewer, C. Zalewski, H. J. Kim, B. Solomon, B. P. Brooks, L. H. Gerber, M. L. Turner, D. L. Domingo, T. C. Hart, J. Graf, J. C. Reynolds, A. Gropman, J. A. Yanovski, M. Gerhard-Herman, F. S. Collins, E. G. Nabel, R. O. Cannon, 3rd, W. A. Gahl, and W. J. Intronc. 2008. Phenotype and course of Hutchinson-Gilford progeria syndrome. *N Engl J Med* 358 (6):592-604.
- Miles, P. D., K. Treuner, M. Latronica, J. M. Olefsky, and C. Barlow. 2007. Impaired insulin secretion in a mouse model of ataxia telangiectasia. *Am J Physiol Endocrinol Metab* 293 (1):E70-4.
- Minamino, T., M. Orimo, I. Shimizu, T. Kunieda, M. Yokoyama, T. Ito, A. Nojima, A. Nabetani, Y. Oike, H. Matsubara, F. Ishikawa, and I. Komuro. 2009. A crucial role for adipose tissue p53 in the regulation of insulin resistance. *Nature medicine* 15 (9):1082-7.
- Mitchell, J. R., M. Verweij, K. Brand, M. van de Ven, N. Goemaere, S. van den Engel, T. Chu, F. Forrer, C. Muller, M. de Jong, W. van Ijcken, J. N. Ijzermans, J. H. Hoeijmakers, and R. W. de Bruin. 2009. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell*.

- Monnat, R. J., Jr. 2010. Human RECQ helicases: roles in DNA metabolism, mutagenesis and cancer biology. *Semin Cancer Biol* 20 (5):329-39.
- Morice-Picard, F., M. Cario-Andre, H. Rezvani, D. Lacombe, A. Sarasin, and A. Taieb. 2009. New clinico-genetic classification of trichothiodystrophy. *Am J Med Genet A* 149A (9):2020-30.
- Mostoslavsky, R., K. F. Chua, D. B. Lombard, W. W. Pang, M. R. Fischer, L. Gellon, P. Liu, G. Mostoslavsky, S. Franco, M. M. Murphy, K. D. Mills, P. Patel, J. T. Hsu, A. L. Hong, E. Ford, H. L. Cheng, C. Kennedy, N. Nunez, R. Bronson, D. Frendewey, W. Auerbach, D. Valenzuela, M. Karow, M. O. Hottiger, S. Hursting, J. C. Barrett, L. Guarente, R. Mulligan, B. Demple, G. D. Yancopoulos, and F. W. Alt. 2006. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 124 (2):315-29.
- Murai, M., Y. Enokido, N. Inamura, M. Yoshino, Y. Nakatsu, G. T. van der Horst, J. H. Hoeijmakers, K. Tanaka, and H. Hatanaka. 2001. Early postnatal ataxia and abnormal cerebellar development in mice lacking Xeroderma pigmentosum Group A and Cockayne syndrome Group B DNA repair genes. *Proceedings of the National Academy of Sciences of the United States of America* 98 (23):13379-84.
- Nance, M. A., and S. A. Berry. 1992. Cockayne syndrome: review of 140 cases. *Am J Med Genet* 42 (1):68-84.
- Nevedomskaya, E., A. Meissner, S. Goral, M. de Waard, Y. Ridwan, G. Zondag, I. van der Pluijm, A. M. Deelder, and O. A. Mayboroda. 2010. Metabolic profiling of accelerated aging ERCC1 d/- mice. *J Proteome Res* 9 (7):3680-7.
- Niedernhofer, L. J., G. A. Garinis, A. Raams, A. S. Lalai, A. R. Robinson, E. Appeldoorn, H. Odijk, R. Oostendorp, A. Ahmad, W. van Leeuwen, A. F. Theil, W. Vermeulen, G. T. van der Horst, P. Meinecke, W. J. Kleijer, J. Vijg, N. G. Jaspers, and J. H. Hoeijmakers. 2006. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. *Nature* 444 (7122):1038-43.
- O'Driscoll, M., and P. A. Jeggo. 2008. The role of the DNA damage response pathways in brain development and microcephaly: insight from human disorders. *DNA repair* 7 (7):1039-50.
- O'Driscoll, M., V. L. Ruiz-Perez, C. G. Woods, P. A. Jeggo, and J. A. Goodship. 2003. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat Genet* 33 (4):497-501.
- Ozdirim, E., M. Topcu, A. Ozon, and A. Cila. 1996. Cockayne syndrome: review of 25 cases. *Pediatr Neurol* 15 (4):312-6.
- Pasquier, L., V. Laugel, L. Lazaro, H. Dollfus, H. Journal, P. Edery, A. Goldenberg, D. Martin, D. Heron, M. Le Merrer, P. Rustin, S. Odent, A. Munnich, A. Sarasin, and V. Cormier-Daire. 2006. Wide clinical variability among 13 new Cockayne syndrome cases confirmed by biochemical assays. *Arch Dis Child* 91 (2):178-82.
- Pendas, A. M., Z. Zhou, J. Cadinanos, J. M. Freije, J. Wang, K. Hulthenby, A. Astudillo, A. Wernerson, F. Rodriguez, K. Tryggvason, and C. Lopez-Otin. 2002. Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice. *Nat Genet* 31 (1):94-9.
- Peretz, S., R. Jensen, R. Baserga, and P. M. Glazer. 2001. ATM-dependent expression of the insulin-like growth factor-I receptor in a pathway regulating radiation response. *Proceedings of the National Academy of Sciences of the United States of America* 98 (4):1676-81.

- Powley, I. R., A. Kondrashov, L. A. Young, H. C. Dobbyn, K. Hill, I. G. Cannell, M. Stoneley, Y. W. Kong, J. A. Cotes, G. C. Smith, R. Wek, C. Hayes, T. W. Gant, K. A. Spriggs, M. Bushell, and A. E. Willis. 2009. Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. *Genes & development* 23 (10):1207-20.
- Randle, P. J., P. B. Garland, C. N. Hales, and E. A. Newsholme. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1 (7285):785-9.
- Rapin, I., K. Weidenheim, Y. Lindenbaum, P. Rosenbaum, S. N. Merchant, S. Krishna, and D. W. Dickson. 2006. Cockayne syndrome in adults: review with clinical and pathologic study of a new case. *J Child Neurol* 21 (11):991-1006.
- Rincon, M., R. Muzumdar, G. Atzmon, and N. Barzilai. 2004. The paradox of the insulin/IGF-1 signaling pathway in longevity. *Mechanisms of ageing and development* 125 (6):397-403.
- Rodier, F., J. P. Coppe, C. K. Patil, W. A. Hoeijmakers, D. P. Munoz, S. R. Raza, A. Freund, E. Campeau, A. R. Davalos, and J. Campisi. 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nature cell biology* 11 (8):973-9.
- Roy, L. M., P. Jaruga, T. G. Wood, A. K. McCullough, M. Dizdaroglu, and R. S. Lloyd. 2007. Human polymorphic variants of the NEIL1 DNA glycosylase. *The Journal of biological chemistry* 282 (21):15790-8.
- Sampath, H., A. K. Batra, V. Vartanian, J. R. Carmical, D. Prusak, I. B. King, B. Lowell, L. F. Earley, T. G. Wood, D. L. Marks, A. K. McCullough, and R. Stephen L. 2011. Variable penetrance of metabolic phenotypes and development of high-fat diet-induced adiposity in NEIL1-deficient mice. *Am J Physiol Endocrinol Metab* 300 (4):E724-34.
- Schmidt, A., A. Chakravarty, E. Brommer, B. D. Fenne, T. Siebler, P. De Meyts, and W. Kiess. 2002. Growth failure in a child showing characteristics of Seckel syndrome: possible effects of IGF-I and endogenous IGFBP-3. *Clin Endocrinol (Oxf)* 57 (2):293-9.
- Schneider, J. G., B. N. Finck, J. Ren, K. N. Standley, M. Takagi, K. H. Maclean, C. Bernal-Mizrachi, A. J. Muslin, M. B. Kastan, and C. F. Semenkovich. 2006. ATM-dependent suppression of stress signaling reduces vascular disease in metabolic syndrome. *Cell metabolism* 4 (5):377-89.
- Schumacher, B., I. van der Pluijm, M. J. Moorhouse, T. Kosteas, A. R. Robinson, Y. Suh, T. M. Breit, H. van Steeg, L. J. Niedernhofer, W. van Ijcken, A. Bartke, S. R. Spindler, J. H. Hoeijmakers, G. T. van der Horst, and G. A. Garinis. 2008. Delayed and accelerated aging share common longevity assurance mechanisms. *PLoS Genet* 4 (8):e1000161.
- Schwer, B., B. Schumacher, D. B. Lombard, C. Xiao, M. V. Kurtev, J. Gao, J. I. Schneider, H. Chai, R. T. Bronson, L. H. Tsai, C. X. Deng, and F. W. Alt. 2010. Neural sirtuin 6 (Sirt6) ablation attenuates somatic growth and causes obesity. *Proceedings of the National Academy of Sciences of the United States of America* 107 (50):21790-4.
- Shackelford, R. E. 2005. Pharmacologic manipulation of the ataxia-telangiectasia mutated gene product as an intervention in age-related disease. *Med Hypotheses* 65 (2):363-9.
- Stokes, M. P., J. Rush, J. Macneill, J. M. Ren, K. Sprott, J. Nardone, V. Yang, S. A. Beausoleil, S. P. Gygi, M. Livingstone, H. Zhang, R. D. Polakiewicz, and M. J. Comb. 2007. Profiling of UV-induced ATM/ATR signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America* 104 (50):19855-60.

- Sun, X. Z., Y. N. Harada, R. Zhang, C. Cui, S. Takahashi, and Y. Fukui. 2003. A genetic mouse model carrying the nonfunctional xeroderma pigmentosum group G gene. *Congenit Anom (Kyoto)* 43 (2):133-9.
- Susa, D., J. R. Mitchell, M. Verweij, M. van de Ven, H. Roest, S. van den Engel, I. Bajema, K. Mangundap, J. N. Ijzermans, J. H. Hoeijmakers, and R. W. de Bruin. 2009. Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. *Aging Cell* 8 (2):192-200.
- Suzuki, A., G. Kusakai, A. Kishimoto, Y. Shimojo, T. Ogura, M. F. Lavin, and H. Esumi. 2004. IGF-1 phosphorylates AMPK-alpha subunit in ATM-dependent and LKB1-independent manner. *Biochemical and biophysical research communications* 324 (3):986-92.
- Tian, M., R. Shinkura, N. Shinkura, and F. W. Alt. 2004. Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. *Mol Cell Biol* 24 (3):1200-5.
- Tieu, K., C. Perier, C. Caspersen, P. Teismann, D. C. Wu, S. D. Yan, A. Naini, M. Vila, V. Jackson-Lewis, R. Ramasamy, and S. Przedborski. 2003. D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. *The Journal of clinical investigation* 112 (6):892-901.
- Tyner, S. D., S. Venkatachalam, J. Choi, S. Jones, N. Ghebraniou, H. Igelmann, X. Lu, G. Soron, B. Cooper, C. Brayton, S. Hee Park, T. Thompson, G. Karsenty, A. Bradley, and L. A. Donehower. 2002. p53 mutant mice that display early ageing-associated phenotypes. *Nature* 415 (6867):45-53.
- Umehara, F., M. Abe, M. Nakagawa, S. Izumo, K. Arimura, K. Matsumuro, and M. Osame. 1993. Werner's syndrome associated with spastic paraparesis and peripheral neuropathy. *Neurology* 43 (6):1252-4.
- van de Ven, M., J. O. Andressoo, V. B. Holcomb, P. Hasty, Y. Suh, H. van Steeg, G. A. Garinis, J. H. Hoeijmakers, and J. R. Mitchell. 2007. Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. *Mechanisms of ageing and development* 128 (1):58-63.
- van de Ven, M., J. O. Andressoo, V. B. Holcomb, M. von Lindern, W. M. Jong, C. I. De Zeeuw, Y. Suh, P. Hasty, J. H. Hoeijmakers, G. T. van der Horst, and J. R. Mitchell. 2006. Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. *PLoS Genet* 2 (12):e192.
- van de Ven, Marieke, Jaan-Olle Andressoo, Valerie B. Holcomb, Marieke von Lindern, Willeke Jong, Chris I. De Zeeuw, Yousin Suh, Paul Hasty, Jan H. J. Hoeijmakers, Gijsbertus T. J. van der Horst, and James R. Mitchell. 2006. Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. *PLoS Genetics* preprint (2006):e192.
- van der Horst, G. T., L. Meira, T. G. Gorgels, J. de Wit, S. Velasco-Miguel, J. A. Richardson, Y. Kamp, M. P. Vreeswijk, B. Smit, D. Bootsma, J. H. Hoeijmakers, and E. C. Friedberg. 2002. UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. *DNA repair* 1 (2):143-57.
- van der Horst, G. T., H. van Steeg, R. J. Berg, A. J. van Gool, J. de Wit, G. Weeda, H. Morreau, R. B. Beems, C. F. van Kreijl, F. R. de Gruijl, D. Bootsma, and J. H. Hoeijmakers. 1997. Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. *Cell* 89 (3):425-35.
- van der Pluijm, I., G. A. Garinis, R. M. Brandt, T. G. Gorgels, S. W. Wijnhoven, K. E. Diderich, J. de Wit, J. R. Mitchell, C. van Oostrom, R. Beems, L. J. Niedernhofer, S.

- Velasco, E. C. Friedberg, K. Tanaka, H. van Steeg, J. H. Hoeijmakers, and G. T. van der Horst. 2007. Impaired genome maintenance suppresses the growth hormone--insulin-like growth factor 1 axis in mice with Cockayne syndrome. *PLoS biology* 5 (1):e2.
- Varela, I., J. Cadinanos, A. M. Pendas, A. Gutierrez-Fernandez, A. R. Folgueras, L. M. Sanchez, Z. Zhou, F. J. Rodriguez, C. L. Stewart, J. A. Vega, K. Tryggvason, J. M. Freije, and C. Lopez-Otin. 2005. Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. *Nature* 437 (7058):564-8.
- Varghese, R. S., A. Cheema, P. Cheema, M. Bourbeau, L. Tuli, B. Zhou, M. Jung, A. Dritschilo, and H. W. Ressom. 2010. Analysis of LC-MS data for characterizing the metabolic changes in response to radiation. *J Proteome Res* 9 (5):2786-93.
- Vartanian, V., B. Lowell, I. G. Minko, T. G. Wood, J. D. Ceci, S. George, S. W. Ballinger, C. L. Corless, A. K. McCullough, and R. S. Lloyd. 2006. The metabolic syndrome resulting from a knockout of the NEIL1 DNA glycosylase. *Proceedings of the National Academy of Sciences of the United States of America* 103 (6):1864-9.
- Verstraeten, V. L., J. L. Broers, F. C. Ramaekers, and M. A. van Steensel. 2007. The nuclear envelope, a key structure in cellular integrity and gene expression. *Curr Med Chem* 14 (11):1231-48.
- Vogel, H., D. S. Lim, G. Karsenty, M. Finegold, and P. Hasty. 1999. Deletion of Ku86 causes early onset of senescence in mice. *Proc Natl Acad Sci U S A* 96 (19):10770-5.
- Weeda, G., I. Donker, J. de Wit, H. Morreau, R. Janssens, C. J. Vissers, A. Nigg, H. van Steeg, D. Bootsma, and J. H. J. Hoeijmakers. 1997. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr Biol* 7 (6):427-39.
- Weidenheim, K. M., D. W. Dickson, and I. Rapin. 2009. Neuropathology of Cockayne syndrome: Evidence for impaired development, premature aging, and neurodegeneration. *Mechanisms of ageing and development* 130 (9):619-36.
- Wijnhoven, S. W., R. B. Beems, M. Roodbergen, J. van den Berg, P. H. Lohman, K. Diderich, G. T. van der Horst, J. Vijg, J. H. Hoeijmakers, and H. van Steeg. 2005. Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. *DNA repair* 4 (11):1314-24.
- Wong, H. K., M. Muftuoglu, G. Beck, S. Z. Imam, V. A. Bohr, and D. M. Wilson, 3rd. 2007. Cockayne syndrome B protein stimulates apurinic endonuclease 1 activity and protects against agents that introduce base excision repair intermediates. *Nucleic acids research* 35 (12):4103-13.
- Wong, R. H., I. Chang, C. S. Hudak, S. Hyun, H. Y. Kwan, and H. S. Sul. 2009. A role of DNA-PK for the metabolic gene regulation in response to insulin. *Cell* 136 (6):1056-72.
- Yang, D. Q., and M. B. Kastan. 2000. Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. *Nature cell biology* 2 (12):893-8.
- Zhang, X. D., Z. H. Qin, and J. Wang. 2010. The role of p53 in cell metabolism. *Acta Pharmacol Sin* 31 (9):1208-12.
- Zhong, L., A. D'Urso, D. Toiber, C. Sebastian, R. E. Henry, D. D. Vadysirisack, A. Guimaraes, B. Marinelli, J. D. Wikstrom, T. Nir, C. B. Clish, B. Vaitheesvaran, O. Iliopoulos, I. Kurland, Y. Dor, R. Weissleder, O. S. Shirihai, L. W. Ellisen, J. M. Espinosa, and R. Mostoslavsky. 2010. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 140 (2):280-93.

Part 5

Infection, Inflammation and DNA Repair

The Role of DNA Repair Pathways in Adeno-Associated Virus Infection and Viral Genome Replication / Recombination / Integration¹

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1. Introduction

Cellular DNA is constantly being damaged not only by extrinsic factors such as ionizing radiation and environmental carcinogens but also by intrinsic agents such as reactive oxygen species arising during normal cellular metabolism. Of the myriad of DNA lesions, inflicted by extrinsic and intrinsic genome damaging agents, DNA double strand break (DSB) is the most threatening. Replication fork arrest at DNA lesions could also be a threat since stalled replication forks, if fail to restart appropriately, induce DNA strand breaks. When cells encounter such strand breaks and other types of DNA damage, they mount a DNA damage response (DDR) (Harper & Elledge, 2007) that senses DNA damage and initiates a cascade of signal transduction pathways consequently culminating in cell cycle arrest, DNA repair and/or apoptosis when the DNA lesions become irreparable. Although cells are equipped with such DNA damage sensing and repair machinery primarily to handle damaged cellular DNA, triggers and receivers of DDR are not necessarily the cells' own genetic materials. DDR can also be provoked by essentially non-damaged DNA exogenously introduced into cells, most commonly viral genetic materials in nature and recombinant DNA (*e.g.*, viral vectors for gene delivery) in laboratory.

During virus-host interaction, viruses manipulate DDR upon infection of cells in a way that benefits their life cycles, while host cells fight against them to eliminate the invaders. DDR is detrimental to viral life cycles in many instances; therefore, DDR is often viewed as an innate antiviral host defense mechanism. For example, adenoviruses express viral proteins that block

¹ Abbreviations: AAV, adeno-associated virus; ATM, Ataxia telangiectasia mutated; ATR, Ataxia telangiectasia and Rad3 related; ATR-IP, ATR-interacting protein; BLM, Bloom syndrome protein; CARE, the cis-acting replication element within the p5 promoter; DDR, DNA damage response; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; ds, double-stranded; DSB, double strand break; HR, homologous recombination; MRN, Mre11/Rad50/NBS1; NHEJ, non-homologous end joining; rAAV, recombinant AAV; RBS, Rep-binding site; RPA, replication protein A; SCID, severe combined immune deficiency; ss, single-stranded; TopBP1, DNA topoisomerase II-binding protein 1; WRN, Warner protein; wtAAV, wild type AAV. The demarcation between wtAAV and rAAV is often not important. If this is the case, AAV without wt or r prefix is used.

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the cellular non-homologous end joining (NHEJ) pathway, which, unless inactivated, concatemerizes viral genomes and prohibits viral genome packaging into virions (Evans & Hearing, 2005; Stracker et al., 2002). Viruses may also take advantage of DDR in their life cycle as seen in retroviruses, which exploit the NHEJ pathway to complete insertion of their genomic materials into host cellular DNA (Daniel et al., 1999; Li et al., 2001). Similar but distinct types of "intervention" by viruses on DDR have been found in many other viruses (Lilley et al., 2007; Weitzman et al., 2004, 2010). Thus, understanding DDR and DNA repair machinery is imperative for elucidating the biology of viruses and viral vectors, and conversely, studying virus biology provides new insights into fundamental biological processes elicited by DNA damage. In this context, interactions between viruses and host DDR and DNA repair machinery have recently gained attention and established a new area of basic research. Importantly, this field of study is relevant to gene therapy research in overcoming its limitations and drawbacks and improving the current molecular therapy approaches.

Adeno-associated virus (AAV) represents a good example for exploring this new research field, which studies the interactions between viruses and DNA repair machinery. AAV has become increasingly popular as a promising gene delivery vehicle. Wild type AAV (wtAAV) is replication defective and recombinant AAV (rAAV) is devoid of virally encoded genes. Despite their replication-defective nature and/or lack of expression of viral proteins, there are significant interactions between virus and host DNA repair machinery, which determine the fates of the virus and the host cells following infection. In this chapter, we provide an overview of how wtAAV and rAAV alter the fate of the host cells through DDR, and how DDR processes the viral genomic DNA by exerting DNA repair machinery to establish the lytic and latent life cycles of wtAAV and transduction of rAAV.

2. Adeno-associated virus (AAV)

Adeno-associated virus (AAV) is a non-enveloped replication-defective animal virus of approximately 20 nm in diameter (Figure 1a). It belongs to Dependovirus, a genus of the family Parvoviridae, which has a viral capsid in the simplest icosahedral shape composed of 60 units of viral structural proteins. Productive AAV replication requires co-infection of a helper virus such as adenoviruses and herpesviruses. A virion has an approximately 5-kb single-stranded DNA genome of either plus or minus polarity at an equal probability. AAV serotype 2 and many other serotypes are prevalent in human populations worldwide and up to 80% of adult humans have been infected with AAV in their childhood (Boutin et al., 2010; Calcedo et al., 2009; Erles et al., 1999). AAV is generally considered as a non-pathogenic virus, and clinical relevance of AAV infection in humans appears to be limited to male infertility (Erles et al., 2001), early miscarriage in pregnant women (Burguete et al., 1999; Pereira et al., 2010) and protection against cervical cancer (Su & Wu, 1996; Walz & Schlehofer, 1992) although some studies have shown negative results (Strickler et al., 1999). The current relevance of AAV in biological and medical research primarily stems from its benefits as a tool for gene delivery and genetic engineering of the cellular genome and as a refined agent for inducing DDR without damaging the cellular genome (Table 1).

2.1 Wild type adeno-associated virus serotype 2 (wtAAV2)

2.1.1 Structural organization of wtAAV2

AAV was first identified as a contaminant in adenovirus stocks in early 1960s (Atchison et al., 1965). Since infectious wild type AAV2 (wtAAV2) clones were generated from

AAV-derived agents and tools	Applications
rAAV of any serotypes	<ul style="list-style-type: none"> • Delivery of exogenous genetic materials to cells with no toxicity • Targeted genetic manipulation of cells (<i>i.e.</i>, precise introduction of insertion, deletion or a small mutation at a defined location in the cellular genome in a predicted manner) • Introduction of DDR without damaging the cellular genome • Identification of DNA breakage sites in the cellular genome
wtAAV2	<ul style="list-style-type: none"> • Introduction of DDR without damaging the cellular genome • Tumor cell-specific killing
Rep68/78	<ul style="list-style-type: none"> • Site-specific insertion of exogenous genetic materials at the AAVS1 site in the human chromosome 19q13.42

Table 1. AAV as biological agents and tools

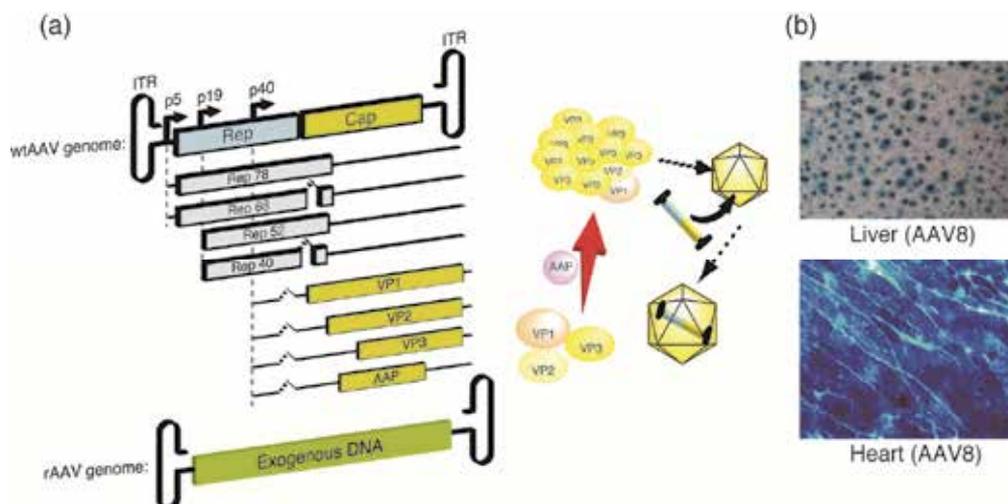


Fig. 1. Wild-type AAV (wtAAV) and recombinant AAV (rAAV). (a) Structural organization of wtAAV and rAAV. wtAAV genome is a single-stranded DNA with ITRs at the both ends. The two viral genes (the *rep* and *cap* genes) encode five non-structural (Rep and AAP) and three structural (VP) proteins, and they are controlled by the three viral promoters (p5, p19, and p40). AAV virion particle consists of VP1, VP2, and VP3. AAP supports assembly of VP proteins. rAAV genome is devoid of the viral components except for the ITRs, and contains an exogenous DNA of interest. (b) Representative photomicrographs of XGal-stained sections of the murine liver and heart transduced AAV8-EF1 α -nls lacZ and AAV8-CMV-lacZ vectors at a high dose (7.2×10^{12} viral particles per mouse), respectively. All the hepatocytes and cardiomyocytes express the lacZ marker gene product, which turns transduced cells blue by the XGal staining.

recombinant plasmids (Samulski et al., 1982), AAV2 has been most extensively studied for viral capsid structure, genome organization, virally encoded protein functions, AAV life cycle and infection pathways (reviewed in Berns & Parrish, 2007; Carter et al., 2009; Smith & Kotin, 2002). WtAAV2 has a single-stranded DNA genome of 4679 nucleotides (nt) in length (GenBank accession no. AF043303) that comprises the region encoding 2 viral genes (*e.g.*, the *rep* and the *cap* genes), their promoters (p5, p19 and p40 promoters), a polyadenylation signal, and two 145-nt inverted terminal repeats (ITR) forming T-shaped DNA hairpins at each viral genome terminus (Figure 1a). WtAAV2 expresses a total of 3 structural proteins (VP1, VP2 and VP3 from the *cap* gene) and 5 non-structural proteins (Rep40, Rep52, Rep68, and Rep78 from the *rep* gene and AAP from an alternative open reading frame from the *cap* gene). VP proteins form the AAV viral capsids while Rep proteins play roles in viral genome replication, packaging and site-specific viral genome integration at the AAVS1 site in the human chromosome 19 (Kotin et al., 1990, 1992). AAP protein (AAP stands for **assembly-activating protein**), which was identified in 2010, plays a role in directing VP proteins to nucleolus, the organelle where new AAV virions assemble (Sonntag et al., 2010).

2.1.2 The viral life cycle of wtAAV2

The life cycle of wtAAV2 consists of the lytic and the latent phases. Following infection through its surface receptors including heparan sulfate proteoglycan (Summerford & Samulski, 1998), wtAAV2 viral particles are carried to the nucleus where single-stranded viral genomes are released from virions into nucleoplasm. When an adenovirus co- or super-infects cells that are infected with wtAAV2, adenovirus helper functions are supplied and wtAAV2 enters the lytic phase where productive viral genome replication takes place. Adenoviral E1a, E1b55k, E4orf6, DNA-binding protein (DPB), and virus-associated RNA I (VAI-RNA) have been identified as the helper functions required for the growth of wtAAV2 and rAAV (Berns & Parrish, 2007; Geoffroy & Salvetti, 2005). In the lytic cycle, there are significant interactions between viral components and host DDR mediated by adenoviral E1 and E4 gene products and AAV large Rep proteins (*i.e.*, Rep68/78). The interactions are primarily aimed at blocking the cell cycle and suppressing the NHEJ DNA repair pathway (more details are described in 3.2.).

In the absence of helper virus co-infection, most of the viral genomes are lost during cell division in dividing cells because they do not replicate or segregate together with the cellular genome into daughter cells. However, a certain proportion of AAV genomes establishes a latent phase by integration into the cellular genome, particularly at the AAVS1 site located within the human chromosome 19q13.42 (Kotin et al., 1992; Samulski et al., 1991). The AAVS1 site has a 33-nt DNA sequence within the myosin binding subunit (MBS) 85 gene and this short DNA sequence serves as the target for the site-specific integration (Linden et al., 1996b; Tan et al., 2001). The site-specific integration process requires expression of Rep68/78, which binds to a GCTC repeat element termed Rep binding site (RBS) and creates a nick at a nearby 3'-CCGGT/TG-5', designated terminal resolution site (*trs*). A set of these two recognition sequences is located within the AAV-ITR and the AAVS1 site (Brister & Muzyczka, 1999; McCarty et al., 1994). Several cellular factors have been shown to modulate Rep68/78-mediated site-specific integration (Figure 2b), although the experimental observations appear to be in conflict in some aspects. High mobility group protein 1 (HMG1) binds to Rep78,

enhances its RBS binding and nicking activities, and promotes site-specific integration at the AAVS1 site (Costello et al., 1997). In addition, the human immunodeficiency virus type 1 (HIV-1) TAR RNA binding protein 185 (TRP-185) binds to the RBS within the AAVS1 site, interact with Rep68, enhances Rep68's helicase activity, and controls selection of AAV genome integration sites within the AAVS1 locus (Figure 2b) (Yamamoto et al., 2007). This mode of latency is unique to wtAAV2 among the animal viruses, and is the case at least in cultured cells. In latently-infected human tissues, a majority of wtAAV2 genomes persist as circular genomes and no site-specific integration has been demonstrated even by sensitive PCR-based assays (Schnepp et al., 2005); therefore, the significance of the site-specific integration of wtAAV2 in natural infection in humans remains elusive.

2.1.3 wtAAV2 viral components that evoke DDR

Among the viral components, large Rep proteins, the cis-acting replication element (CARE) within the p5 promoter (Fragkos et al., 2008; Francois et al., 2005; Nony et al., 2001; Tullis & Shenk, 2000), AAV-ITR, and the unusual single-stranded nature of the viral genome are particularly important in AAV-evoked DDR and interaction with DNA repair machinery. These elements could potentially activate DDR without AAV viral genome replication.

2.2 Recombinant AAV (rAAV) vectors

Recombinant AAV (rAAV) vectors are genetically-engineered viral agents that carry heterologous DNA to be delivered to target cells and are devoid of all the viral genome sequence except for the 145-nt (ITR) at each genome terminus. Until early 2000s, rAAV vectors were primarily derived from AAV2 due to the limited availability of alternative serotypes at that time. rAAV2 vectors have a broad host range and outstanding ability to deliver genes of interest to both dividing and non-dividing cells of various types *in vitro*. However, rAAV2 exhibits limited transduction efficiency in tissues and organs *in vivo* when administered in experimental animals. This drawback of rAAV2 has recently been overcome by the discovery of new serotypes exemplified by AAV serotypes 8 and 9 (AAV8 and AAV9) (Gao et al., 2002, 2004). rAAV vectors derived from serotypes alternative to AAV2 have become widely available at present and been shown to exhibit unprecedented robust transduction in various tissues and organs by intravascular injection of the vector (Figure 1b) (Foust et al., 2009; Ghosh et al., 2007; Inagaki et al., 2006; Nakai et al., 2005a; Sarkar et al., 2006; Vandendriessche et al., 2007; Wang et al., 2005). It should be noted that alternative serotype rAAV vectors are in general those containing a rAAV2 viral genome encapsidated with an alternative serotype viral coat (*i.e.*, pseudoserotyped rAAV2 vectors) (Rabinowitz et al., 2002). Therefore, they are often referred to AAV2/8 and AAV2/9 (genotype/serotype) when rAAV2 genome is contained in AAV8 and AAV9 viral coats, respectively. In addition to the exploitation of AAV capsids derived from various serotypes and variants present in nature, recent advances in genetic engineering of viral capsids aiming for the creation of specific cell type/tissue-targeting vectors have significantly broadened the utility of this vector system (Asokan et al., 2010; Excoffon et al., 2009; Koerber et al., 2009; Yang et al., 2009). Double-stranded (ds) rAAV vectors and gene targeting rAAV vectors are also worthy of note. Ds rAAV contains a ds viral genome in place of a single-stranded (ss) DNA

(McCarty, 2008). Because ds rAAV vectors overcome the rate-limiting step in transduction, *i.e.*, conversion from ss to ds DNA in infected cells, they exhibit a 1-2 log higher transduction efficiency than that achievable with the conventional ss vectors (McCarty et al., 2003; Wang et al., 2003). Gene-targeting rAAV vectors have the ability to introduce genomic alterations precisely and site-specifically at high frequencies of up to 1% (Russell & Hirata, 1998; Vasileva & Jessberger, 2005), which is several logs higher than that achievable by the conventional homologous recombination (HR) approaches (Thomas & Capecchi, 1987) (please refer to 3.5 for more details). Nonetheless, even if rAAV vectors are devoid of several key components that trigger DDR (*i.e.*, large Rep expression and the CARE), establishment of rAAV transduction heavily relies on the interactions between rAAV viral genomes and DNA repair machinery irrespective of serotypes or nature of viral genomes (*i.e.*, ss rAAV or ds rAAV).

3. AAV and DNA repair pathways

An overview of AAV and DNA repair pathways is summarized in Figure 2.

3.1 AAV-evoked DDR

3.1.1 Earlier evidence for the role of DDR in the AAV genome processing

Although the interplay between virus and DDR is a relatively new area of research, earlier studies indicated potential roles of DDR in the AAV life cycle or viral genome processing. The first indicative evidence came from the observation that cells treated with a wide variety of genotoxic agents including UV irradiation and carcinogens such as hydroxyurea could support wtAAV2 genome replication in the absence of helper virus co-infection (Yakinoglu et al., 1988; Jakobson et al., 1987, 1989). Subsequently, such treatment was found to augment rAAV2 transduction efficiency in both dividing and non-dividing cells with the latter showing a more dramatic enhancing effect (Alexander et al., 1994; Russell et al., 1995). These earlier observations suggested that activated DNA repair pathways following DNA damage induced by genotoxic treatment somehow facilitated the conversion of rAAV genomes from ss to ds DNA by second-strand synthesis (Figure 2f, g and h) (Ferrari et al., 1996; Fisher et al., 1996). As mentioned earlier, the formation of ds AAV genomes is a critical step for wtAAV to initiate productive infection and for rAAV to undergo abortive infection and express transgene products. A better response to the treatment in non-dividing cells conforms to the idea that DNA repair pathways are constitutively activated to a greater extent in dividing cells. Such activation is required to repair DNA replication errors that occur naturally and unavoidably. Although the underlying mechanism of this effect still remains elusive, one can speculate that up-regulation of DNA repair pathways increases the pool of cellular factors required for AAV genome processing. Alternatively, factors inhibitory for wtAAV genome replication or rAAV transduction may become sequestered from AAV genomes to multiple DNA repair foci formed on the damaged cellular genome (Figure 2f and g). A recent observation that the MRN complex and ATM, the major DDR proteins, have an inhibitory effect on rAAV transduction supports the latter model (Cataldi & McCarty 2010; Cervelli et al., 2008; Choi et al., 2006; Sanlioglu et al., 2000; Schwartz et al., 2007).

3.1.2 DNA repair proteins associated with AAV genomes in cells

AAV is composed of only two elements, VP proteins that form viral capsids and a single-stranded viral genomic DNA. VP proteins primarily determine biological properties of various AAV serotypes; *i.e.*, how AAV particles reach cells, enter cells, traffic in cytoplasm and nucleoplasm, and uncoat virion shells to release viral genomes. At present there is no evidence indicating that the above-mentioned AAV infection pathways driven by the capsid trigger DDR. AAV-evoked DDR is all about the cellular responses against AAV viral genomes except for wtAAV2, which expresses Rep68/78 proteins that also trigger DDR. It is plausible that the structure of single-stranded DNA with T-shaped hairpin termini, which is unusual and is not present in the cellular genome, is recognized as damaged DNA and triggers DDR. Direct evidence for the association of AAV genomes with DNA repair machinery has obtained in chromatin immunoprecipitation (ChIP) studies where AAV genomes and their associated cellular factors were crosslinked by formalin and precipitated together using antibodies specific to DNA repair proteins. To date, the MRN complex, Ku86, Rad52, RPA and DNA polymerase delta have been identified as factors bound to ss AAV genomes (Cervelli et al., 2008; Jurvansuu et al., 2005; Zentilin et al., 2001). In addition, immunofluorescence microscopy has revealed that the MRN complex, ATR, TopBP1, BLM, Brca1, Rad17, RPA, and Rad51 are recruited to the discrete nuclear foci where AAV genomes accumulate (Cervelli et al., 2008; Jurvansuu et al., 2005). Table 2 summarizes the roles of DNA repair proteins in AAV infection/transduction, AAV genome self-circularization, and AAV genome integration into the host genome.

3.1.3 AAV genome activates ATR-mediated DDR

Although not exclusive, the DNA repair proteins found to be associated with AAV genomes described in 3.1.2 are those involved in the ATR-mediated DDR that is triggered by stalled replication forks (Figure 2d and e) (reviewed in Branzei & Foiani, 2010 and Shiotani & Zou, 2009). At stalled replication forks, ss DNA regions become coated with RPA. RPA then recruits ATR-ATRIP and the Rad17 complex to the damaged site. The Rad17 complex subsequently recruits the ring-shaped trimeric Rad9/Rad1/Hus1 (9-1-1) complex, and finally ATR-ATRIP kinase becomes activated by TopBP1 recruited to the site and sends a DNA damage checkpoint signal (Figure 2d and e). In addition, Mre11 has also been reported to relocate to stalled replication forks to a limited extent (Mirzoeva & Petrini, 2003). AAV-ITR exhibits a close structural similarity to stalled replication forks in that it contains both ss DNA regions and ss DNA-ds DNA junctions. This strongly supports a model in which AAV-ITR is recognized as a stalled replication fork and triggers the checkpoint response via ATR kinase. The actual activation of the ATR pathway by AAV genomes has been confirmed by the demonstration that the ATR-downstream effector proteins; *i.e.*, Chk1 and RPA, become phosphorylated in cells infected with wtAAV2 or UV-irradiated wtAAV2, both of which are devoid of the ability to replicate or express viral genes in the system used for the experiment (Fragkos et al., 2008; Ingemarsdotter et al., 2010; Jurvansuu et al., 2005, 2007). Interestingly, rAAV2 genome devoid of the 55-nt CARE within the p5 promoter does not evoke the ATR-mediated checkpoint signal, and it has been shown that co-existence of both ITR and CARE in an AAV genome is essential for the activation (Fragkos et al., 2008). The consequence of the AAV genome-evoked ATR-mediated DDR is G2/M cell cycle arrest in wild type cells, while it leads to cell death in p53-deficient cells (Ingemarsdotter et al., 2010; Jurvansuu et al., 2007) (please see 3.1.4. for more details). Cell cycle arrest in the late S and/or G2 phases following infection of wtAAV2 or UV-irradiated wtAAV2 was observed

Protein	Effects of deficiency ²						Note	References ³
	In vitro			In vivo				
	T	C	I	T	C	I		
Artemis				↓	↓			1
ATM	↑	↓/↑	↑		↓			2, 3, 4, 5
ATR	↑/→	→	→				wtAAV2-evoked signal ↑	5
BLM		↓						4
Chk1							wtAAV2-evoked signal ↑	14, 15
DNA-PKcs	↓	↓	↓/↑	↓	↓	↑/→	rAAV2 genome replication ↓ when deficient wtAAV2 genome replication ↑ when deficient	1, 4, 5, 6, 7, 8, 15
Ku70/80(86)	↑	→					wtAAV2 genome replication ↓ when deficient Targeting efficiency ↑ when deficient	3, 4
ligase IV		→	↓					4, 8
MDC1	↑							9
MRN	↑	↓			→			9, 4, 10
Rad52	↓			→	→			3, 16
Rad54B							Targeting efficiency ↓ when deficient	11
Rad54L							Targeting efficiency ↓ when deficient	11
WRN		↓						4
XRCC3		→					Targeting efficiency ↓ when deficient	4, 11
pRb							Induction of cell death when deficient	12, 13
p21							Induction of cell death when deficient	12, 13
p53							Induction of cell death when deficient	12, 13

Table 2. DNA repair and AAV

in an earlier study although how and what DDR is involved was not known at that time (Winocour et al., 1988).

3.1.4 AAV genome-evoked DDR leading to cell death

A unique aspect of AAV-evoked DDR is the ability to induce cell death without productive viral genome amplification, viral gene expression or cellular DNA damage, when cells are devoid of p53 expression. In 2001, Raj et al. reported an unexpected experimental observation that wtAAV2 infection of an osteosarcoma cell line that lacks expression of functional p53 leads to cell death through apoptosis or mitotic catastrophe, whereas the wild type control cells merely undergo a transient cell cycle arrest in the G2 phase (Raj et al., 2001). Mitotic catastrophe is an ill-defined term describing an apoptosis-like cell death during mitosis that takes place even in the presence of unrepaired DNA damage (Castedo et al., 2004; Vakifahmetoglu et al., 2008). This p53 deficiency-dependent cell killing effect was

² Observed effects caused by deficiency or knockdown are summarized. T, transduction efficiency; C, AAV genome self- circularization efficiency; I, AAV genome integration efficiency. In the table, arrows indicate an increase (↑), a decrease (↓) or no change (→).

³ References cited are as follows: 1, Inagaki et al., 2007b; 2, Sanlioglu et al., 2000; 3, Zentilin et al., 2001; 4, Choi et al., 2006; 5, Cataldi et al., 2010; 6, Song et al., 2006; 7, Choi et al., 2010; 8, Daya et al., 2009; 9, Cervelli et al., 2008; 10, Schwartz et al., 2007; 11, Vasileva et al., 2006; 12, Garner et al., 2007; 13, Raj et al., 2001; 14, Schwartz et al., 2009; 15, Collaco et al., 2009; 16, Nakai, unpublished.

also observed when cells were infected with UV-irradiated wtAAV2 or microinjected with a 145-nt AAV2-ITR oligonucleotide, demonstrating that the unusual structure of the AAV2-ITR sequence itself is the culprit (Raj et al., 2001). Initially it was presumed that infection of wtAAV2 activates the ATM-p53-mediated DDR, which in turn increases and decreases the levels of p21 and CDC25C, respectively, resulting in the G2 arrest (Raj et al., 2001). Although the mechanism of p53 deficiency-dependent cell killing by AAV genomes still remains elusive, a series of subsequent studies on this phenomenon has revealed at least three potentially independent AAV-evoked pathways leading to cell death: the pathways involving (1) p53-p21-pRb, (2) p84N5 via caspase 6, and (3) ATR-Chk1. In the first mechanism, AAV-evoked DDR signal is transduced to a potent antiapoptotic proteins, pRb, via p53 and p21. Therefore, cells defective in this pathway fail to transduce the DDR signal to pRb, leading to apoptosis (Garner et al., 2007). In the second mechanism, functional defect of the p53-p21-pRb pathway allows activation of the nuclear domain protein p84N5, which otherwise is inhibited by association with pRb (Doostzadeh-Cizeron et al., 1999). The activated p84N5 then induces apoptosis via caspase-6 (Garner et al., 2007). In the third mechanism, AAV genomes activate ATR, which in turn phosphorylates Chk1, causing a transient cell cycle arrest in the G2 phase. In the absence of p53, cells fail to sustain the G2 arrest following degradation of the unstable Chk1, progress suicidally into mitosis, and die via mitotic catastrophe associated with centriole overduplication and the subsequent formation of multipolar mitotic spindles (Ingemarsdotter et al., 2010; Jurvansuu et al., 2007). Whether all of the pathways or only some of them are triggered by AAV genomes remains unknown at present.

3.1.5 AAV2 Rep68/78-evoked DDR

In addition to AAV genome as a trigger of DDR, AAV2 large Rep proteins (*i.e.*, Rep68/78) themselves also evoke DDR independent of AAV genome. In the lytic phase of the AAV life cycle where cells are co-infected with a helper virus, Rep proteins are strongly expressed and exert many functions in the network of cellular proteins and viral factors derived from adenovirus or other helpers. Rep proteins can also be expressed without helper virus infection but only to a limited extent due in part to the large Repls' ability to negatively regulate their own promoter (p5) and the promoter for the small Rep proteins (p19) (Beaton et al., 1989; Kyostio et al., 1994). The significance of Rep68/78 expression in the absence of helper viruses reside in a series of the AAVS1-targeting approaches that exploit wtAAV2's ability to introduce exogenously derived DNA into the AAVS1 site in a site-specific manner (Figure 2c) (Henckaerts & Linden, 2010; Linden et al., 1996a). In these approaches, a donor vector in any context (*e.g.*, plasmid DNA, adenoviral vectors or rAAV) containing a gene of interest and RBS is delivered to human cells where AAV2 large Rep expression is supplied by the same vector or a separate one. The AAV2-ITR sequence is commonly used as an RBS-containing cis element; however, the p5 promoter also serves as an alternative (Philpott et al., 2002).

It has been known that Rep68/78 shows significant cellular toxicity due to the strong antiproliferative action of the protein (Yang et al., 1994). Rep78 completely blocks the cell cycle in the S phase (Saudan et al., 2000). Studies have shown that Rep78 exerts two independent but complementing DDR-associated cellular signal transduction pathways to arrest the cell cycle. The two pathways are the pRb pathway and the ATM-Chk2 pathway (Berthet et al., 2005). In the first pRb pathway, Rep78 expression leads to an increased level of the cyclin-dependent kinase inhibitor p21 and accumulation of hypophosphorylated pRb,

the active form of pRb protein (Berthet et al., 2005; Saudan et al., 2000) (Figure 2c). Consequently, cellular proteins that control cell cycle progression such as cyclin A, cyclin B1, and Cdc2, are down-regulated, resulting in slowing down the cell cycle (Saudan et al., 2000). Supporting this model, this effect is substantially attenuated in pRb-deficient mouse embryonic fibroblasts (Saudan et al., 2000). An increased amount of p21 might explain the inability to phosphorylate pRb upon large Rep expression, but the transcriptional activation of p21 has been shown to occur via a p53-independent pathway (Hermanns et al., 1997). In the second ATM-Chk2 pathway, the DNA-nicking activity of Rep68/78 creates multiple damaged sites in the cellular genome, which activates the ATM-Chk2 pathway and arrests the cell cycle (Figure 2c) (Berthet et al., 2005). Large Rep proteins create a break in only one strand of two-stranded DNA, which is not a type of damage that usually activates ATM. It is currently unknown how Rep68/78-induced DNA damage triggers this pathway. Worthy to note, activation of either one of the above-mentioned two pathways by itself is not sufficient for the complete block of the cell cycle, which is attainable by Rep68/78 expression (Berthet et al., 2005). It appears that there would be many other Rep68/78-associated DNA repair pathways that have yet to be identified. This is because a recent study using a tandem affinity purification (TAP) approach has demonstrated physical interaction of Rep78 with many DNA repair-associated proteins including DNA-dependent protein kinase catalytic subunit (DNA-PKcs), minichromosome maintenance (MCM) proteins, Ku70/80, proliferating cell nuclear antigen (PCNA), RPA, and structural maintenance of chromosome 2 (SMC2) (Nash et al., 2009).

3.2 wtAAV2 genome replication and DNA repair pathways

In the lytic phase of the wtAAV2 life cycle, viral genome replication requires co-infection of a helper virus. Since human adenoviruses have been most extensively studied in the context of AAV virology, this section specifically focuses on the interplay between wtAAV2, human adenoviruses, and DNA repair machinery. The adenoviral components required in the lytic infection are E1a, E1b55k, E4orf6, DBP, and VAI. A series of adenovirus/AAV co-infection studies has provided significant insights into how DDR and DNA repair machinery play roles in AAV genome replication in the presence of adenovirus helper functions (Collaco et al., 2009; Schwartz et al., 2009). It has been shown that adenoviral E1b55k/E4orf6 degrades the MRN complex via the ubiquitin-proteasome pathway and E4orf3 mislocalizes the MRN complex to aggresome, abrogating the MRN function in triggering the ATM and ATR pathways (Figure 2a) (Collaco et al., 2009). In addition, E4orf6 dissociates ligase IV from ligase IV/XRCC complex and degrades it (Jayaram et al., 2008). The main consequence of this adenoviral manipulation of DDR is inhibition on NHEJ, which prevents concatemeric adenoviral genome formation and promotes adenoviral genome replication and packaging. Although it remains elusive how beneficial the inhibition of NHEJ is in the AAV lytic life cycle, the significance of E1b/E4-mediated degradation of MRN complex in the wtAAV2 lytic cycle has been revealed by the observation that the MRN complex binds to AAV-ITR and inhibits wtAAV2 genome replication and rAAV vector transduction (Cervelli et al., 2008; Schwartz et al., 2007). Along the same line, the observation that cells deficient in ATM exhibit a higher rAAV transduction efficiency (Sanlioglu et al., 2000) might be explainable on the assumption that lack of ATM would be an equivalent to inactivation of the MRN complex because the MRN complex serves as a damage sensor that activates the ATM pathway (Carson et al., 2003). It is tempting to propose that dislocation of the MRN complex and other inhibitory factors from AAV genomes to the sites in the cellular genome where

genome integrity is more severely threatened, is the mechanism for the augmentation of wtAAV2 genome replication and rAAV vector transduction by genotoxic treatment (Figure 2g). Suppression of the NHEJ pathway that involves DNA-PKcs and Ku proteins, however, may or may not be beneficial, because one study has shown that deficiency of these proteins both resulted in impaired rAAV2 genome replication (Choi et al., 2010) whereas another study has reported that siRNA-mediated knockdown of DNA-PKcs enhanced wtAAV2 genome replication (Collaco et al., 2009).

In addition to the adenovirus-evoked DDR, productive wtAAV2 viral genome replication triggers DDR distinct from that observed in adenovirus only infection (Collaco et al., 2009; Schwartz et al., 2009). Adenovirus-wtAAV2 co-infection results in much more pronounced activation of ATM and the checkpoint kinases, Chk1 and Chk2. This activation occurs independently of the MRN complex; therefore, the activation sustains even if MRN complex starts being degraded by adenoviral E1b/E4 proteins (Collaco et al., 2009). Other DDR substrate proteins RPA, NBS1 and H2AX become phosphorylated as the lytic phase progresses (Collaco et al., 2009; Schwartz et al., 2009). It has been shown that AAV genome replication is essential and sufficient to induce the DDR signal transduction cascade observed in the adenovirus co-infection, and Rep proteins does not play a role in the activation of DDR (Collaco et al., 2009; Schwartz et al., 2009). Among the three phosphatidylinositol 3-kinase-like kinases (PIKKs) that initiate signal transduction (*i.e.*, ATM, ATR and DNA-PKcs), ATM and DNA-PKcs are the primary kinases that phosphorylate downstream DDR substrates, and ATR appears to play only a minor role in the lytic phase of the AAV life cycle (Collaco et al., 2009; Schwartz et al., 2009). Although the significance of the DDR in the AAV lytic cycle remains unclear, the activation of the ATM pathway appears to be beneficial for AAV genome replication (Collaco et al., 2009).

3.3 rAAV genome recombination and DNA repair pathways

3.3.1 rAAV genome processing is mediated solely by DNA repair machinery

After entering nuclei, rAAV virion shells break down, releasing single-stranded (ss) vector genomes into nucleoplasm, which subsequently convert to various forms of double-stranded (ds) genomes (Deyle & Russell, 2009; Schultz & Chamberlain, 2008). It should be noted that rAAV does not express any viral gene products that can process viral genomes such as recombinases and integrases; therefore the processing of viral genomes must heavily depend on DNA repair machinery. In addition, unlike the battle between adenovirus and the host DNA repair systems as described in 3.2, rAAV has no means to manipulate DNA repair pathways once viral genomes evoke DDR. Unless rAAV genomes have been processed to completion into stable ds DNA with no free ends, DDR would remain activated due to the continued presence of viral DNA in an unusual structure presenting a single strand with free ends. In mammalian cells, extrachromosomal free DNA ends at ds rAAV genome termini as well as those in ds linear plasmid DNA, when exogenously delivered, appear to be removed primarily by ligating two free ends and making a single continuous ds DNA strand via NHEJ and/or occasionally HR rather than by DNA degradation (Nakai et al., 2003b; Nakai, unpublished observation). In this sense, the rAAV genomes processed into various forms in their latency could be viewed as byproducts that have been created and disposed of by a cellular defense mechanism against potentially toxic exogenous agents.

3.3.2 Single-to-double-stranded rAAV genome conversion and DNA repair machinery

How ss rAAV genomes become ds DNA is not completely understood but the process involves the following two mechanisms; second-strand synthesis (Ferrari et al., 1996; Fisher et al., 1996; Zhong et al., 2008; Zhou et al., 2008) and annealing of plus and minus strands (Hauck et al., 2004; Nakai et al., 2000). It has been shown that, upon rAAV infection, the MRN complex becomes activated, physically associates with AAV2-ITR and inhibits wtAAV2 replication and rAAV transduction (Figure 2a and h) (Cervelli et al., 2008; Schwartz et al., 2007); therefore, MRN appears to have some role in the conversion of ss to ds DNA. ATM has also been suggested to be a cellular factor that inhibits the single-to-double-stranded genome conversion because transduction efficiency with ss rAAV is significantly enhanced in ATM-deficient cells *in vitro* (Figure 2h) (Sanlioglu et al., 2000). However, a recent study has proposed an ATM-mediated gene silencing model rather than the mechanism involving the second-strand synthesis to explain the ATM's inhibitory effect. This model stems from the observation that, in the absence of ATM, ds rAAV transduction was enhanced as well, indicating that an alternative mechanism other than second-strand synthesis is involved (Cataldi & McCarty, 2010). Another factor that is known to inhibit this process is tyrosine-phosphorylated FKBP52, which binds to AAV-ITR and inhibits second-strand synthesis (Qing et al., 2001). Its dephosphorylation by T-cell protein tyrosine phosphatase (TC-PTP) dissociates FKBP52 from AAV-ITR, allowing the formation of ds genomes (Qing et al., 2003). *In vitro* AAV replication studies have identified the DNA polymerase that catalyzes second-strand synthesis as DNA polymerase δ (Nash et al., 2007), which is a polymerase that fills a single-stranded DNA gap created during the nuclear excision repair (Torres-Ramos et al., 1997). Physical association of DNA polymerase δ and AAV genome has also been demonstrated (Jurvansuu et al., 2005). At present it remains elusive whether and how the above-mentioned signal kinases (*i.e.*, MRN and ATM) and effectors (FKBP52 and DNA polymerase δ) are linked in the rAAV-evoked DDR.

3.3.3 Extrachromosomal rAAV genome recombination and DNA repair machinery

In addition to the above-mentioned single-to-double-stranded genome conversion, rAAV genomes are further processed into the following stable ds forms by intra- or intermolecular DNA recombination mediated solely by DNA repair machinery, and establish the latent infection. The viral genome forms in the latent phase include ds circular monomers, large concatemers (circular and/or linear), and rAAV proviral genomes that are stably integrated into the host cellular genome at low frequencies (Deyle & Russell, 2009; Schultz & Chamberlain, 2008). It has not been determined when the rAAV genome recombination takes place, which may be either before, at, or after completion of the single-to-double-stranded genome conversion. In dividing cells, extrachromosomal genomes are lost because they do not replicate episomally, whereas they can be stabilized and maintained as chromatin in quiescent cells in animal tissues (Penaud-Budloo et al., 2008). Earlier studies indicated that the formation of large concatemeric rAAV genomes is important for transgene expression; however, accumulated observations might favor a model in which extrachromosomal circular monomer genomes, not large concatemers or integrated forms, are primarily responsible for persistent and stable transgene expression in rAAV-transduced animal tissues (Nakai et al., 2001; Nakai et al., 2002; Nathwani et al., 2011).

In extrachromosomal rAAV genome recombination, AAV-ITR plays a pivotal role in mediating recombination. Although it has yet to be elucidated how DDR is evoked by rAAV

genomes in the context of rAAV genome recombination, it is not unreasonable to speculate that the T-shaped hairpin structure within the AAV-ITR and/or ss DNA-ds DNA junctions in the stem of the hairpin DNA trigger DDR. A set of DNA repair proteins, which includes DNA-PKcs, Artemis, ATM, MRN, BLM, and WRN (Figure 2h), has been found to be involved in rAAV genome recombination (Cataldi & McCarty, 2010; Choi et al., 2006; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Sanlioglu et al., 2000; Song et al., 2001). Deficiency of these proteins impairs intramolecular recombination of ss rAAV and/or ds rAAV genomes via the AAV-ITR sequence. DNA-PKcs and Artemis are the two major components in the classical NHEJ pathway of DSB repair. Artemis, when activated by DNA-PKcs, possesses an endonuclease activity and resolves DNA hairpin loops and flaps formed at broken DNA ends to facilitate ds DNA end joining (Ma et al., 2005). BLM and WRN are members of the RecQ family of DNA helicases. They unwind ds DNA to ensure the formation of proper recombination intermediates, and mediate a various types of DNA transactions, mainly HR (Bernstein et al., 2010). MRN is a multifaceted protein complex that functions as a primary sensor of DSB, binds DNA lesion, recruits ATM, and processes DNA ends by utilizing the Mre11 endo- and exo-nuclease activity that creates recombinogenic 3' single-stranded tails (Williams et al., 2010). The initial study of the structure of ITR-ITR junction sequences revealed that the majority of the recombination junctions in ds circular monomer genomes exhibited a 165-nt double-D ITR structure, the hallmark of HR (Duan et al., 1999; Xiao et al., 1997). This indicates that HR is the major pathway for intra- and inter-molecular genome recombination events. Supporting this view, Rad52, which is a key player in HR, was identified as a protein that binds to rAAV genomes in cultured cells (Zentilin et al., 2001). Interestingly, deficiency of Rad52 does not affect rAAV transduction efficiency or genome processing in murine liver (Nakai, unpublished observation). It remains possible that HR plays a major role in rAAV genome recombination at least under certain cellular environment; however, accumulated observations by us and others rather support a model in which NHEJ is the major pathway for extrachromosomal rAAV genome recombination. In the absence of DNA-PKcs or Artemis, intramolecular recombination is significantly impaired in cultured cells and animal tissues (Cataldi & McCarty, 2010; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Song et al., 2001), and the footprints on junction DNA are quite consistent with NHEJ-mediated recombination, showing nucleotide deletions of various degrees with occasional microhomology at junctions (Inagaki et al., 2007b). Interestingly, intra- and inter-molecular recombination events that form ds circular monomers and ds concatemers, respectively, are differentially regulated by different DNA repair pathways (Figure 2h). Intramolecular recombination heavily depends on the Artemis/DNA-PKcs-dependent NHEJ pathway, while the NHEJ pathways that mediate intermolecular recombination are redundant because intermolecular recombination occurs efficiently in the absence of DNA-PKcs or Artemis (Inagaki et al., 2007b). The DNA-PKcs or Artemis-independent NHEJ might be those involving ATM and/or MRN (Cataldi & McCarty, 2010; Choi et al., 2006; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Sanlioglu et al., 2000; Song et al., 2001). Alternatively, HR might be the major pathway for intermolecular rAAV recombination. This model stems from the observation that recombination between two homologous AAV-ITRs derived from the same serotype is preferred to that between two non-homologous AAV-ITRs derived from different serotypes (Yan et al., 2007). The ATR pathway does not appear to be involved in extrachromosomal rAAV genome recombination (Cataldi & McCarty, 2010).

How DNA-PKcs and Artemis process rAAV genome termini and mediate recombination has been extensively studied in the context of murine tissues. In DNA-PKcs or Artemis-deficient SCID mice, ds linear rAAV genomes with covalently closed hairpin caps at genome termini accumulate in rAAV-transduced tissues (Figure 3b). In SCID mouse thymi, V(D)J recombination is impaired resulting in accumulation of covalently-sealed hairpin intermediates at V(D)J coding ends in the T cell receptor gene (Rooney et al., 2002; Roth et al., 1992) (Figure 3a). These two phenomena are essentially the same in that if hairpin structures at DNA ends are not cleaved by the Artemis/DNA-PKcs endonuclease activity, covalently closed DNA ends accumulate without undergoing further recombination. Therefore, intramolecular recombination most likely uses the same Artemis/DNA-PKcs-dependent NHEJ pathway used for V(D)J recombination. It is not easy to determine GC-rich AAV-ITR hairpin DNA structures at sequencing levels; however this shortcoming has been overcome by exploiting the bisulfite PCR technique. Utilizing this method, the primary cleavage site by the Artemis/DNA-PKcs endonuclease activity has been mapped to the 5' end of the 3-base AAA loop at the AAV-ITR hairpin tips (Figure 3b) (Inagaki et al., 2007b). In DNA-PKcs-deficient SCID mouse tissues, the relative proportion of rAAV genome recombination junctions exhibiting the hallmark of HR increases, indicating compensatory activation of HR in the absence of DNA-PKcs in quiescent cells in animal tissues (Nakai, unpublished observation). In this regard, worthy of note are the following observations made by us and others that DNA repair pathways might somehow be linked to epigenetic modifications of rAAV genomes. We have found that the cytomegalovirus (CMV) immediately early gene promoter in rAAV genome can be significantly silenced in Artemis- or DNA-PKcs-deficient mouse muscle (Nakai, unpublished observation). Recently, Cataldi et al. reported that the CMV promoter is somewhat silenced in ATM-proficient murine fibroblasts compared to that in ATM-deficient cells (Cataldi & McCarty, 2010). These observations imply that rAAV genome recombination via NHEJ generates more functionally active genomes than HR presumably due to a difference in epigenetic modifications of rAAV genomes (Cataldi & McCarty, 2010).

3.4 rAAV genome integration and DNA repair pathways

rAAV is devoid of Rep68/78 expression; therefore, it lacks the ability to integrate into the cellular genome site specifically. In addition, rAAV does not harness machinery designed specifically for integration into the cellular genome. rAAV vectors are generally considered as episomal vectors, but they do integrate into the cellular genome of both dividing and non-dividing cells at low frequencies (Deyle & Russell, 2009; McCarty et al., 2004). This process is entirely dependent on the host cellular DNA repair machinery. Although it is not easy to determine the frequency of rAAV genome integration in each case and it may vary depending on the amount of rAAV genomes delivered to cells, integration has been reported to occur at approximately ~0.1% of total input rAAV genomes (Russell et al., 1994) or up to ~4% of cell population in rAAV-infected cultured cells (Cataldi & McCarty, 2010), or at approximately 0.1% of rAAV-transduced hepatocytes when rAAV is injected into newborn mice (Inagaki et al., 2008). rAAV genome integration occurs at nonrandom sites in both cultured cells and somatic cells in animals. The preferred genomic sites for integration include the 45s pre-ribosomal RNA gene, transcriptionally active genes, DNA palindromes, CpG islands, and the neighborhood of transcription start sites (Inagaki et al., 2007a; Miller et al., 2005; Nakai et al., 2003a). Although the mechanism of integration remains largely unknown, it has been presumed that input rAAV genomes are fortuitously captured at pre-

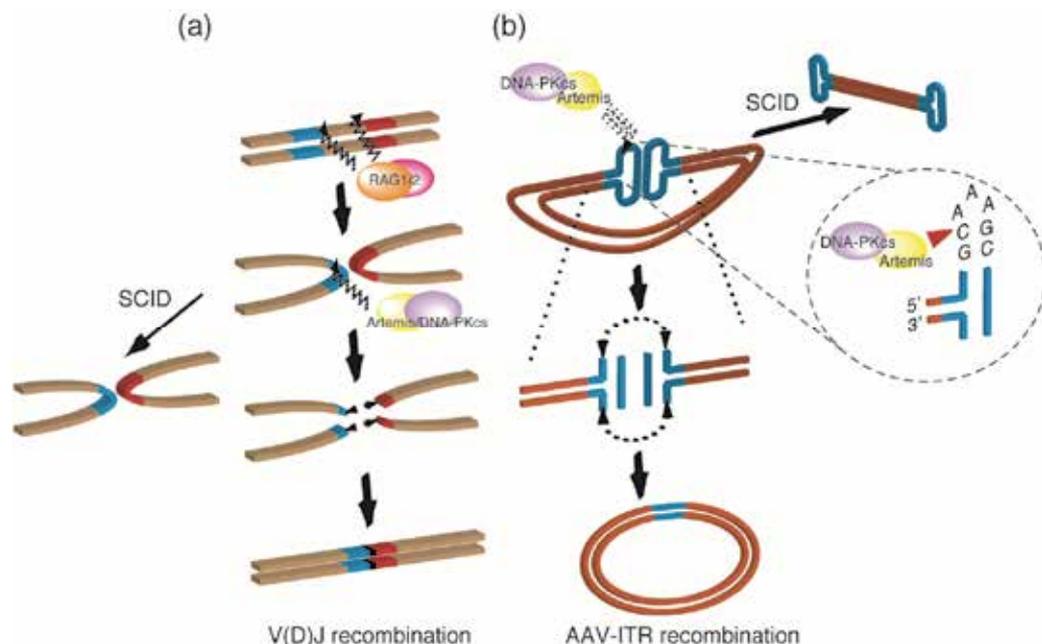


Fig. 3. A similarity of Artemis / DNA-PKcs-mediated hairpin cleavage in V(D)J recombination and AAV-ITR recombination. (a) During V(D)J recombination, the recombination activating gene products, Rag1 and Rag2 endonucleases, cleave the immunoglobulin and T cell receptor genes, forming covalently closed hairpin loops at cleaved DNA ends. Artemis/DNA-PKcs complex resolves the hairpin loops, which triggers the subsequent recombination between the two coding ends via the classical NHEJ DNA repair pathway. In cells deficient in either Artemis or DNA-PKcs (SCID phenotype), hairpin coding ends remain unrecombined and accumulate. (b) The same Artemis/DNA-PKcs-dependent NHEJ pathway mediates intramolecular AAV-ITR recombination, forming circular monomer genomes. Intermolecular AAV-ITR recombination occurs independently of Artemis/DNA-PKcs. A red arrowhead indicates the primary target for Artemis/DNA-PKcs-mediated cleavage. In SCID mouse tissues, ds linear rAAV genomes with covalently closed AAV-ITR hairpin caps accumulate.

existing breaks in the cellular genome when the DNA breaks are repaired by DNA repair machinery, which establishes rAAV integration. This model has been supported by the observations that rAAV genome integrations are frequently found at I-SceI-induced DSBs in the cellular genome (Miller et al., 2004) and genotoxic treatments can increase integration rates (Russell et al., 1995). Clinically, rAAV vectors are generally considered to be safe; however, one study has shown that vector genome integration could cause insertional mutagenesis leading to hepatocarcinogenesis in a mouse model (Donsante et al., 2007). The detailed analyses of rAAV vector genome-cellular genome junction sequences in cultured cells and murine tissues have provided significant insights into which and how DNA repair pathways play roles in rAAV integration (Inagaki et al., 2007a; Miller et al., 2005; Nakai et al., 2005b). rAAV integration does not take place in a neat cut-and-paste fashion and always accompanies various degrees of deletions in rAAV genome terminal sequences and the cellular genomes around integration sites. Complex genomic

rearrangements are not rare and integration often causes a chromosomal translocation. All of these observations fit very well with a model in which NHEJ mediates rAAV integration. A series of studies has shown that DNA-PKcs has negative or positive effects on integration depending on the experimental systems used (Figure 2i). In a cell-free *in vitro* rAAV integration system, ss rAAV integration frequency increases and decreases by the addition of DNA-PKcs antibody and purified DNA-PKcs, respectively, leading to a conclusion that DNA-PKcs inhibits rAAV integration (Song et al., 2004). Whereas, in a cell culture system using DNA-PKcs-proficient M059K and deficient M059J cells, DNA-PKcs has been shown to enhance integration of both ss rAAV and ds rAAV (Cataldi & McCarty, 2010; Daya et al., 2009). In the context of animal experiment, Song et al. have exploited a two-thirds partial hepatectomy approach and shown that rAAV genomes integrate in DNA-PKcs-deficient SCID mouse livers at a significantly greater frequency than that of wild type control animals (*i.e.*, >50% in SCID versus <10% in wild type mice) (Song et al., 2004). Whereas, our most recent study has indicated that this effect could be observed at a limited range of liver transduction levels, and deficiency of DNA-PKcs may not have a generalized effect on rAAV integration frequency (Adachi & Nakai, unpublished observation). Nonetheless, a high-throughput ss rAAV integration site analysis in mouse liver, muscle and heart has successfully identified many rAAV integration sites in both wild type and SCID mouse tissues. This indicates that DNA-PKcs itself does not play a direct role in the process of rAAV genome integration (Figure 2i) (Inagaki et al., 2007a). Other DNA repair proteins that might participate in the rAAV genome integration process include ATM, which also shows varying effects on integration in cell culture experiments depending on the types of cells and rAAV (*i.e.*, ss versus ds rAAV) (Cataldi & McCarty, 2010; Sanlioglu et al., 2000). Interestingly, our study has implied that rAAV genomes more preferably integrate in the cellular genome than remain as extrachromosomal genomes when murine hepatocytes receive a minimum rAAV dose to establish latency (Adachi & Nakai, unpublished observation). This observation indicates that different DDRs are evoked and recruit different DNA repair machinery depending on the amount of DDR triggers in a cell. Collectively, at this point, there is no consensus model that explains which and how DNA repair pathways mediate rAAV integration. As for the integration of rAAV at the AAVS1 site in the presence of Rep68/78 expression, DNA-PKcs enhances site-specific integration of ss rAAV but not ds rAAV, indicating differential effects of DNA repair proteins in the Rep-mediated integration (Daya et al., 2009).

3.5 rAAV-mediated gene targeting and DNA repair pathways

HR mediated by the conventional vector systems occurs with efficiencies of a range of 10^{-6} to 10^{-7} . In this regard, rAAV has become increasingly popular as the most efficient tool to precisely introduce defined DNA modifications at the target site in the cellular genome with remarkably high efficiencies of up to 1% in the cell population (Hendrie & Russell, 2005; Khan et al., 2011; Russell & Hirata, 1998; Vasileva & Jessberger, 2005). Targeting efficiencies could be increased further by 60-100 fold or more by introducing a DSB at the target site with a site-specific endonuclease (Miller et al., 2003; Porteus et al., 2003). This system, named the gene targeting rAAV vector system, has been applied in various disciplines, not only for gene therapy (Chamberlain et al., 2004) but also for generating knockout animals (Sun et al., 2008) and other types of basic research (Khan et al., 2011). Gene targeting rAAV serves as a donor vector that carries a DNA segment homologous to the chromosomal target sequence with a desired modification being introduced. The length of the homology arms can be 1.7

kb or potentially shorter, which is an advantage over the conventional targeting vectors that require a longer homologous DNA sequence (Hirata & Russell, 2000). Despite significant advance in the applications of the system, the underlying mechanism for rAAV-mediated gene targeting is poorly understood. As described above, rAAV does not harness any machinery designed specifically for mediating highly efficient gene targeting. The unusual structure of viral genome DNA is the only element that makes the system much more efficient than the conventional approaches.

The mechanism of rAAV-mediated gene targeting has just begun to be partly elucidated. Studies have indicated that the single-stranded nature of gene-targeting rAAV is key to efficient gene targeting reactions. Experimental evidence has come from the observation that, when mixtures of gene-targeting ss rAAV and ds rAAV vectors were used, gene correction rates correlated with the amounts of ss rAAV but not ds rAAV within the mixtures (Hirata & Russell, 2000). Another study took advantage of recombinant minute virus of mice (rMVM), a rAAV-like parvovirus-based vector that predominantly packages viral genomes of minus polarity and does rarely undergo second-strand synthesis to form ds viral genomes. When reporter cells were infected with gene-targeting rMVM vectors containing either the coding or noncoding strand of a transgene cassette, a significant difference in targeting efficiencies was revealed between the two, indicating that ss viral genomes are the substrate (Hendrie et al., 2003). However, a recent study points out limitations in the previously used assay systems and argues against the above model because ds rAAV has also been found to mediate gene targeting at a higher level compared with the ss rAAV control (Hirsch et al., 2010). Although the nature of gene targeting substrates may be a subject of debate, it is clear that rAAV genome integration and rAAV-mediated gene targeting use different DNA repair pathways. Genotoxic treatment, which significantly augments rAAV genome integrations, does not affect gene targeting efficiency (Hirata & Russell, 2000). In addition, rAAV gene targeting occurs preferentially in S-phase cells and does not take place at an appreciable level in terminally differentiated murine skeletal muscle fibers (Liu et al., 2004; Trobridge et al., 2005). Moreover, the cell cycle dependence has not clearly been demonstrated in rAAV integration and a study has demonstrated a readily appreciable level of rAAV integration in terminally differentiated cardiomyocytes and skeletal myofibers (Inagaki et al., 2007a). Collectively, NHEJ appears to be the major DNA repair pathway involved in rAAV integration while rAAV-mediated gene targeting uses HR. It has been demonstrated that RAD51/RAD54 pathway of HR is required for efficient rAAV-mediated gene targeting (Figure 2j), and deficiency of either of the NHEJ proteins, DNA-PKcs and Ku70, enhances the targeting rates (Fattah et al., 2008; Vasileva et al., 2006). Although the DNA-PKcs effect appears to be a cell-type dependent phenomenon (Fattah et al., 2008), the observations underscore the significant contribution of the HR pathways in rAAV-mediated gene targeting. Manipulation of HR and NHEJ pathways with small molecules will offer a novel and effective means to further improve rAAV-mediated gene targeting approaches to genetically engineer cellular genomes.

4. AAV as a tool for studying damaged DNA sites, DDR, and DNA repair pathways

AAV has provided the most powerful means to deliver genetic materials to a broad range of cell and tissue/organ types without toxicity and to introduce sequence modifications at defined locations. What has made AAV more attractive is its utility as an unprecedented research tool to study molecular and cellular biology, where gene delivery is not a primary

goal. As described in 3.1.3 and 3.1.4, AAV has been successfully exploited as a refined agent that can trigger DDR toward cell cycle arrest and apoptosis. AAV can deliver an element that triggers DDR (*e.g.*, stalled replication forks) extrachromosomally with minimal transcriptional responses (McCaffrey et al., 2008) and without damaging the cellular genome. Although the phenomena observed in the AAV-based system may not necessarily recapitulate what takes place when the cellular DNA is damaged, it is assumed that molecularly defined extrachromosomal DDR triggers would provide a simple and less complicated means to study cellular responses to DNA damage. In addition, AAV has been exploited to study potential differences in DNA repair pathways among various tissues in the context of living animals. This type of study has demonstrated that, in hepatocytes, there is significant redundancy of Artemis/DNA-PKcs-independent NHEJ pathways that process hairpin DNA ends, while such redundancy is not observed in skeletal myofibers or cardiomyocytes in mice (Inagaki et al., 2007b). Moreover, AAV has recently emerged as a powerful tool to identify DNA sites damaged either endogenously or exogenously by genotoxic treatment or agents. Using rAAV as a tool to label pre-existing damaged DNA sites, a study has shown that DNA palindromes with an arm length of ≥ 20 base pairs in the cellular genome represent the sites susceptible to breakage in mouse tissues (Inagaki et al., 2007a). Another study has taken a similar AAV-based labeling approach and demonstrated frequent off-target cleavage of the cellular genome by a rare cutting endonuclease, I-SceI, following expression of I-SceI in cells (Petek et al., 2010). Perhaps applications of AAV in biological and medical research will not be limited to the disciplines described above and will continue to expand with the advent of novel rAAV vector technologies.

5. Conclusions

The virus-host interaction from a viewpoint of viral components and DNA repair machinery is an emerging research area that would offer unprecedented means to study both virology and molecular and cellular biology. The interaction in this aspect is most studied with adenoviruses, herpesviruses, and retroviruses including human immunodeficiency virus. These viruses have evolved sophisticated machinery to benefit them by manipulating or controlling DDR, DNA repair machinery, and the cell cycle. In this regard, AAV (*i.e.*, wtAAV and rAAV) represents a unique viral agent in that Rep proteins are the sole viral components that interact with DNA repair machinery and rAAV expresses no such component. Despite the seemingly simple nature of AAV, there are significant virus-host interactions that involve DDR and DNA repair machinery in AAV infection, and we have just begun to appreciate them as summarized in this chapter. There has been an increasing interest in AAV primarily as a promising gene delivery vector and more recently as a new tool to study DNA damage, DDR, and DNA repair machinery. Studying AAV from various scientific aspects including virology, immunology, physiology, gene therapy, DNA damage, DDR, DNA repair, genomic instability, carcinogenesis, and so on, would significantly advance our knowledge about AAV and could solve unanswered fundamental biological questions that are difficult to address by the conventional approaches.

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7. References

- Alexander, I. E., Russell, D. W. & Miller, A. D. (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *Journal of Virology*, Vol.68, No.12, pp. 8282-8287, ISSN 0022-538X
- Asokan, A., Conway, J. C., Phillips, J. L., Li, C., Hegge, J., Sinnott, R., Yadav, S., DiPrimio, N., Nam, H. J., Agbandje-McKenna, M., McPhee, S., Wolff, J. & Samulski, R. J. (2010). Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nature Biotechnology*, Vol.28, No.1, pp. 79-82, ISSN 1546-1696
- Atchison, R. W., Casto, B. C. & Hammon, W. M. (1965). Adenovirus-associated defective virus particles. *Science*, Vol.149, pp. 754-756, ISSN 0036-8075
- Beaton, A., Palumbo, P. & Berns, K. I. (1989). Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in trans by the rep protein. *Journal of Virology*, Vol.63, No.10, pp. 4450-4454, ISSN 0022-538X
- Berns, K. I. & Parrish C. R.. (2007). Parvoviridae, In *Fields VIROLOGY*, vol.2, 5th ed., D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (eds.), pp. 2437-2477, LIPPINCOTT WILLIAMS & WILKINS, ISBN 0-7817-1832-5, Philadelphia, PA.
- Bernstein, K. A., Gangloff, S. & Rothstein, R. (2010). The recq DNA helicases in DNA repair. *Annual Review of Genetics*, Vol.44, pp. 393-417, ISSN 1545-2948
- Berthet, C., Raj, K., Saudan, P. & Beard, P. (2005). How adeno-associated virus rep78 protein arrests cells completely in s phase. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.102, No.38, pp. 13634-13639, ISSN 0027-8424
- Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M. F. & Masurier, C. (2010). Prevalence of serum igg and neutralizing factors against adeno-associated virus (aav) types 1, 2, 5, 6, 8, and 9 in the healthy population: Implications for gene therapy using aav vectors. *Human Gene Therapy*, Vol.21, No.6, pp. 704-712, ISSN 1557-7422
- Branzei, D. & Foiani, M. (2010). Maintaining genome stability at the replication fork. *Nature Reviews Molecular Cell Biology*, Vol.11, No.3, pp. 208-219, ISSN 1471-0080
- Brister, J. R. & Muzyczka, N. (1999). Rep-mediated nicking of the adeno-associated virus origin requires two biochemical activities, DNA helicase activity and transesterification. *Journal of Virology*, Vol.73, No.11, pp. 9325-9336, ISSN 0022-538X
- Burguete, T., Rabreau, M., Fontanges-Darriet, M., Roset, E., Hager, H. D., Koppel, A., Bischof, P. & Schlehofer, J. R. (1999). Evidence for infection of the human embryo with adeno-associated virus in pregnancy. *Human Reproduction*, Vol.14, No.9, pp. 2396-2401, ISSN 0268-1161
- Calcedo, R., Vandenberghe, L. H., Gao, G., Lin, J. & Wilson, J. M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *Journal of Infectious Diseases*, Vol.199, No.3, pp. 381-390, ISSN 0022-1899

- Carson, C. T., Schwartz, R. A., Stracker, T. H., Lilley, C. E., Lee, D. V. & Weitzman, M. D. (2003). The mre11 complex is required for atm activation and the g2/m checkpoint. *EMBO Journal*, Vol.22, No.24, pp. 6610-6620, ISSN 0261-4189
- Carter, B. J., Burstein, H. & Peluso, R. W. (2009). Adeno-associated virus and AAV vectors for gene delivery, In: Gene and cell therapy, N. S. Templeton, (ed.), pp. 115-157, CRC press, ISBN 978-0-8493-8768-5, Boca Raton, FL.
- Castedo, M., Perfettini, J. L., Roumier, T., Andreau, K., Medema, R. & Kroemer, G. (2004). Cell death by mitotic catastrophe: A molecular definition. *Oncogene*, Vol.23, No.16, pp. 2825-2837, ISSN 0950-9232
- Costello, E., Saudan, P., Winocour, E., Pizer, L. & Beard, P. (1997). High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (rep) and promotes rep-mediated site-specific cleavage of DNA, atpase activity and transcriptional repression. *EMBO Journal*, Vol. 16, No. 19, pp. 5943-5954, ISSN 0261-4189
- Cataldi, M. P. & McCarty, D. M. (2010). Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. *Journal of Virology*, Vol.84, No.17, pp. 8673-8682, ISSN 1098-5514
- Cervelli, T., Palacios, J. A., Zentilin, L., Mano, M., Schwartz, R. A., Weitzman, M. D. & Giacca, M. (2008). Processing of recombinant aav genomes occurs in specific nuclear structures that overlap with foci of DNA-damage-response proteins. *Journal of Cell Science*, Vol.121, No.Pt 3, pp. 349-357, ISSN 0021-9533
- Chamberlain, J. R., Schwarze, U., Wang, P. R., Hirata, R. K., Hankenson, K. D., Pace, J. M., Underwood, R. A., Song, K. M., Sussman, M., Byers, P. H. & Russell, D. W. (2004). Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science*, Vol.303, No.5661, pp. 1198-1201, ISSN 1095-9203
- Choi, V. W., McCarty, D. M. & Samulski, R. J. (2006). Host cell DNA repair pathways in adeno-associated viral genome processing. *Journal of Virology*, Vol.80, No.21, pp. 10346-10356, ISSN 0022-538X
- Choi, Y. K., Nash, K., Byrne, B. J., Muzyczka, N. & Song, S. (2010). The effect of DNA-dependent protein kinase on adeno-associated virus replication. *PLoS ONE*, Vol.5, No.12, pp. e15073, ISSN 1932-6203
- Collaco, R. F., Bevington, J. M., Bhrigu, V., Kalman-Maltese, V. & Trempe, J. P. (2009). Adeno-associated virus and adenovirus coinfection induces a cellular DNA damage and repair response via redundant phosphatidylinositol 3-like kinase pathways. *Virology*, Vol.392, No.1, pp. 24-33, ISSN 1096-0341
- Daniel, R., Katz, R. A. & Skalka, A. M. (1999). A role for DNA-pk in retroviral DNA integration. *Science*, Vol.284, No.5414, pp. 644-647, ISSN 0036-8075
- Daya, S., Cortez, N. & Berns, K. I. (2009). Adeno-associated virus site-specific integration is mediated by proteins of the nonhomologous end-joining pathway. *Journal of Virology*, Vol.83, No.22, pp. 11655-11664, ISSN 1098-5514
- Deyle, D. R. & Russell, D. W. (2009). Adeno-associated virus vector integration. *Current Opinion Molecular Therapy*, Vol.11, No.4, pp. 442-447, ISSN 2040-3445
- Donsante, A., Miller, D. G., Li, Y., Vogler, C., Brunt, E. M., Russell, D. W. & Sands, M. S. (2007). Aav vector integration sites in mouse hepatocellular carcinoma. *Science*, Vol.317, pp. 477, ISSN 1095-9203

- Doostzadeh-Cizeron, J., Evans, R., Yin, S. & Goodrich, D. W. (1999). Apoptosis induced by the nuclear death domain protein p84n5 is inhibited by association with rb protein. *Molecular Biology of the Cell*, Vol.10, No.10, pp. 3251-3261, ISSN 1059-1524
- Duan, D., Yan, Z., Yue, Y. & Engelhardt, J. F. (1999). Structural analysis of adeno-associated virus transduction circular intermediates. *Virology*, Vol.261, No.1, pp. 8-14, ISSN 0042-6822
- Duan, D., Yue, Y. & Engelhardt, J. F. (2003). Consequences of DNA-dependent protein kinase catalytic subunit deficiency on recombinant adeno-associated virus genome circularization and heterodimerization in muscle tissue. *Journal of Virology*, Vol.77, No.8, pp. 4751-4759, ISSN 0022-538X
- Erles, K., Sebokova, P. & Schlehofer, J. R. (1999). Update on the prevalence of serum antibodies (igg and igm) to adeno-associated virus (aav). *Journal of Medical Virology*, Vol.59, No.3, pp. 406-411, ISSN 0146-6615
- Erles, K., Rohde, V., Thaele, M., Roth, S., Edler, L. & Schlehofer, J. R. (2001). DNA of adeno-associated virus (aav) in testicular tissue and in abnormal semen samples. *Human Reproduction*, Vol.16, No.11, pp. 2333-2337, ISSN 0268-1161
- Evans, J. D. & Hearing, P. (2005). Relocalization of the mre11-rad50-nbs1 complex by the adenovirus e4 orf3 protein is required for viral replication. *Journal of Virology*, Vol.79, No.10, pp. 6207-6215, ISSN 0022-538X
- Excoffon, K. J., Koerber, J. T., Dickey, D. D., Murtha, M., Keshavjee, S., Kaspar, B. K., Zabner, J. & Schaffer, D. V. (2009). Directed evolution of adeno-associated virus to an infectious respiratory virus. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.106, No.10, pp. 3865-3870, ISSN 1091-6490
- Fattah, F. J., Lichter, N. F., Fattah, K. R., Oh, S. & Hendrickson, E. A. (2008). Ku70, an essential gene, modulates the frequency of raav-mediated gene targeting in human somatic cells. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.105, No.25, pp. 8703-8708, ISSN 1091-6490
- Ferrari, F. K., Samulski, T., Shenk, T. & Samulski, R. J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *Journal of Virology*, Vol.70, No.5, pp. 3227-3234, ISSN 0022-538X
- Fisher, K. J., Gao, G. P., Weitzman, M. D., DeMatteo, R., Burda, J. F. & Wilson, J. M. (1996). Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *Journal of Virology*, Vol.70, No.1, pp. 520-532, ISSN 0022-538X
- Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M. & Kaspar, B. K. (2009). Intravascular aav9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology*, Vol.27, No.1, pp. 59-65, ISSN 1546-1696
- Fragkos, M., Breuleux, M., Clement, N. & Beard, P. (2008). Recombinant adeno-associated viral vectors are deficient in provoking a DNA damage response. *Journal of Virology*, Vol.82, No.15, pp. 7379-7387, ISSN 1098-5514
- Francois, A., Guilbaud, M., Awedikian, R., Chadeuf, G., Moullier, P. & Salvetti, A. (2005). The cellular tata binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. *Journal of Virology*, Vol.79, No.17, pp. 11082-11094, ISSN 0022-538X
- Gao, G., Vandenberghe, L. H., Alvira, M. R., Lu, Y., Calcedo, R., Zhou, X. & Wilson, J. M. (2004). Clades of adeno-associated viruses are widely disseminated in human tissues. *Journal of Virology*, Vol.78, No.12, pp. 6381-6388, ISSN 0022-538X

- Gao, G. P., Alvira, M. R., Wang, L., Calcedo, R., Johnston, J. & Wilson, J. M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.*, Vol.99, No.18, pp. 11854-11859, ISSN 0027-8424
- Garner, E., Martinon, F., Tschopp, J., Beard, P. & Raj, K. (2007). Cells with defective p53-p21-prb pathway are susceptible to apoptosis induced by p84n5 via caspase-6. *Cancer Research*, Vol.67, No.16, pp. 7631-7637, ISSN 0008-5472
- Geoffroy, M. C. & Salvetti, A. (2005). Helper functions required for wild type and recombinant adeno-associated virus growth. *Current Gene Therapy*, Vol.5, No.3, pp. 265-271, ISSN 1566-5232
- Ghosh, A., Yue, Y., Long, C., Bostick, B. & Duan, D. (2007). Efficient whole-body transduction with trans-splicing adeno-associated viral vectors. *Molecular Therapy*, Vol.15, No.4, pp. 750-755, ISSN 1525-0016
- Harper, J. W. & Elledge, S. J. (2007). The DNA damage response: Ten years after. *Molecular Cell*, Vol.28, No.5, pp. 739-745, ISSN 1097-2765
- Hauck, B., Zhao, W., High, K. & Xiao, W. (2004). Intracellular viral processing, not single-stranded DNA accumulation, is crucial for recombinant adeno-associated virus transduction. *Journal of Virology*, Vol.78, No.24, pp. 13678-13686, ISSN 0022-538X
- Henckaerts, E. & Linden, R. M. (2010). Adeno-associated virus: A key to the human genome? *Future Virology*, Vol.5, No.5, pp. 555-574, ISSN 1746-0808
- Hendrie, P. C., Hirata, R. K. & Russell, D. W. (2003). Chromosomal integration and homologous gene targeting by replication-incompetent vectors based on the autonomous parvovirus minute virus of mice. *Journal of Virology*, Vol.77, No.24, pp. 13136-13145, ISSN 0022-538X
- Hendrie, P. C. & Russell, D. W. (2005). Gene targeting with viral vectors. *Molecular Therapy*, Vol.12, No.1, pp. 9-17, ISSN 1525-0016
- Hermanns, J., Schulze, A., Jansen-Db1urr, P., Kleinschmidt, J. A., Schmidt, R. & zur Hausen, H. (1997). Infection of primary cells by adeno-associated virus type 2 results in a modulation of cell cycle-regulating proteins. *Journal of Virology*, Vol.71, No.8, pp. 6020-6027, ISSN 0022-538X
- Hirata, R. K. & Russell, D. W. (2000). Design and packaging of adeno-associated virus gene targeting vectors. *Journal of Virology*, Vol.74, No.10, pp. 4612-4620, ISSN 0022-538X
- Hirsch, M. L., Green, L., Porteus, M. H. & Samulski, R. J. (2010). Self-complementary aav mediates gene targeting and enhances endonuclease delivery for double-strand break repair. *Gene Therapy*, Vol.17, No.9, pp. 1175-1180, ISSN 1476-5462
- Inagaki, K., Fuess, S., Storm, T. A., Gibson, G. A., McTiernan, C. F., Kay, M. A. & Nakai, H. (2006). Robust systemic transduction with aav9 vectors in mice: Efficient global cardiac gene transfer superior to that of aav8. *Molecular Therapy*, Vol.14, No.1, pp. 45-53, ISSN 1525-0016
- Inagaki, K., Lewis, S. M., Wu, X., Ma, C., Munroe, D. J., Fuess, S., Storm, T. A., Kay, M. A. & Nakai, H. (2007a). DNA palindromes with a modest arm length of greater, similar 20 base pairs are a significant target for recombinant adeno-associated virus vector integration in the liver, muscles, and heart in mice. *Journal of Virology*, Vol.81, No.20, pp. 11290-11303, ISSN 0022-538X
- Inagaki, K., Ma, C., Storm, T. A., Kay, M. A. & Nakai, H. (2007b). The role of DNA-pkcs and artemis in opening viral DNA hairpin termini in various tissues in mice. *Journal of Virology*, Vol.81, No.20, pp. 11304-11321, ISSN 0022-538X

- Inagaki, K., Piao, C., Kotchey, N. M., Wu, X. & Nakai, H. (2008). Frequency and spectrum of genomic integration of recombinant adeno-associated virus serotype 8 vector in neonatal mouse liver. *Journal of Virology*, Vol.82, No.19, pp. 9513-9524, ISSN 1098-5514
- Ingemarsdotter, C., Keller, D. & Beard, P. (2010). The DNA damage response to non-replicating adeno-associated virus: Centriole overduplication and mitotic catastrophe independent of the spindle checkpoint. *Virology*, Vol.400, No.2, pp. 271-286, ISSN 1096-0341
- Jayaram, S., Gilson, T., Ehrlich, E. S., Yu, X. F., Ketner, G. & Hanakahi, L. (2008). E1b 55k-independent dissociation of the DNA ligase iv/xrcc4 complex by e4 34k during adenovirus infection. *Virology*, Vol.382, No.2, pp. 163-170, ISSN 1096-0341
- Jurvansuu, J., Raj, K., Stasiak, A. & Beard, P. (2005). Viral transport of DNA damage that mimics a stalled replication fork. *Journal of Virology*, Vol.79, No.1, pp. 569-580, ISSN 0022-538X
- Jurvansuu, J., Fragkos, M., Ingemarsdotter, C. & Beard, P. (2007). Chk1 instability is coupled to mitotic cell death of p53-deficient cells in response to virus-induced DNA damage signaling. *Journal of Molecular Biology*, Vol.372, No.2, pp. 397-406, ISSN 0022-2836
- Khan, I. F., Hirata, R. K. & Russell, D. W. (2011). Aav-mediated gene targeting methods for human cells. *Nature Protocol*, Vol.6, No.4, pp. 482-501, ISSN 1750-2799
- Koerber, J. T., Klimczak, R., Jang, J. H., Dalkara, D., Flannery, J. G. & Schaffer, D. V. (2009). Molecular evolution of adeno-associated virus for enhanced glial gene delivery. *Molecular Therapy*, Vol.17, No.12, pp. 2088-2095, ISSN 1525-0024
- Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M. & Berns, K. I. (1990). Site-specific integration by adeno-associated virus. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.87, No.6, pp. 2211-2215, ISSN 0027-8424
- Kotin, R. M., Linden, R. M. & Berns, K. I. (1992). Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO Journal*, Vol. 11, No. 13, pp. 5071-5078, ISSN 0261-4189
- Kyostio, S. R., Owens, R. A., Weitzman, M. D., Antoni, B. A., Chejanovsky, N. & Carter, B. J. (1994). Analysis of adeno-associated virus (aav) wild-type and mutant rep proteins for their abilities to negatively regulate aav p5 and p19 mrna levels. *Journal of Virology*, Vol.68, No.5, pp. 2947-2957, ISSN 0022-538X
- Li, L., Olvera, J. M., Yoder, K. E., Mitchell, R. S., Butler, S. L., Lieber, M., Martin, S. L. & Bushman, F. D. (2001). Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. *EMBO Journal*, Vol.20, No.12, pp. 3272-3281, ISSN 0261-4189
- Lilley, C. E., Schwartz, R. A. & Weitzman, M. D. (2007). Using or abusing: Viruses and the cellular DNA damage response. *Trends in Microbiology*, Vol.15, No.3, pp. 119-126, ISSN 0966-842X
- Linden, R. M., Ward, P., Giraud, C., Winocour, E. & Berns, K. I. (1996a). Site-specific integration by adeno-associated virus. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.93, No.21, pp. 11288-11294, ISSN 0027-8424
- Linden, R. M., Winocour, E. & Berns, K. I. (1996b). The recombination signals for adeno-associated virus site-specific integration. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.93, No.15, pp. 7966-7972, ISSN 0027-8424
- Liu, X., Yan, Z., Luo, M., Zak, R., Li, Z., Driskell, R. R., Huang, Y., Tran, N. & Engelhardt, J. F. (2004). Targeted correction of single-base-pair mutations with adeno-associated virus

- vectors under nonselective conditions. *Journal of Virology*, Vol.78, No.8, pp. 4165-4175, ISSN 0022-538X
- Ma, Y., Schwarz, K. & Lieber, M. R. (2005). The artemis:DNA-pkcs endonuclease cleaves DNA loops, flaps, and gaps. *DNA Repair*, Vol.4, No.7, pp. 845-851, ISSN 1568-7864
- McCaffrey, A. P., Fawcett, P., Nakai, H., McCaffrey, R. L., Ehrhardt, A., Pham, T. T., Pandey, K., Xu, H., Feuss, S., Storm, T. A. & Kay, M. A. (2008). The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Molecular Therapy*, Vol.16, No.5, pp. 931-941, ISSN 1525-0024
- McCarty, D. M., Pereira, D. J., Zolotukhin, I., Zhou, X., Ryan, J. H. & Muzyczka, N. (1994). Identification of linear DNA sequences that specifically bind the adeno-associated virus rep protein. *Journal of Virology*, Vol.68, No.8, pp. 4988-4997, ISSN 0022-538X
- McCarty, D. M., Fu, H., Monahan, P. E., Toulson, C. E., Naik, P. & Samulski, R. J. (2003). Adeno-associated virus terminal repeat (tr) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Therapy*, Vol.10, No.26, pp. 2112-2118, ISSN 0969-7128
- McCarty, D. M., Young, S. M., Jr. & Samulski, R. J. (2004). Integration of adeno-associated virus (aav) and recombinant aav vectors. *Annual Review of Genetics*, Vol.38, pp. 819-845, ISSN 0066-4197
- McCarty, D. M. (2008). Self-complementary aav vectors; advances and applications. *Molecular Therapy*, Vol.16, No.10, pp. 1648-1656, ISSN 1525-0024
- Miller, D. G., Petek, L. M. & Russell, D. W. (2003). Human gene targeting by adeno-associated virus vectors is enhanced by DNA double-strand breaks. *Molecular and Cellular Biology*, Vol.23, No.10, pp. 3550-3557, ISSN 0270-7306
- Miller, D. G., Petek, L. M. & Russell, D. W. (2004). Adeno-associated virus vectors integrate at chromosome breakage sites. *Nature Genetics*, Vol.36, No.7, pp. 767-773, ISSN 1061-4036
- Miller, D. G., Trobridge, G. D., Petek, L. M., Jacobs, M. A., Kaul, R. & Russell, D. W. (2005). Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. *Journal of Virology*, Vol.79, No.17, pp. 11434-11442, ISSN 0022-538X
- Mirzoeva, O. K. & Petrini, J. H. (2003). DNA replication-dependent nuclear dynamics of the mre11 complex. *Molecular Cancer Research*, Vol.1, No.3, pp. 207-218, ISSN 1541-7786
- Nakai, H., Storm, T. A. & Kay, M. A. (2000). Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *Journal of Virology*, Vol.74, No.20, pp. 9451-9463, ISSN 0022-538X
- Nakai, H., Yant, S. R., Storm, T. A., Fuess, S., Meuse, L. & Kay, M. A. (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *Journal of Virology*, Vol.75, No.15, pp. 6969-6976, ISSN 0022-538X
- Nakai, H., Thomas, C. E., Storm, T. A., Fuess, S., Powell, S., Wright, J. F. & Kay, M. A. (2002). A limited number of transducible hepatocytes restricts a wide-range linear vector dose response in recombinant adeno-associated virus-mediated liver transduction. *Journal of Virology*, Vol.76, No.22, pp. 11343-11349, ISSN 0022-538X
- Nakai, H., Montini, E., Fuess, S., Storm, T. A., Grompe, M. & Kay, M. A. (2003a). Aav serotype 2 vectors preferentially integrate into active genes in mice. *Nature Genetics*, Vol.34, No.3, pp. 297-302, ISSN 1061-4036

- Nakai, H., Storm, T. A., Fuess, S. & Kay, M. A. (2003b). Pathways of removal of free DNA vector ends in normal and DNA-pkcs-deficient scid mouse hepatocytes transduced with raav vectors. *Human Gene Therapy*, Vol.14, No.9, pp. 871-881, ISSN 1043-0342
- Nakai, H., Fuess, S., Storm, T. A., Muramatsu, S., Nara, Y. & Kay, M. A. (2005a). Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *Journal of Virology*, Vol.79, No.1, pp. 214-224, ISSN 0022-538X
- Nakai, H., Wu, X., Fuess, S., Storm, T. A., Munroe, D., Montini, E., Burgess, S. M., Grompe, M. & Kay, M. A. (2005b). Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. *Journal of Virology*, Vol.79, No.6, pp. 3606-3614, ISSN 0022-538X
- Nash, K., Chen, W., McDonald, W. F., Zhou, X. & Muzyczka, N. (2007). Purification of host cell enzymes involved in adeno-associated virus DNA replication. *Journal of Virology*, Vol.81, No.11, pp. 5777-5787, ISSN 0022-538X
- Nash, K., Chen, W., Salganik, M. & Muzyczka, N. (2009). Identification of cellular proteins that interact with the adeno-associated virus rep protein. *Journal of Virology*, Vol.83, No.1, pp. 454-469, ISSN 1098-5514
- Nathwani, A. C., Rosales, C., McIntosh, J., Rastegarlar, G., Nathwani, D., Raj, D., Nawathe, S., Waddington, S. N., Bronson, R., Jackson, S., Donahue, R. E., High, K. A., Mingozzi, F., Ng, C. Y., Zhou, J., Spence, Y., McCarville, M. B., Valentine, M., Allay, J., Coleman, J., Sleep, S., Gray, J. T., Nienhuis, A. W. & Davidoff, A. M. (2011). Long-term safety and efficacy following systemic administration of a self-complementary aav vector encoding human fix pseudotyped with serotype 5 and 8 capsid proteins. *Molecular Therapy*, pp., ISSN 1525-0024
- Nony, P., Tessier, J., Chadeuf, G., Ward, P., Giraud, A., Dugast, M., Linden, R. M., Moullier, P. & Salvetti, A. (2001). Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. *Journal of Virology*, Vol.75, No.20, pp. 9991-9994, ISSN 0022-538X
- Penaud-Budloo, M., Le Guiner, C., Nowrouzi, A., Toromanoff, A., Cherel, Y., Chenuaud, P., Schmidt, M., von Kalle, C., Rolling, F., Moullier, P. & Snyder, R. O. (2008). Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle. *Journal of Virology*, Vol.82, No.16, pp. 7875-7885, ISSN 1098-5514
- Pereira, C. C., de Freitas, L. B., de Vargas, P. R., de Azevedo, M. L., do Nascimento, J. P. & Spano, L. C. (2010). Molecular detection of adeno-associated virus in cases of spontaneous and intentional human abortion. *Journal of Medical Virology*, Vol.82, No.10, pp. 1689-1693, ISSN 1096-9071
- Petek, L. M., Russell, D. W. & Miller, D. G. (2010). Frequent endonuclease cleavage at off-target locations *in vivo*. *Molecular Therapy*, Vol.18, No.5, pp. 983-986, ISSN 1525-0024
- Philpott, N. J., Gomos, J., Berns, K. I. & Falck-Pedersen, E. (2002). A p53 integration efficiency element mediates rep-dependent integration into aav1 at chromosome 19. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.99, No.19, pp. 12381-12385, ISSN 0027-8424
- Porteus, M. H., Cathomen, T., Weitzman, M. D. & Baltimore, D. (2003). Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. *Molecular and Cellular Biology*, Vol.23, No.10, pp. 3558-3565, ISSN 0270-7306
- Qing, K., Hansen, J., Weigel-Kelley, K. A., Tan, M., Zhou, S. & Srivastava, A. (2001). Adeno-associated virus type 2-mediated gene transfer: Role of cellular fkbp52 protein in

- transgene expression. *Journal of Virology*, Vol.75, No.19, pp. 8968-8976, ISSN 0022-538X
- Qing, K., Li, W., Zhong, L., Tan, M., Hansen, J., Weigel-Kelley, K. A., Chen, L., Yoder, M. C. & Srivastava, A. (2003). Adeno-associated virus type 2-mediated gene transfer: Role of cellular t-cell protein tyrosine phosphatase in transgene expression in established cell lines *in vitro* and transgenic mice *in vivo*. *Journal of Virology*, Vol.77, No.4, pp. 2741-2746, ISSN 0022-538X
- Rabinowitz, J. E., Rolling, F., Li, C., Conrath, H., Xiao, W., Xiao, X. & Samulski, R. J. (2002). Cross-packaging of a single adeno-associated virus (aav) type 2 vector genome into multiple aav serotypes enables transduction with broad specificity. *Journal of Virology*, Vol.76, No.2, pp. 791-801, ISSN 0022-538X
- Raj, K., Ogston, P. & Beard, P. (2001). Virus-mediated killing of cells that lack p53 activity. *Nature*, Vol.412, No.6850, pp. 914-917, ISSN 0028-0836
- Rooney, S., Sekiguchi, J., Zhu, C., Cheng, H. L., Manis, J., Whitlow, S., DeVido, J., Foy, D., Chaudhuri, J., Lombard, D. & Alt, F. W. (2002). Leaky scid phenotype associated with defective v(dj) coding end processing in artemis-deficient mice. *Molecular Cell*, Vol.10, No.6, pp. 1379-1390, ISSN 1097-2765
- Roth, D. B., Menetski, J. P., Nakajima, P. B., Bosma, M. J. & Gellert, M. (1992). V(dj) recombination: Broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. *Cell*, Vol.70, No.6, pp. 983-991, ISSN 0092-8674
- Russell, D. W., Miller, A. D. & Alexander, I. E. (1994). Adeno-associated virus vectors preferentially transduce cells in s phase. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.91, No.19, pp. 8915-8919, ISSN 0027-8424
- Russell, D. W., Alexander, I. E. & Miller, A. D. (1995). DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.92, No.12, pp. 5719-5723, ISSN 0027-8424
- Russell, D. W. & Hirata, R. K. (1998). Human gene targeting by viral vectors [see comments]. *Nature Genetics*, Vol.18, No.4, pp. 325-330, ISSN 1061-4036
- Samulski, R. J., Berns, K. I., Tan, M. & Muzyczka, N. (1982). Cloning of adeno-associated virus into pbr322: Rescue of intact virus from the recombinant plasmid in human cells. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 79, No. 6, pp. 2077-2081, ISSN 0027-8424
- Samulski, R. J., Zhu, X., Xiao, X., Brook, J. D., Housman, D. E., Epstein, N. & Hunter, L. A. (1991). Targeted integration of adeno-associated virus (aav) into human chromosome 19 [published erratum appears in *embo j* 1992 mar;11(3):1228]. *EMBO Journal*, Vol.10, No.12, pp. 3941-3950, ISSN 0261-4189
- Sanlioglu, S., Benson, P. & Engelhardt, J. F. (2000). Loss of atm function enhances recombinant adeno-associated virus transduction and integration through pathways similar to uv irradiation. *Virology*, Vol.268, No.1, pp. 68-78, ISSN 0042-6822
- Sarkar, R., Mucci, M., Addya, S., Tetreault, R., Bellinger, D. A., Nichols, T. C. & Kazazian, H. H., Jr. (2006). Long-term efficacy of adeno-associated virus serotypes 8 and 9 in hemophilia a dogs and mice. *Human Gene Therapy*, Vol.17, No.4, pp. 427-439, ISSN 1043-0342
- Saudan, P., Vlach, J. & Beard, P. (2000). Inhibition of s-phase progression by adeno-associated virus rep78 protein is mediated by hypophosphorylated prb. *EMBO Journal*, Vol.19, No.16, pp. 4351-4361, ISSN 0261-4189

- Schnepp, B. C., Jensen, R. L., Chen, C. L., Johnson, P. R. & Clark, K. R. (2005). Characterization of adeno-associated virus genomes isolated from human tissues. *Journal of Virology*, Vol.79, No.23, pp. 14793-14803, ISSN 0022-538X
- Schultz, B. R. & Chamberlain, J. S. (2008). Recombinant adeno-associated virus transduction and integration. *Molecular Therapy*, Vol.16, No.7, pp. 1189-1199, ISSN 1525-0024
- Schwartz, R. A., Palacios, J. A., Cassell, G. D., Adam, S., Giacca, M. & Weitzman, M. D. (2007). The mre11/rad50/nbs1 complex limits adeno-associated virus transduction and replication. *Journal of Virology*, Vol.81, No.23, pp. 12936-12945, ISSN 1098-5514
- Schwartz, R. A., Carson, C. T., Schubert, C. & Weitzman, M. D. (2009). Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. *Journal of Virology*, Vol.83, No.12, pp. 6269-6278, ISSN 1098-5514
- Shiotani, B. & Zou, L. (2009). Atr signaling at a glance. *Journal of Cell Science*, Vol.122, No.Pt 3, pp. 301-304, ISSN 0021-9533
- Smith, R. H. & Kotin, R. M. (2002). Adeno-associated virus, In: Mobile DNA II, N. L. Craig, R. Craigie, M. Gellert, A. M. Lambowitz (eds.), pp. 905-923, ASM press, ISBN 1-55581-209-0, Herndon, VA.
- Song, S., Laipis, P. J., Berns, K. I. & Flotte, T. R. (2001). Effect of DNA-dependent protein kinase on the molecular fate of the raav2 genome in skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.98, No.7, pp. 4084-4088, ISSN 0027-8424
- Song, S., Lu, Y., Choi, Y. K., Han, Y., Tang, Q., Zhao, G., Berns, K. I. & Flotte, T. R. (2004). DNA-dependent pk inhibits adeno-associated virus DNA integration. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.101, No.7, pp. 2112-2116, ISSN 0027-8424
- Sonntag, F., Schmidt, K. & Kleinschmidt, J. A. (2010). A viral assembly factor promotes aav2 capsid formation in the nucleolus. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.107, No.22, pp. 10220-10225, ISSN 1091-6490
- Stracker, T. H., Carson, C. T. & Weitzman, M. D. (2002). Adenovirus oncoproteins inactivate the mre11-rad50-nbs1 DNA repair complex. *Nature*, Vol.418, No.6895, pp. 348-352, ISSN 0028-0836
- Strickler, H. D., Viscidi, R., Escoffery, C., Rattray, C., Kotloff, K. L., Goldberg, J., Manns, A., Rabkin, C., Daniel, R., Hanchard, B., Brown, C., Hutchinson, M., Zanizer, D., Palefsky, J., Burk, R. D., Cranston, B., Clayman, B. & Shah, K. V. (1999). Adeno-associated virus and development of cervical neoplasia. *Journal of Medical Virology*, Vol.59, No.1, pp. 60-65, ISSN 0146-6615
- Su, P. F. & Wu, F. Y. (1996). Differential suppression of the tumorigenicity of hela and siha cells by adeno-associated virus. *British Journal of Cancer*, Vol.73, No.12, pp. 1533-1537, ISSN 0007-0920
- Summerford, C. & Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *Journal of Virology*, Vol.72, No.2, pp. 1438-1445, ISSN 0022-538X
- Sun, X., Yan, Z., Yi, Y., Li, Z., Lei, D., Rogers, C. S., Chen, J., Zhang, Y., Welsh, M. J., Leno, G. H. & Engelhardt, J. F. (2008). Adeno-associated virus-targeted disruption of the cftr gene in cloned ferrets. *Journal of Clinical Investigation*, Vol.118, No.4, pp. 1578-1583, ISSN 0021-9738
- Tan, I., Ng, C. H., Lim, L. & Leung, T. (2001). Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of

- actin cytoskeleton. *Journal of Biological Chemistry*, Vol.276, No.24, pp. 21209-21216, ISSN 0021-9258
- Thomas, K. R. & Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*, Vol.51, No.3, pp. 503-512, ISSN 0092-8674
- Torres-Ramos, C. A., Prakash, S. & Prakash, L. (1997). Requirement of yeast DNA polymerase delta in post-replicative repair of uv-damaged DNA. *Journal of Biological Chemistry*, Vol.272, No.41, pp. 25445-25448, ISSN 0021-9258
- Trobridge, G., Hirata, R. K. & Russell, D. W. (2005). Gene targeting by adeno-associated virus vectors is cell-cycle dependent. *Human Gene Therapy*, Vol.16, No.4, pp. 522-526, ISSN 1043-0342
- Tullis, G. E. & Shenk, T. (2000). Efficient replication of adeno-associated virus type 2 vectors: A cis-acting element outside of the terminal repeats and a minimal size. *Journal of Virology*, Vol.74, No.24, pp. 11511-11521, ISSN 0022-538X
- Vakifahmetoglu, H., Olsson, M. & Zhivotovsky, B. (2008). Death through a tragedy: Mitotic catastrophe. *Cell Death and Differentiation*, Vol.15, No.7, pp. 1153-1162, ISSN 1350-9047
- Vandendriessche, T., Thorrez, L., Acosta-Sanchez, A., Petrus, I., Wang, L., Ma, L., L, D. E. W., Iwasaki, Y., Gillijns, V., Wilson, J. M., Collen, D. & Chuah, M. K. (2007). Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. Lentiviral vectors for hemophilia b gene therapy. *Journal of Thrombosis Haemostasis*, Vol.5, No.1, pp. 16-24, ISSN 1538-7933
- Vasileva, A. & Jessberger, R. (2005). Precise hit: Adeno-associated virus in gene targeting. *Nature Reviews: Microbiology*, Vol.3, No.11, pp. 837-847, ISSN 1740-1526
- Vasileva, A., Linden, R. M. & Jessberger, R. (2006). Homologous recombination is required for aav-mediated gene targeting. *Nucleic Acids Res*, Vol.34, No.11, pp. 3345-3360, ISSN 1362-4962
- Walz, C. & Schlehofer, J. R. (1992). Modification of some biological properties of hela cells containing adeno-associated virus DNA integrated into chromosome 17. *Journal of Virology*, Vol.66, No.5, pp. 2990-3002, ISSN 0022-538X
- Wang, Z., Ma, H. I., Li, J., Sun, L., Zhang, J. & Xiao, X. (2003). Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Therapy*, Vol.10, No.26, pp. 2105-2111, ISSN 0969-7128
- Wang, Z., Zhu, T., Qiao, C., Zhou, L., Wang, B., Zhang, J., Chen, C., Li, J. & Xiao, X. (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nature Biotechnology*, Vol.23, No.3, pp. 321-328, ISSN 1087-0156
- Weitzman, M. D., Carson, C. T., Schwartz, R. A. & Lilley, C. E. (2004). Interactions of viruses with the cellular DNA repair machinery. *DNA Repair*, Vol.3, No.8-9, pp. 1165-1173, ISSN 1568-7864
- Weitzman, M. D., Lilley, C. E. & Chaurushiya, M. S. (2010). Genomes in conflict: Maintaining genome integrity during virus infection. *Annual Review of Microbiology*, Vol.64, pp. 61-81, ISSN 1545-3251
- Williams, G. J., Lees-Miller, S. P. & Tainer, J. A. (2010). Mre11-rad50-nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. *DNA Repair*, Vol.9, No.12, pp. 1299-1306, ISSN 1568-7856
- Winocour, E., Callahan, M. F. & Huberman, E. (1988). Perturbation of the cell cycle by adeno-associated virus. *Virology*, Vol.167, No.2, pp. 393-399, ISSN 0042-6822

- Xiao, X., Xiao, W., Li, J. & Samulski, R. J. (1997). A novel 165-base-pair terminal repeat sequence is the sole cis requirement for the adeno-associated virus life cycle. *Journal of Virology*, Vol.71, No.2, pp. 941-948, ISSN 0022-538X
- Yakinoglu, A. O., Heilbronn, R., Burkle, A., Schlehofer, J. R. & zur Hausen, H. (1988). DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. *Cancer Research*, Vol.48, No.11, pp. 3123-3129, ISSN 0008-5472
- Yakobson, B., Koch, T. & Winocour, E. (1987). Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. *Journal of Virology*, Vol.61, No.4, pp. 972-981, ISSN 0022-538X
- Yakobson, B., Hrynko, T. A., Peak, M. J. & Winocour, E. (1989). Replication of adeno-associated virus in cells irradiated with uv light at 254 nm. *Journal of Virology*, Vol.63, No.3, pp. 1023-1030, ISSN 0022-538X
- Yamamoto, N., Suzuki, M., Kawano, M. A., Inoue, T., Takahashi, R. U., Tsukamoto, H., Enomoto, T., Yamaguchi, Y., Wada, T. & Handa, H. (2007). Adeno-associated virus site-specific integration is regulated by trp-185. *Journal of Virology*, Vol. 81, No. 4, pp. 1990-2001, ISSN 0022-538X
- Yan, Z., Lei-Butters, D. C., Zhang, Y., Zak, R. & Engelhardt, J. F. (2007). Hybrid adeno-associated virus bearing nonhomologous inverted terminal repeats enhances dual-vector reconstruction of minigenes *in vivo*. *Human Gene Therapy*, Vol.18, No.1, pp. 81-87, ISSN 1043-0342
- Yang, L., Jiang, J., Drouin, L. M., Agbandje-McKenna, M., Chen, C., Qiao, C., Pu, D., Hu, X., Wang, D. Z., Li, J. & Xiao, X. (2009). A myocardium tropic adeno-associated virus (aav) evolved by DNA shuffling and *in vivo* selection. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.106, No.10, pp. 3946-3951, ISSN 1091-6490
- Yang, Q., Chen, F. & Trempe, J. P. (1994). Characterization of cell lines that inducibly express the adeno-associated virus rep proteins. *Journal of Virology*, Vol.68, No.8, pp. 4847-4856, ISSN 0022-538X
- Zentilin, L., Marcello, A. & Giacca, M. (2001). Involvement of cellular double-stranded DNA break binding proteins in processing of the recombinant adeno-associated virus genome. *Journal of Virology*, Vol.75, No.24, pp. 12279-12287, ISSN 0022-538X
- Zhong, L., Zhou, X., Li, Y., Qing, K., Xiao, X., Samulski, R. J. & Srivastava, A. (2008). Single-polarity recombinant adeno-associated virus 2 vector-mediated transgene expression *in vitro* and *in vivo*: Mechanism of transduction. *Molecular Therapy*, Vol.16, No.2, pp. 290-295, ISSN 1525-0024
- Zhou, X., Zeng, X., Fan, Z., Li, C., McCown, T., Samulski, R. J. & Xiao, X. (2008). Adeno-associated virus of a single-polarity DNA genome is capable of transduction *in vivo*. *Molecular Therapy*, Vol.16, No.3, pp. 494-499, ISSN 1525-0024

Integration of the DNA Damage Response with Innate Immune Pathways

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1. Introduction

Genotoxic or replicative stress triggers a DNA damage response (DDR) that induces cell cycle arrest, DNA repair or – if the damage is too severe – apoptosis. The DDR has been suggested to represent a barrier against tumorigenesis by preventing the uncontrolled proliferation of cells with genomic instability or harmful mutations. Recent studies have uncovered novel links of the DDR to innate immune signaling pathways. The activation of NF- κ B in response to DNA damage is mediated by ATM (ataxia telangiectasia mutated)-dependent phosphorylation of NEMO, resulting in the induction of the classical NF- κ B pathways. Furthermore links between the DDR and various members of the type I interferon (IFN) pathway have been uncovered. The DDR also increases the sensitivity of cells to immune cell-mediated killing by inducing the expression of surface ligands for activating immune receptors. Here, we review how the DDR links to innate immune pathways and the potential role of these interactions in cancer and viral infection.

2. The DNA damage response (DDR)

The genome integrity is constantly challenged by environmental genotoxic agents (chemicals, ultra-violet, viral infection etc.) and endogenous genotoxic stress (replication, oxidative stress, etc.) (Lindahl, 1993; Nyberg et al., 2002; Kunkel, 2004). DNA damage may also be caused by reactive oxygen species and nitrogen compounds produced by neutrophils and macrophages at sites of inflammation (deRoja-Walker T et al., 1995; Kawanishi et al., 2006). These DNA lesions or aberrations can block transcription and genome replication, and if not repaired, lead to mutations or large-scale genome aberrations that threaten the survival of the individual cells and the whole organism (Jackson & Bartek, 2009). To cope with genomic DNA damage, organisms have evolved a repertoire of surveillance and repair mechanisms to detect and combat the deleterious effects of damaged DNA (Zhou & Elledge, 2000). The DDR is composed of sensor protein kinases that are recruited to the sites of DNA damage, the signal transducer proteins that propagate the signal downstream, and the effector proteins which activate the appropriate responses such as DNA repair, cell cycle arrest and apoptosis (Gasser et al., 2007) (Figure 1).

2.1 ATM and ATR

The diversity in the types of DNA lesions necessitates specific protein complexes to detect and initiate the correct repair programme. Studies on the biochemistry of specific DNA

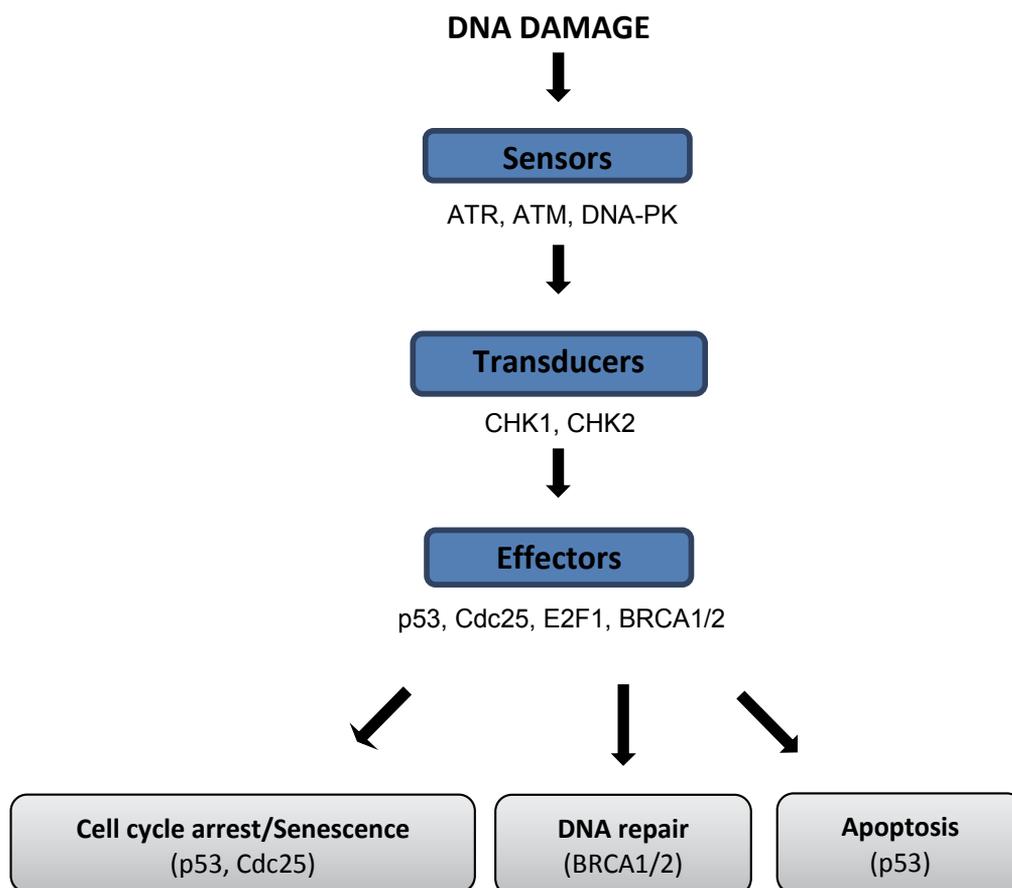


Fig. 1. Schematic diagram of the DNA damage response (DDR). The DDR is initiated by the sensors ATM and/or ATR depending of the nature of the DNA damage inducing cell cycle arrest and DNA repair or apoptosis if the damage is beyond repair.

lesions are complicated by the fact that the DNA damaging agents or ionizing radiation may each generate multiple types of DNA damage, and possibly recruiting several DNA damage sensors (Zhou & Elledge, 2000). The chemotherapeutic drug doxorubicin, for example, is a DNA topoisomerase II inhibitor that not only creates DNA strand-breaks, but also forms doxorubicin-DNA adducts, which induce torsional stress in the DNA structure (Swift et al., 2006). The different types of DNA damage eventually induce the activation of the phosphoinositide-3-kinase (PI3K)-related protein kinases (PIKKs) ATM and ATR (ATM- and Rad3-related) (Yang et al., 2003). Single-stranded DNA resulting from various types of genotoxic stress is bound by ssDNA-binding proteins such as RPA (replication protein A) (Wold, 1997). ATR and the ATR-interacting protein (ATRIP) then localizes to the RPA-coated ssDNA (Zou & Elledge, 2003). ATR also responds to stalled DNA replication forks in humans and mice (Cortez et al., 2001; Brown & Baltimore, 2000). On the other hand, ATM is recruited by double-stranded (ds) DNA breaks resulting primarily from ionizing radiation and oxidative stress (Zhou & Elledge, 2000; Shiloh, 2003). ATM is also activated by programmed dsDNA breaks during V(D)J recombination, an important process in the

generation of the diverse repertoire of immunoglobulins and T-cell receptors in B and T lymphocytes (Perkins et al., 2002; Dujka et al., 2009). It is currently not clear how ATM and ATR initiate the correct repair programmes for the wide variety of DNA lesions. Most likely different types of DNA damage recruit specific proteins (e.g. BRCA-1, H2AX, Nbs1, 53BP1 etc.) that interact with ATM and ATR thereby initiating a DNA damage-specific repair programme (Yang et al., 2003; Matsuoka et al., 2007).

2.2 DNA-PK

The DNA-dependent protein kinase (DNA-PK), a member of the PI3K superfamily, is a nuclear serine/threonine kinase composed of the catalytic subunit (DNA-PKcs) and the DNA-binding Ku70/80 subunit (Carter et al., 1990; Kurimasa et al., 1999). Similar to ATM, DNA-PK is involved in the detection of dsDNA breaks and is activated by DNA damage following ionizing radiation, UV radiation and V(D)J recombination events (Kurimasa et al., 1999; Yang et al., 2003). The binding of Ku70/Ku80 to dsDNA breaks is required for the activation of DNA-PKcs and the subsequent ligation of the double-stranded DNA ends by other protein components of the NHEJ (non-homologous end-joining) machinery (Kurimasa et al., 1999; Walker et al., 2001). Recent data suggest that DNA-PK and ATM are partially redundant in their function (Stiff et al., 2004). DNA-PK has been reported to be able to phosphorylate H2AX in ATM-deficient cells after treatment with ionizing radiation (Stiff et al., 2004). Furthermore, association of ATR and DNA-PK is observed during UV irradiation and the activation of DNA-PK is impaired when ATR is inhibited (Yajima et al., 2006). Taken together, DNA-PK appears to co-operate with ATM and ATR to initiate the DDR.

2.3 CHK1 and CHK2

ATM and ATR partially coordinate the DDR through the signal transducers CHK1 and CHK2 (Zhou & Elledge, 2000; Gasser 2007). CHK1 and CHK2 contain conserved kinase domains but differ in their function and structure (reviewed in Bartek et al., 2001; McGowan 2002). The main functions of CHK1 and CHK2 are to reinforce signals from ATM and ATR (Matsuoka et al., 1998; Abraham, 2001; Shiloh, 2003). CHK1 is an unstable protein that is specifically expressed during the S and G2 phases of cell cycle (Lukas et al., 2001). Interestingly, while CHK1 is expressed and activated in unperturbed cell cycles, stalled replication forks and ssDNA breaks enhance its activity further (Kaneko et al., 1999; Zhao et al., 2002; Sørensen et al., 2003). In contrast, CHK2 is expressed throughout the cell cycle (Lukas et al., 2001) but is activated specifically in response to dsDNA breaks. Similar to ATM, CHK2 activation depends on its dimerization and autophosphorylation (Cai et al., 2009). Historically, it was thought that ATR specifically activates CHK1, while CHK2 activation depends on ATM, but recent data suggests that a certain degree of redundancy exists in the ability of ATR and ATM to activate the signal transducers. For example it was reported that the phosphorylation of CHK1 in response to ionizing radiation depends on ATM (Gatei et al., 2003; Sørensen et al., 2003). CHK1 and CHK2 share many overlapping substrates and are therefore often found to be functionally redundant (Bartek et al., 2001; McGowan, 2002). The targets of CHK1 and CHK2 regulate many fundamental cellular functions such as cell cycle, DNA repair and apoptosis (Figure 1). For example, both CHK2 and CHK1 reduce the activity of cyclin-dependent kinases (CDKs) such as CDK2 and CDK1. The resulting inhibition of these G1/S- and G2/M-promoting CDKs results in cell cycle delays (Falck et al, 2001; Zhao & Piwnicka-Worms, 2001). Despite their functional redundancy, mouse studies have revealed striking differences for CHK1 and CHK2 during

development (Liu et al., 2000; Takai et al., 2000). CHK1-deficient mice are embryonic lethal in contrast to CHK2-deficient mice (Liu et al., 2000; Takai et al., 2000; Hirao et al., 2000; Takai et al., 2002). CHK2 is required for radiation-induced, p53-dependent apoptosis and the stability of p53 is reduced in mice lacking CHK2 (Hirao et al., 2000; Takai et al., 2002). Nevertheless both CHK1 and CHK2 have been shown to be able to phosphorylate p53 on several sites in response to DNA damage (Hirao et al., 2000; Shieh et al., 2000; Ou et al., 2005).

2.4 p53

Whether the DNA repair machinery or cellular apoptosis is initiated as a consequence of DNA damage is a crucial decision of the DDR. Although not fully understood, a large body of evidence hints at p53 playing a critical role in this important decision (reviewed in Vousden & Lu, 2002; Das et al., 2008). Depending on the nature of the DNA damage, p53 is phosphorylated on several serine residues by ATM (Banin et al., 1998; Khanna et al., 1998), ATR (Tibbetts et al., 1999) or DNA-PK (Shieh et al., 1997; Achanta et al., 2001). In addition the signal transducers CHK1 and CHK2 directly bind and phosphorylate p53 (Hirao et al., 2000; Ou et al., 2005, Dumaz & Meek, 1999). The phosphorylation of p53 at Ser20 is known to be important for destabilizing the interaction of p53 with its inhibitor MDM2 (Shieh et al., 1997; Unger et al., 1999). In addition, studies have demonstrated that p53 function is modulated by acetylation in response to DNA damage (Ou et al., 2005). These posttranslational modifications allow p53 to induce the expression of its target genes such as p21, a CDK2 inhibitor implicated in G1/S transition (Wade Harper et al., 1993; Chen et al., 1995). In case of irreparable DNA damage, p53 induces a differential set of target genes leading to the activation of both the mitochondrial and CD95-FasL apoptotic pathways (Kastan et al., 1991; Lowe et al., 1993b; Bennett et al., 1998; Chipuk et al., 2003; Mihara et al., 2003).

Recent studies demonstrated that p53 is activated early in tumorigenesis as a result of oncogene expression. Oncogene activation is thought to induce "replication stress" leading to the collapse of DNA replication forks and the formation of dsDNA breaks (Halazonetis et al., 2008). It has been suggested that p53 acts as an anti-cancer barrier in precancerous lesions. In support of this hypothesis, functional inactivation of p53 has been observed in 50% of all human cancers (Hanahan & Weinberg, 2000). Thus, oncogene-induced DNA damage may explain two key features of cancer: the high frequency of p53 mutations and the resulting genomic instability, which is often observed in cells lacking p53 (reviewed in Jackson & Bartek, 2009).

3. DDR and the immune system

As early as the 19th century it was recognized that some tumors are infiltrated by innate and adaptive immune cells (reviewed in Dvorak, 1986). In recent years new data suggests that DDR can initiate an immune response. As discussed below in more detail, the DDR directly activates a variety of transcription factors such as NF- κ B and interferon regulatory factors (IRFs). These transcription factors induce the expression of various immune genes, including inflammatory cytokines and chemokines. In addition, the DDR and oxidative stress induce the expression of a number of ligands for activating immune receptors such as NKG2D and DNAM-1, which are mainly expressed by cytotoxic immune cells such as T cells and NK cells.

3.1 NF- κ B

Transcription factors belonging to the NF- κ B family are mostly nuclear proteins that were initially reported to bind to the promoter of the κ immunoglobulin gene in B cells upon lipopolysaccharide (LPS) stimulation (Sen & Baltimore, 1986). It is now recognized that these transcription factors regulate many key aspects of innate immune signaling (Baeuerle & Henkel, 1994; Pahl, 1999). The NF- κ B subunits are usually sequestered in the cytoplasm through their interactions with inhibitory I κ B proteins. Phosphorylation of I κ B proteins by the I κ B kinase (IKK) complex, consisting of IKK α , IKK β and the scaffold protein NEMO/IKK γ , leads to the degradation of I κ B (Scheidereit 2006). Upon I κ B degradation, NF- κ B subunits translocate to the nucleus (Scheidereit 2006) and modulate the expression of NF- κ B target genes such as IL-6 (Libermann & Baltimore, 1990), IL-8 (Kunsch et al., 1994), and IL-1 β (Cogswell et al., 199). The picture is complicated by the fact that NF- κ B complexes consist of homodimers or heterodimers of five NF- κ B family proteins: p65 (Rel-A), Rel-B, c-Rel, p50 and p52 (Hayden & Ghosh, 2008). The Rel subfamily of NF- κ B proteins possess C-terminal transactivation domains (TADs) that promote target gene expression when bound to κ B sites as heterodimers with either p50 or p52 (Ghosh et al., 1998). The p50/p65 heterodimer is the main activating NF- κ B dimer in many cells, and the combinatorial diversity of heterodimers confers specificity in gene activation under specific physiological conditions (Ghosh et al., 1998). In contrast, the p52 and p50 homodimers inhibit transcription (Ghosh & Karin, 2002).

NF- κ B is also activated in response to DNA damage (Brach et al., 1991; Simon et al., 1994). In ATM-deficient mice, NF- κ B activation is impaired after irradiation (Li et al., 2001). Similarly, in DNA-PK-deficient cells, the activation of NF- κ B was impaired upon irradiation (Basu et al., 1998). The activation of NF- κ B following DNA damage mainly results in survival signals (Wang et al., 1998; Wang et al., 1999) that could provide a time window for cells to repair damaged DNA (Beg & Baltimore, 1996; Wang et al., 1996).

3.1.1 NEMO

Recent insights into the molecular mechanisms leading to NF- κ B activation in response to DNA damage indicate an important role for NEMO (Huang et al., 2000; Huang et al., 2002; Huang et al., 2003). The reconstitution of NEMO-deficient cells with wild-type NEMO restored NF- κ B activation in response to DNA damage (Huang et al., 2002). The dsDNA breaks promote the SUMO (small ubiquitin-like modifier) modification of nuclear NEMO, which prevents its nuclear export (Huang et al., 2003; Janssens et al., 2005). At the same time, activated ATM phosphorylates SUMOylated NEMO leading to the removal of SUMO and the attachment of ubiquitin (Wu et al., 2006). These modifications allow NEMO to enter in a complex with ATM to be exported to the cytoplasm, where ATM mediates K63-linked polyubiquitination of ELKS and TRAF6 (Hinz et al., 2010; Wu et al., 2010). In addition NEMO is monoubiquitinated on lysine 285 via cIAP1 (Hinz et al., 2010). The polyubiquitinated complex activates IKK ϵ in a TAK1-dependent manner. Activated IKK ϵ then phosphorylates I κ B α leading to K48-linked polyubiquitination and the subsequent degradation of I κ B α by the proteasome (Figure 2 and Scheidereit 2006). The free NF- κ B (p50/p65) dimer undergoes nuclear translocation and induces the transcription of pro-survival genes (Beg & Baltimore, 1996; Wang et al., 1998).

The activation of NF- κ B in tumor cells in response to constitutive genotoxic stress has been suggested to be tumor-promoting (Annunziata et al., 2007; Grosjean-Raillard et al., 2009;

Meylan et al., 2009). The ATM-NEMO-NF- κ B pathway is constitutively activated in acute myeloid leukemia (AML) cell lines, samples from high-risk myelodysplastic syndrome (MDS) and AML patients (Grosjean-Raillard et al., 2009), multiple myeloma (Annunziata et al., 2007) and lung adenocarcinomas (Meylan et al., 2009). The pharmacological inhibition or knockdown of ATM in AML cell lines ablated ATM-NEMO interactions, downregulated NF- κ B and induced apoptosis (Grosjean-Raillard et al., 2009). In summary, the constitutive activation of the DDR in early cancer not only induces cell cycle arrest, thereby establishing a barrier to cancer progression, but also promotes the survival of cancer cells by the activation of NF- κ B (Bartkova et al., 2005; Gorgoulis et al., 2005).

Apart from promoting tumorigenesis, DDR-mediated NF- κ B activation also plays an important role in lymphocyte development and survival (Bredemeyer et al., 2008). DsDNA breaks are generated as result of recombinase activating gene (RAG) expression during V(D)J recombination in pre-B cells. The subsequent activation of the ATM-NEMO-NF- κ B pathway is critical for the expression of genes involved in lymphocyte development, survival and function (Bredermeyer et al., 2008).

3.2 Interferon regulatory factors

IRFs are a class of transcription factors that have diverse roles in immune responses (Honda & Taniguchi, 2006). There are nine members in the mammalian IRF family. Each IRF contains a well-conserved DNA-binding domain, which recognizes a consensus DNA sequence known as the IFN-stimulated response element (ISRE) (Honda & Taniguchi, 2006). ISRE sequences are found on the promoter regions of type I interferons (IFN- α and IFN- β) and other pro-inflammatory genes, thus making IRFs the essential mediators of IFN- α/β and other pro-inflammatory cytokines (Tanaka et al., 1993; Taniguchi et al., 2001). IRFs are well known to be activated upon binding of invariant microbial motifs, often referred to as pattern-associated molecular patterns (PAMPs), to pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Akira et al., 2006; Creagh & O'Neill, 2006; Kanneganti et al., 2007; Yoneyama and Fujita, 2007). However, some IRFs are also activated in response to genotoxic stress as discussed below in more detail (Taniguchi et al., 2001).

3.2.1 IRF-1 and IRF-2

The link between IRFs and the DDR was first shown for IRF-1. IRF-1 was found to be essential for the apoptosis of T lymphocytes and embryonic fibroblasts in response to ionizing radiation or chemotherapeutic agents (Tanaka et al., 1994; Tamura et al., 1995). Recent studies have shown that the overexpression of IRF-1 results in the apoptosis of cancer cells through both cell intrinsic mitochondrial and extrinsic death ligand pathways (Strang et al., 2007; Gao et al., 2010). Furthermore, ATM-deficient cells derived from patients with ataxia telangiectasia (AT) fail to induce IRF-1 mRNA transcription in response to DNA damage (Pamment et al., 2002). The reconstitution of ATM restored IRF-1 induction in response to radiation (Pamment et al., 2002). These findings suggest that IRF-1 participates in DDR-mediated cell cycle arrest, although the precise molecular mechanisms still need to be established in more details (Tanaka et al., 1996).

3.2.2 IRF-3

IRF-3 interacts with CREB-binding protein (CBP)/p300 co-activators to form a dsDNA-activated transcription factor 1 (DRAF-1) complex which binds to the ISRE of type 1

interferons and other interferon-stimulated genes (ISGs) (Weaver et al., 1998). In contrast to other IRF members, IRF-3 is constitutively expressed in the cytoplasm of most cells (Kumar et al., 2000). IRF-3 is activated through phosphorylation by TANK-binding kinase 1 (TBK1) and/or IKKε leading to its dimerization and translocation to the nucleus (Kim et al., 1999; Fitzgerald et al., 2003). It was later discovered that IRF-3 is also a direct target of DNA-PK (Karpov et al., 2002). Our own data suggest that IRF-3 phosphorylation and activation in response to DNA damaging agents Ara-C or aphidicolin critically depends on ATM and ATR (Lam et al., submitted). Interestingly, IRF-3 may also participate in the DDR-mediated anti-cancer barrier. A dominant negative mutant of IRF-3 promoted the transformation of NIH3T3 cells and tumorigenesis *in vivo* (Kim et al., 2003). The overexpression of IRF-3 inhibited the proliferation of fibroblasts and astrocytes. Interestingly, the IRF-3 induced cell cycle arrest depended on p53 (Kim et al., 2006). Similarly, over-expression of IRF-3 in B16 melanoma cells resulted in growth suppression *in vivo* (Duguay et al., 2002). Recent evidence suggests that IRF-3 also induces apoptosis under certain circumstances such as in response to Sendai virus and NDV virus infection (Heylbroeck et al., 2000; Weaver et al., 2001). The molecular mechanisms of IRF-3-induced apoptosis are not well understood, but may in part rely on the ISRE promoter element present in TNF-related apoptosis-inducing ligand (TRAIL), an important member of the apoptotic machinery (Kirschner et al., 2005). In summary, it is possible that IRF-3 acts as a tumor suppressor gene that partially depends on p53 for its function.

3.2.3 IRF-5

IRF-5 is constitutively expressed in cytoplasm of a variety of cell types, particularly, in cells of lymphoid origins (Barnes et al., 2001; Yanai et al., 2007). IRF-5 is a direct target of p53 (Mori et al., 2002) and IRF-5 transcript levels further increase in response to DNA damage (Barnes et al., 2003; Hu et al., 2005). However, the induction of p53 target genes was not impaired in IRF5-deficient cells (Hu et al., 2005). Overexpression of IRF-5 rendered cells more susceptible to DNA damage-induced apoptosis even in p53-deficient cancer cell lines (Hu et al., 2005). In response to TLR agonists or DNA damage, IRF-5 is activated, possibly by phosphorylation, and translocates to the nucleus. Similar to other IRFs it promotes gene transcription by binding to target ISRE sequences in the regulatory region of target genes (Barnes et al., 2001), such as the interleukin-12b gene (Takaoka et al., 2005). In summary IRF-5 is a type I IFN-responsive p53 target gene that induces the expression of target genes distinct from those of p53.

3.2.4 Type I IFNs

Type I IFNs belong to a multigene family that includes IFN-α and IFN-β. Type I IFNs are expressed rapidly in response to many viral infections (Tanaka et al., 1998). They are best known to induce the expression of genes that increase the resistance of cells to virus infection (Taniguchi & Takaoka, 2002). In addition, type I IFNs were shown to modulate other cellular functions, such as proliferation and apoptosis (reviewed in Chawla et al., 2003). The increased sensitivity of cells to apoptosis in the presence of type I IFNs depends in part on their ability to increase p53 protein levels (Takaoka et al., 2003). Binding of type I IFNs to the IFN receptor activates the receptor-associated kinases Jak1 and Tyk2 leading to phosphorylation of the STAT1 and STAT2 proteins. The activated STAT proteins bind the IFN-regulatory factor 9 (IRF-9) to form the trimeric IFN-stimulated gene factor 3 (ISGF-3)

complex. The ISGF-3 complex translocates to the nucleus and binds to ISREs sites present in the mouse and human p53 genes thereby activating p53 transcription (Takaoka et al., 2003). However, type I IFN treatment is not sufficient to activate p53. It rather enhances the p53 response, thereby rendering cells more sensitive to the DDR (Takaoka et al., 2003; Yuan et al., 2007). In clinical trials type I IFNs have been successfully utilized for both first-line and salvage therapy for a variety of cancers such as human papilloma virus (HPV)-associated cervical cancer, hepatic cancer and leukemias (Parmar & Plataniias, 2003; Wang et al., 2011).

3.3 NKG2D ligands

One of the best-characterized NK cell-activating receptor in the context of cancer is the NKG2D receptor (reviewed in Raulet, 2003). All NK cells constitutively express the NKG2D receptor. In humans its cell surface expression requires association with the adaptor protein DAP10. Engagement of NKG2D leads to cytokine secretion and cytotoxicity (Billadeau et al., 2003; Upshaw et al., 2006). NKG2D recognizes MHC class I chain-related (MIC) A and B proteins and RAET1 (retinoic acid early transcript 1) gene family members in humans (Raulet, 2003, Cosman et al., 2001). No MIC homologs have been found in the mouse genome so far. The mouse *Rae1* genes can be further divided into *Rae1*, *H60* and *Mult1* subfamilies that share little homology but are structurally similar. The *Rae1* subfamily consists of highly related isoforms *Rae1 α* - *Rae1 ϵ* encoded by different genes (Diefenbach et al., 2000; Cerwenka et al., 2000; Raulet, 2003). NKG2D ligand expression has been observed on tumors of many origins, in particular in solid tumors, lymphomas and myeloid leukemia (Groh et al., 1999; Pende et al., 2002; Rohner et al., 2007). NKG2D was shown to be critical for the immunosurveillance of carcinoma, epithelial and lymphoid tumors in mouse models of de novo tumorigenesis. We and others have demonstrated that NKG2D ligand expression can be induced by DNA damage and oxidative stress (Gasser et al., 2005; Peraldi et al., 2009). The upregulation of NKG2D ligands in response to DNA damage critically depends on ATR or ATM, depending on the nature of the DNA damage (Gasser et al., 2005). On tumour cells that constitutively express NKG2D ligands, inhibition of the DDR decreased ligand cell surface expression (Gasser et al., 2005), suggesting that persistent DNA damage in the tumour cells at least partially maintains constitutive NKG2D ligand expression. An important question is if the p53- and the NKG2D-mediated tumor surveillance are linked or provide independent protection against the development of malignant cells. In favor of the latter idea, we found that NKG2D ligands could be induced in cells that lacked p53. While p53 is not required for the expression of NKG2D ligands in tumor cell lines or in cells with DNA damage, it is possible that other p53 family members, along with p53, function in a partially redundant fashion to induce NKG2D ligand expression. Intriguingly, the loss of p53 is implicated in the loss of genomic stability. It is therefore plausible that the resulting genomic lesions may further increase the DDR and upregulate the expression of NKG2D ligands on tumor cells.

3.4 DNAM-1 ligands

Although NKG2D is a major receptor implicated in recognition of cells with damaged DNA, NKG2D blocking experiments suggested that additional immunomodulatory molecules are required (Gasser et al., data not shown). A recent study showed that the DDR also upregulates the expression of DNAM-1 ligands (Soriani et al., 2009). DNAM-1 ligands include CD155 (also called poliovirus receptor, tumor associated antigen 4 and *necl-5*) and

CD112 (Nectin-2) (Bottino et al., 2003). CD112 and CD155 are ubiquitously expressed on most normal cells of neuronal, epithelial endothelial and fibroblast origin, however their expression levels are significantly enhanced in tumor cells including acute myeloid leukemias, neuroblastomas, melanomas and colorectal carcinomas (Castriconi et al., 2004; Carlsten et al., 2007; El-Sherbiny et al., 2007). DNAM-1 is a member of the immunoglobulin superfamily and is constitutively expressed on most immune cells including T cells, NK cells, a subset of B cells and monocytes/macrophages (Shibuya et al., 1996). DNAM-1 is physically and functionally associated with LFA-1, a receptor for ICAM-1 which is also upregulated in response to DNA damage (see below). The expression of CD112 or CD155 on tumor cells induces NK cell- and CD8⁺ T cell-mediated cytotoxicity and cytokine secretion (Bottino et al., 2003). Strikingly, DNAM-1-deficient mice injected with carcinogen-induced tumor cells developed tumors faster and showed higher mortality (Iguchi-Manaka et al., 2008). CD155 is also recognized by CD96, a stimulatory receptor expressed by NK cells and other immune cells (Fuchs et al., 2004). The existence of a dual receptor system recognizing CD155 further suggests an important role of this ligand in NK cell-mediated recognition of tumor cells. In addition to its activating functions, CD155 has recently also been shown to suppress immune cell activation through a third receptor called TIGIT/ VSTM/WUCAM, primarily expressed on T cells and on NK cells (Stanietsky et al., 2009). Moreover, the binding of CD155 to TIGIT on DCs leads to the secretion of IL-10 and inhibition of pro-inflammatory cytokine secretion (Yu et al., 2009). CD155 has higher affinity for TIGIT than DNAM-1. In contrast CD112 preferentially binds to DNAM-1 (Bottino et al., 2003). Hence the over-all avidity of cells for DNAM-1 over TIGIT may ultimately determine if an immune response is initiated or inhibited by DNAM-1 ligands.

3.5 ICAM-1

Intercellular adhesion molecule-1 (ICAM-1, also called CD54) is a cell adhesion molecule, which is expressed by fibroblasts, epithelial, endothelial and immune cells such as lymphocytes and macrophages (Dustin et al., 1986; Rothlein et al., 1986). Binding of ICAM-1 to its receptors LFA-1 and macrophage-1 antigen (Mac-1) expressed on leukocytes is often required to initiate inflammatory and immune responses (Simmons et al., 1988; Diamond et al., 1993; Sleigh et al., 1993). The expression of ICAM-1 is induced by several pro-inflammatory cytokines (Dustin et al., 1986; Pober et al., 1986). However, ICAM-1 expression has also been shown to be upregulated by ionizing radiation in a p53-dependent manner (Hallahan et al., 1996; Gaugler et al., 1997; Hallahan & Virudachalam, 1997). Recently it was discovered that ICAM-1 expression correlates with senescence (see 4.1 and Gourgoulis et al., 2005).

4. The role of DDR in diseases

4.1 Senescence-associated secretory phenotype (SASP)

If low levels of DNA damage persist in cells, the DDR induces an irreversible cell cycle arrest called senescence. Recent data have shown *in vivo* accumulation of senescent cells with age (Herbig et al., 2006; Jeyapalan et al., 2007). Senescent cells secrete a broad spectrum of factors, including the cytokines IL-6, IL-8, transforming growth factor- β (TGF- β), plasminogen activator inhibitor 1 (PAI-1), and others, collectively often referred to as the senescence-associated secretory phenotype (SASP) (Kortlever & Bernards, 2006; Coppé et al., 2009; Rodier et al., 2009). There is good evidence that some of these factors contribute to

senescence entry and maintenance. For instance, the autocrine secretion of IL-6 is required for the establishment of oncogene-induced senescence (Kuilman et al. 2008). Some IRFs, including IRF-1, IRF-5 and IRF-7, have been functionally linked to senescence (Li et al., 2008; Upreti et al., 2010). Some members of the SASP do not function exclusively in a cell-autonomous manner, but they also affect neighboring cells. Paradoxically, their paracrine effects sometimes promote tumorigenesis. IL-6 contributes to tumorigenesis by promoting angiogenesis (Wei et al., 2003; Fan et al., 2008). In addition, IL-6 secretion by HRasV12-transformed cancer cells has been reported to mediate tumour growth (Leslie 2010). Tumor-promoting effects have also been described for other SASP members, such as TGF- β (reviewed in Bierie & Moses, 2006), IL-1 (Dejana et al., 1988; Voronov et al., 2003) and IL-8 (Norgauer et al., 1996). These opposite effects may be explained by differences in cells type, stage of transformation or the mode of signaling (autocrine versus paracrine). It is possible that healthy, normal cells enter senescence in response to oncogene-induced DNA damage, and possibly due to the subsequent SASP, whereas the SASP can promote tumorigenesis in neighboring precancerous lesions harboring specific mutations. DNA damage-induced senescence may therefore have dual roles in preventing and promoting tumorigenesis, depending on the cellular context. Some characteristics of senescent cells, such as the ability of SASP members to modify the extracellular environment, may play a role in aging and age-related pathology (Chung et al., 2009). Of note many of DDR-induced ligands for activating immune receptors, such as NKG2D, DNAM-1 and LFA-1 are upregulated in senescent cells. It remains currently unclear if the underlying DDR in senescent cells is regulating the expression of these ligands or if the expression depends on senescence-specific pathways.

4.2 Cancer

As mentioned earlier, the DDR may represent a major barrier to tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). Replication stress in response to oncogene activation results in the collapse of DNA replication forks. The resulting DNA breaks activate the DDR, leading to either senescence or cellular apoptosis. In addition to these largely cell-intrinsic barrier effects of the DDR, recent evidence suggests that cell-extrinsic barriers could exist, some of which may depend on the immune system. A link between the DDR and immune system was suggested by the upregulation of ligands for the activating immune receptors NKG2D, DNAM-1 and LFA-1 in tumor cells or in cells undergoing genotoxic stress. In addition the DDR also regulates the expression of the apoptosis-inducing death receptor 5 (DR5), a ligand for TRAIL (Wu et al., 1997). NKG2D, DNAM-1 and LFA-1 participate in 'induced self-recognition' of target cells by cytotoxic NK cells (Lakshmikanth et al., 2009). "Induced self-ligands" are absent or only poorly expressed by normal cells, but upregulated on diseased cells (Castriconi et al., 2004; Gasser et al., 2005; Gorgoulis et al., 2005). The activating receptors NKG2D, DNAM-1 and LFA-1 are mainly expressed by natural killer (NK) cells and T cells, which play an important role in the immunity against cancer (Shibuya et al., 1996; Barber et al., 2004; El-Sherbiny et al., 2007). The recognition of tumor cells by NK cells is governed by activating and inhibitory receptor-mediated signals (Gasser & Raulet, 2006). Many of the inhibitory receptors expressed by NK cells are specific for major histocompatibility complex (MHC) class I molecules. MHC class I molecules are expressed by normal cells but are often downregulated from tumour cells. Increased expression of activating ligands by tumor cells can override inhibitory receptor signaling, resulting in NK cell activation and NK cell-mediated lysis of tumor cells. NK cells also

produce pro-inflammatory cytokines such as IFN- γ , which help to initiate an adaptive immune response (reviewed in Kos, 1998). In addition to their role in NK cells, NKG2D, DNAM-1 and LFA-1 provide signals that enhance the activation of specific T cell subsets, such as the cytotoxic CD8⁺ T cells (Shibuya et al., 1996; Barber et al., 2004; Gasser & Raulet, 2006). The qualitative and quantitative effector responses of NK and T cells are regulated by cytokines such as interleukin-2 (IL-2), IL-12, IL-15, IL-18, IL-21, TGF- β and the type I IFNs (Biron et al., 1999). Hence, in addition to the effects described above, the DDR-induced expression of type I interferons may also help in stimulating an immune response through the activation of NK and T cells.

4.3 Viral infections

The DDR is also triggered when cells are infected with certain viruses, including retroviruses such as the human immunodeficiency virus 1 (HIV-1), adenoviruses, herpes simplex viruses 1 and 2 (HSV-1 and 2), cytomegalovirus (CMV), hepatitis B virus, Epstein-Barr virus (EBV) and the human papilloma virus type 16 and 18 (HPV-16 and 18) (Lilley et al., 2007). In many cases, the DDR is triggered in response to viral nucleic acid intermediates produced during the viral "life cycle" (Lilley et al., 2007). The importance of the DDR in preventing virus-induced tumorigenesis is evidenced by the fact that oncogenic viruses infect many cells but rarely lead to tumorigenesis. For example, infectious mononucleosis can be caused by the infection of EBV, but rarely leads to Burkitt's and Hodgkin's lymphoma (Lemon et al., 1977). The ATM-CHK2 pathway is triggered in B cells during a latent EBV infection, which is thought to suppress EBV-induced transformation by inducing cell cycle arrest and apoptosis (Nikitin et al., 2010). Adenovirus infection results in the phosphorylation of ATM and H2AX, the stabilization of p53 and the downregulation of the anti-apoptotic protein myeloid cell leukemia 1 (MCL-1), thereby promoting the induction of apoptosis in virus-infected cells (Debbas & White, 1993; Lowe & Ruley, 1993a; Cuconati et al., 2003). In summary, the DDR is not restricted to controlling tumorigenesis induced by the activation of host oncogenes, but also functions to control the activity of viral genes and may therefore participate in defending organisms from viral infections. In support of this idea, p53-deficient mice show higher viral titer and mortality after vesicular stomatitis virus infection (Takaoka et al., 2003). In another study, the knockdown of p53 in a liver cell line resulted in higher levels of hepatitis C virus replication (Dharel et al., 2008). In addition, p53 was shown to be activated in cells infected with the Newcastle disease virus, herpes simplex virus and influenza virus (Takaoka et al., 2003; Turpin et al., 2005).

Many viruses have developed means to interfere with the DDR, further supporting the idea that the DDR may restrict viral infection and proliferation of infected cells. The adenovirus core protein VII protects the viral genome from the DDR (Karen & Hearing, 2011). Tax, a protein encoded by HTLV-1 attenuates the ATM-mediated DDR by interacting with CHK1 and CHK2 (Park et al., 2004; Park et al., 2005). The activation of the DDR is disrupted by the human CMV through altering the localization of CHK2 by viral structural proteins (Gaspar & Shenk, 2005). During the EBV infection of B cells, the latent EBNA3C protein attenuates the DDR by modulating CHK2 and p53 activity (Nikita et al., 2010). Other proteins (E6 protein of the HPV-16, HPV-18, S40 large T antigen of simian virus etc.) of oncogenic viruses interfere with p53 functions in infected cells (Werness et al., 1990; Kassis et al., 1993).

Despite the potential antiviral properties of the DDR, many viruses have also evolved ways to activate at least part of the DDR for their own replication. In retroviral integration, for instance, the viral integrase cleaves the host DNA to facilitate the integration of the viral

double-stranded cDNA, and as a consequence, leaves a dsDNA break that requires NHEJ repair (Skalka & Katz, 2005). Viral replication of HIV-1 was suppressed when cells were treated with an ATM-specific inhibitor (Lau et al., 2005). Furthermore, HIV-1 encodes a protein, Vpr, which activates the ATR-CHK1 pathway to arrest infected cells in the G2 phase of the cell cycle and to repair dsDNA breaks by homologous recombination (Goh et al., 1998; Roshal et al., 2003; Nakai-Murakami et al., 2006).

The activation of the DDR in response to viral infection renders cell sensitive to immune cell-mediated lysis by upregulating ligands for NKG2D, DNAM-1 and LFA-1. Two recent reports show that HIV-1 ATR-CHK1 activation by the HIV-1 Vpr upregulates the expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing (Richard et al., 2009; Ward et al., 2009). EBV-transformed B-cell lines are relatively resistant to NK cell-mediated lysis possibly as a result of their attenuated DDR in addition to high expression of MHC class I molecules, which inhibit NK cells (Pappworth et al., 2007). However, the reactivation of EBV in transformed B cells renders them susceptible to NK-cell-mediated lysis, which was partially depends on NKG2D and DNAM-1 (Pappworth et al., 2007). NKG2D ligand expression is upregulated upon infection by a number of viruses, such as CMV, HBV, poxvirus and hepatitis C virus, although the role of the DDR in the regulation has yet to be explored in detail.

A number of viruses have developed means to interfere with the expression of ligands for activating receptors. This phenomenon is best characterized for the ligands of NKG2D. Nef (Negative factor) protein encoded by HIV-1 downregulates the expression of NKG2D ligands, HLA-A and HLA-B, to potentially evade recognition by NK cells and HLA-A-/HLA-B- restricted HIV-1-specific cytotoxic T cells (McMichael 1998; Cerboni et al., 2007). Hepatitis C virus impairs the NKG2D-dependent NK cell responses by downregulating NKG2D ligand and receptor expression (Wen et al., 2008). Both murine and human CMV have developed strategies to evade the NKG2D-dependent recognition. The murine CMV encodes the viral glycoproteins m138, m145 and m152 for evasion strategies. The m152 targets Rae1 for degradation (Lodoen et al., 2003), m145 and m138 prevent MULT1 expression (Krmptotic et al., 2005), while m138 cooperates with m155 to impair H60 expression (Lodoen et al., 2004; Lenac et al., 2006). The human CMV (HCMV)-encoded UL16 protein inhibits the expression of MICB, ULBP1, ULBP2 and RAET1G (Dunn et al., 2003; Rölle et al., 2003). The HCMV protein UL142 prevents the expression of some, but not all, alleles of MICA (Chalupny et al., 2006). Some alleles of MICA, such as the prevalent allele of MICA, MICA*008, are resistant to downregulation by HCMV because of a truncation of the cytoplasmic domain (Chalupny et al., 2006). These polymorphisms may reflect a counter-offensive of the host to evade viral protein-mediated inhibition of NKG2D ligand expression. Furthermore, it was recently discovered that a microRNA encoded by HCMV downregulates MICB expression by targeting a specific site in the *MICB* 3' untranslated region (Stern-Ginossar et al., 2007). Finally, the HCMV protein UL141 protein impedes the expression of DNAM-1 ligand CD155 (Tomasec et al., 2005). Interestingly, CD155 functions as a poliovirus receptor, but the role of NK cells or the DDR in poliovirus infection has not been studied in detail. Taken together, the DDR presents a challenge to many viruses as their replication critically depends on certain aspects of the DDR. At the same time, the DDR can induce apoptosis of infected cells or render infected cells sensitive to immune cell-mediated lysis (Figure 2). In response, viruses most likely target the specific effector molecules of the DDR that prevent their subsequent infection of new target cells, while leaving the part of the pathway required for their replication intact.

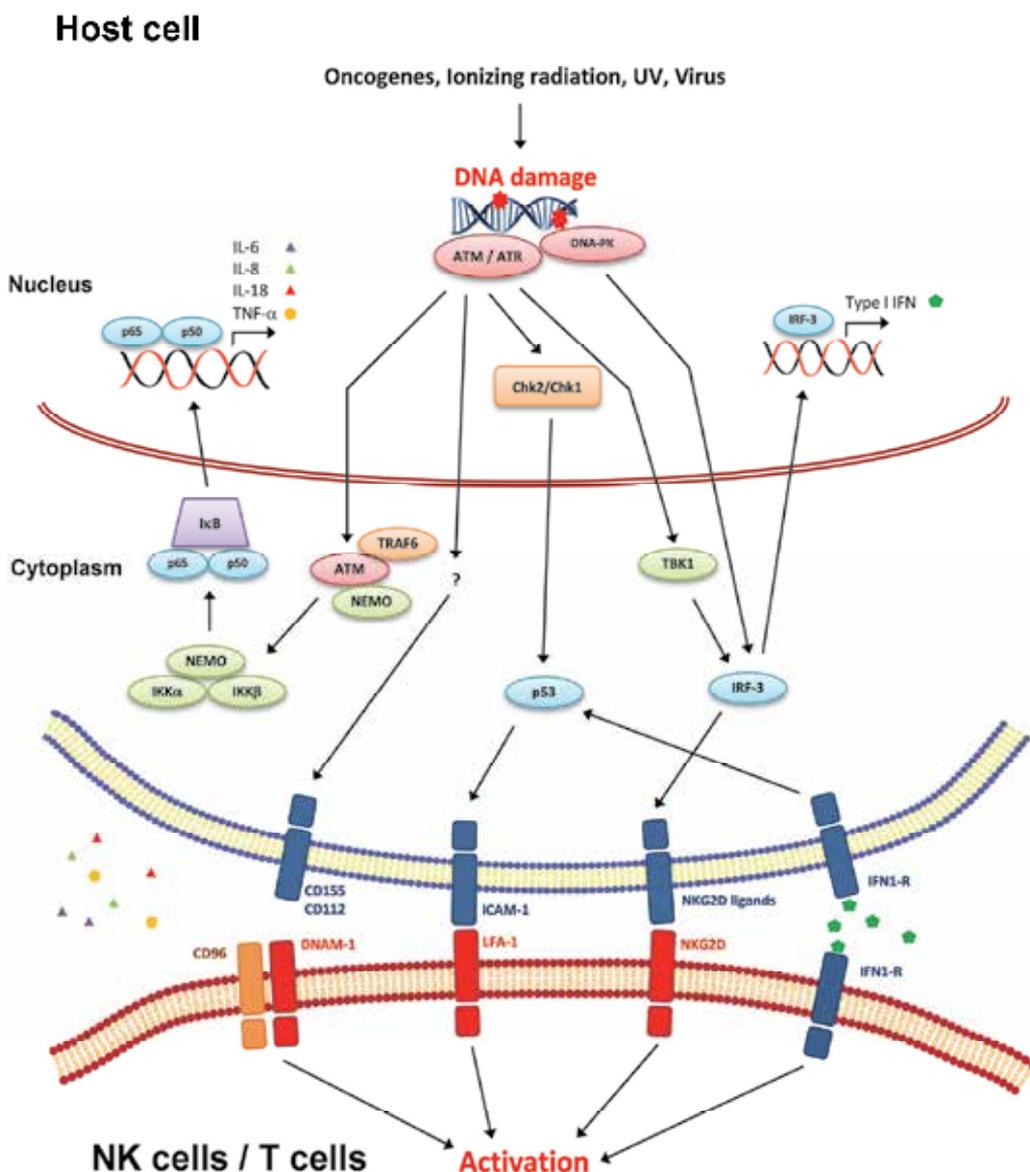


Fig. 2. Potential links between the DDR and the immune system. Activation of the DDR activates cytotoxic natural killer and T cells by inducing the expression of proinflammatory cytokines and ligands for activating immune receptors.

5. Conclusion

The DDR is activated in response to genotoxic stress caused by oncogene activation, viral infection or other environmental insults to the cell. The DDR initiates cell autonomous programmes to try to repair the damaged DNA, or to induce apoptosis if the damage is not repairable, in order to preserve the genome integrity and the survival of the organism.

Recent evidence links the DDR to innate and possibly, adaptive immunity. The activation of an immune response may contribute to the removal of these potentially harmful cells (Figure 2).

6. References

- Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes & Development* 15: 2177-2196
- Achanta G, Pelicano H, Feng L, Plunkett W & Huang P (2001) Interaction of p53 and DNA-PK in Response to Nucleoside Analogues. *Cancer Research* 61: 8723-8729
- Akira S, Uematsu S & Takeuchi O (2006) Pathogen Recognition and Innate Immunity. *Cell* 124: 783-801
- Annunziata CM, Davis RE, Demchenko Y, Bellamy W, Gabrea A, Zhan F, Lenz G, Hanamura I, Wright G & Xiao W (2007) Frequent Engagement of the Classical and Alternative NF- κ B Pathways by Diverse Genetic Abnormalities in Multiple Myeloma. *Cancer Cell* 12: 115-130
- Baeuerle PA & Henkel T (1994) Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol* 12: 141-179
- Banin S, Moyal L, Shieh S, Taya Y, Anderson C, Chessa L, Smorodinsky N, Prives C, Reiss Y & Shiloh Y (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 281: 1674
- Barber DF, Faure M & Long EO (2004) LFA-1 Contributes an Early Signal for NK Cell Cytotoxicity. *The Journal of Immunology* 173: 3653-3659
- Barnes BJ, Kellum MJ, Pinder KE, Frisancho JA & Pitha PM (2003) Interferon Regulatory Factor 5, a Novel Mediator of Cell Cycle Arrest and Cell Death. *Cancer Research* 63: 6424-6431
- Barnes BJ, Moore PA & Pitha PM (2001) Virus-specific Activation of a Novel Interferon Regulatory Factor, IRF-5, Results in the Induction of Distinct Interferon alpha Genes. *J. Biol. Chem.* 276: 23382-23390
- Bartek J, Falck J & Lukas J (2001) Chk2 kinase - a busy messenger. *Nat Rev Mol Cell Biol* 2: 877-886
- Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldborg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J & Bartek J (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434: 864-870
- Basu S, Rosenzweig KR, Youmell M & Price BD (1998) The DNA-Dependent Protein Kinase Participates in the Activation of NF[kappa]B Following DNA Damage. *Biochemical and Biophysical Research Communications* 247: 79-83
- Beg AA & Baltimore D (1996) An Essential Role for NF- κ B in Preventing TNF- α -Induced Cell Death. *Science* 274: 782-784
- Bennett M, Macdonald K, Chan S, Luzio J, Simari R & Weissberg P (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 282: 290
- Bierie B & Moses HL (2006) TGF- β and cancer. *Cytokine & Growth Factor Reviews* 17: 29-40
- Billadeau DD, Upshaw JL, Schoon RA, Dick CJ & Leibson PJ (2003) NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol* 4: 557-564

- Biron CA, Nguyen KB, Pien GC, Cousens LP & Salazar-Mather TP (1999) NATURAL KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate Cytokines. *Annu. Rev. Immunol.* 17: 189-220
- Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, Vitale M, Moretta L, Lopez M & Moretta A (2003) Identification of PVR (CD155) and Nectin-2 (CD112) as Cell Surface Ligands for the Human DNAM-1 (CD226) Activating Molecule. *The Journal of Experimental Medicine* 198: 557-567
- Brach MA, Hass R, Sherman ML, Gunji H, Weichselbaum R & Kufe D (1991) Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. *J Clin Invest* 88: 691-695
- Bredemeyer AL, Helmink BA, Innes CL, Calderon B, McGinnis LM, Mahowald GK, Gapud EJ, Walker LM, Collins JB, Weaver BK, Mandik-Nayak L, Schreiber RD, Allen PM, May MJ, Paules RS, Bassing CH & Sleckman BP (2008) DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. *Nature* 456: 819-823
- Brown EJ & Baltimore D (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* 14: 397-402
- Cai Z, Chehab NH & Pavletich NP (2009) Structure and Activation Mechanism of the CHK2 DNA Damage Checkpoint Kinase. *Molecular Cell* 35: 818-829
- Carlsten M, Björkström NK, Norell H, Bryceson Y, van Hall T, Baumann BC, Hanson M, Schedvins K, Kiessling R, Ljunggren H-G & Malmberg K-J (2007) DNAX Accessory Molecule-1 Mediated Recognition of Freshly Isolated Ovarian Carcinoma by Resting Natural Killer Cells. *Cancer Research* 67: 1317-1325
- Carter T, Vancurová I, Sun I, Lou W & DeLeon S (1990) A DNA-activated protein kinase from HeLa cell nuclei. *Mol Cell Biol* 10: 6460-6471
- Carvalho G, Fabre C, Braun T, Grosjean J, Ades L, Agou F, Tasdemir E, Boehrer S, Israel A, Veron M, Fenaux P & Kroemer G (2007) Inhibition of NEMO, the regulatory subunit of the IKK complex, induces apoptosis in high-risk myelodysplastic syndrome and acute myeloid leukemia. *Oncogene* 26: 2299-2307
- Castriconi R, Dondero A, Corrias MV, Lanino E, Pende D, Moretta L, Bottino C & Moretta A (2004) Natural Killer Cell-Mediated Killing of Freshly Isolated Neuroblastoma Cells. *Cancer Research* 64: 9180-9184
- Cerboni C, Neri F, Casartelli N, Zingoni A, Cosman D, Rossi P, Santoni A & Doria M (2007) Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen Virol* 88: 242-250
- Cerwenka A (2000) Retinoic Acid Early Inducible Genes Define a Ligand Family for the Activating NKG2D Receptor in Mice. *Immunity* 12: 721-727
- Chalupny NJ, Rein-Weston A, Dosch S & Cosman D (2006) Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. *Biochemical and Biophysical Research Communications* 346: 175-181
- Chawla-Sarkar M, Lindner DJ, Liu Y-F, Williams BR, Sen GC, Silverman RH & Borden EC (2003) Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 8: 237-249

- Chen X, Bargonetti J & Prives C (1995) p53, through p21 (WAF1/CIP1), Induces Cyclin D1 Synthesis. *Cancer Research* 55: 4257-4263
- Chipuk JE, Maurer U, Green DR & Schuler M (2003) Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. *Cancer Cell* 4: 371-381
- Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, Carter C, Yu BP & Leeuwenburgh C (2009) Molecular inflammation: Underpinnings of aging and age-related diseases. *Ageing Research Reviews* 8: 18-30
- Cogswell J, Godlevski M, Wisely G, Clay W, Leesnitzer L, Ways J & Gray J (1994) NF-kappa B regulates IL-1 beta transcription through a consensus NF- kappa B binding site and a nonconsensus CRE-like site. *The Journal of Immunology* 153: 712-723
- Coppé J-P, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, Nelson PS, Desprez P-Y & Campisi J (2008) Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLoS Biol* 6: e301
- Cortez D, Guntuku S, Qin J & Elledge SJ (2001) ATR and ATRIP: Partners in Checkpoint Signaling. *Science* 294: 1713-1716
- Cosman D, Müllberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M & Chalupny NJ (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14: 123-133
- Creagh EM & O'Neill LAJ (2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends in Immunology* 27: 352-357
- Cuconati A, Mukherjee C, Perez D & White E (2003) DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev.* 17: 2922-2932
- Debbas M & White E (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes & Development* 7: 546-554
- Dejana E, Bertocchi F, Bortolami MC, Regonesi A, Tonta A, Breviario F & Giavazzi R (1988) Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. *J. Clin. Invest.* 82: 1466-1470
- deRojas-Walker T, Tamir S, Ji H, Wishnok JS & Tannenbaum SR (1995) Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem. Res. Toxicol* 8: 473-477
- Das S, Boswell SA, Aaronson SA & Lee SW (2008) p53 promoter selection: Choosing between life and death. *cc* 7: 154-157
- Dharel N, Kato N, Muroyama R, Taniguchi H, Otsuka M, Wang Y, Jazag A, Shao R, Chang J, Adler MK, Kawabe T & Omata M (2008) Potential contribution of tumor suppressor p53 in the host defense against hepatitis C virus. *Hepatology* 47: 1136-1149
- Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL & Springer TA (1993) The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *The Journal of Cell Biology* 120: 1031-1043
- Diefenbach A, Jamieson AM, Liu SD, Shastri N & Raulet DH (2000) Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* 1: 119-126

- Duguay D, Mercier F, Stagg J, Martineau D, Bramson J, Servant M, Lin R, Galipeau J & Hiscott J (2002) In Vivo Interferon Regulatory Factor 3 Tumor Suppressor Activity in B16 Melanoma Tumors. *Cancer Research* 62: 5148 -5152
- Dujka ME, Puebla-Osorio N, Tavana O, Sang M & Zhu C (2009) ATM and p53 are essential in the cell-cycle containment of DNA breaks during V(D)J recombination in vivo. *Oncogene* 29: 957-965
- Dumaz N & Meek DW (1999) Serine 15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *EMBO J* 18: 7002-7010
- Dunn C, Chalupny NJ, Sutherland CL, Dosch S, Sivakumar PV, Johnson DC & Cosman D (2003) Human Cytomegalovirus Glycoprotein UL16 Causes Intracellular Sequestration of NKG2D Ligands, Protecting Against Natural Killer Cell Cytotoxicity. *The Journal of Experimental Medicine* 197: 1427 -1439
- Dustin ML, Rothlein R, Bhan AK, Dinarello CA & Springer TA (1986) Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol* 137: 245-254
- Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med* 315: 1650-1659
- El-Sherbiny YM, Meade JL, Holmes TD, McConagle D, Mackie SL, Morgan AW, Cook G, Feyler S, Richards SJ, Davies FE, Morgan GJ & Cook GP (2007) The Requirement for DNAM-1, NKG2D, and Nkp46 in the Natural Killer Cell-Mediated Killing of Myeloma Cells. *Cancer Research* 67: 8444 -8449
- Falck J, Mailand N, Syljuasen RG, Bartek J & Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 410: 842-847
- Fan Y, Ye J, Shen F, Zhu Y, Yeghiazarians Y, Zhu W, Chen Y, Lawton MT, Young WL & Yang G-Y (2008) Interleukin-6 stimulates circulating blood-derived endothelial progenitor cell angiogenesis in vitro. *J Cereb Blood Flow Metab* 28: 90-98
- Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao S-M & Maniatis T (2003) IKK ϵ and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4: 491-496
- Fuchs A, Cella M, Giurisato E, Shaw AS & Colonna M (2004) Cutting Edge: CD96 (Tactile) Promotes NK Cell-Target Cell Adhesion by Interacting with the Poliovirus Receptor (CD155). *The Journal of Immunology* 172: 3994 -3998
- Gao J, Senthil M, Ren B, Yan J, Xing Q, Yu J, Zhang L & Yim J (2010) IRF-1 transcriptionally up-regulates PUMA which mediates the mitochondrial apoptotic pathway in IRF-1 induced apoptosis in cancer cells. *Cell Death Differ* 17: 699-709
- Gatei M, Sloper K, Sørensen C, Syljuäsen R, Falck J, Hobson K, Savage K, Lukas J, Zhou B-B, Bartek J & Khanna KK (2003) Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation. *Journal of Biological Chemistry* 278: 14806 -14811
- Gaspar M & Shenk T (2006) Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins. *Proceedings of the National Academy of Sciences of the United States of America* 103: 2821 -2826
- Gasser S (2007) DNA - damage response and development of targeted cancer treatments. *Annals of Medicine* 39: 457

- Gasser S & Raulet DH (2006) Activation and self-tolerance of natural killer cells. *Immunological Reviews* 214: 130-142
- Gasser S, Orsulic S, Brown EJ & Raulet DH (2005) The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436: 1186-1190
- Gaugler, C. Squiban, A. Van Der Mee M-H (1997) Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro. *Int J Radiat Biol* 72: 201-209
- Ghosh S & Karin M (2002) Missing Pieces in the NF- κ B Puzzle. *Cell* 109: S81-S96
- Ghosh S, May MJ & Kopp EB (1998) NF- κ B AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses. *Annu. Rev. Immunol.* 16: 225-260
- Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, Hahn BH & Emerman M (1998) HIV-1 Vpr increases viral expression by manipulation of the cell cycle: A mechanism for selection of Vpr in vivo. *Nat Med* 4: 65-71
- Gorgoulis VG, Pratsinis H, Zacharatos P, Demoliou C, Sigala F, Asimacopoulos PJ, Papavassiliou AG & Kletsas D (2005) p53-Dependent ICAM-1 overexpression in senescent human cells identified in atherosclerotic lesions. *Lab Invest* 85: 502-511
- Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH & Spies T (1999) Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$ T cells of MICA and MICB. *Proceedings of the National Academy of Sciences* 96: 6879 -6884
- Grosjean-Raillard J, Tailler M, Ades L, Perfettini J-L, Fabre C, Braun T, De Botton S, Fenaux P & Kroemer G (2008) ATM mediates constitutive NF-[kappa]B activation in high-risk myelodysplastic syndrome and acute myeloid leukemia. *Oncogene* 28: 1099-1109
- Halazonetis T, Gorgoulis V & Bartek J (2008) An oncogene-induced DNA damage model for cancer development. *Science* 319: 1352
- Hallahan DE & Virudachalam S (1997) Ionizing Radiation Mediates Expression of Cell Adhesion Molecules in Distinct Histological Patterns within the Lung. *Cancer Research* 57: 2096 -2099
- Hallahan D, Kuchibhotla J & Wyble C (1996) Cell Adhesion Molecules Mediate Radiation-induced Leukocyte Adhesion to the Vascular Endothelium. *Cancer Research* 56: 5150 -5155
- Hanahan D & Weinberg RA (2000) The Hallmarks of Cancer. *Cell* 100: 57-70
- Hayden MS & Ghosh S (2008) Shared Principles in NF- κ B Signaling. *Cell* 132: 344-362
- Herbig U, Ferreira M, Condel L, Carey D & Sedivy JM (2006) Cellular Senescence in Aging Primates. *Science* 311: 1257
- Heylbroeck C, Balachandran S, Servant MJ, DeLuca C, Barber GN, Lin R & Hiscott J (2000) The IRF-3 transcription factor mediates Sendai virus-induced apoptosis. *J. Virol* 74: 3781-3792
- Hinz M, Stilmann M, Arslan SÇ, Khanna KK, Dittmar G & Scheidereit C (2010) A Cytoplasmic ATM-TRAF6-cIAP1 Module Links Nuclear DNA Damage Signaling to Ubiquitin-Mediated NF- κ B Activation. *Molecular Cell* 40: 63-74
- Hirao A, Kong Y-Y, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ & Mak TW (2000) DNA Damage-Induced Activation of p53 by the Checkpoint Kinase Chk2. *Science* 287: 1824-1827

- Honda K & Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6: 644-658
- Hu G, Mancl ME & Barnes BJ (2005) Signaling through IFN Regulatory Factor-5 Sensitizes p53-Deficient Tumors to DNA Damage-Induced Apoptosis and Cell Death. *Cancer Res* 65: 7403-7412
- Huang TT, Feinberg SL, Suryanarayanan S & Miyamoto S (2002) The Zinc Finger Domain of NEMO Is Selectively Required for NF- κ B Activation by UV Radiation and Topoisomerase Inhibitors. *Mol. Cell. Biol.* 22: 5813-5825
- Huang TT, Wuerzberger-Davis SM, Seufzer BJ, Shumway SD, Kurama T, Boothman DA & Miyamoto S (2000) NF- κ B Activation by Camptothecin. *Journal of Biological Chemistry* 275: 9501-9509
- Huang TT, Wuerzberger-Davis SM, Wu Z-H & Miyamoto S (2003) Sequential Modification of NEMO/IKK γ by SUMO-1 and Ubiquitin Mediates NF- κ B Activation by Genotoxic Stress. *Cell* 115: 565-576
- Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S-ichiro, Yasui T, Kikutani H, Shibuya K & Shibuya A (2008) Accelerated tumor growth in mice deficient in DNAM-1 receptor. *The Journal of Experimental Medicine* 205: 2959-2964
- Jackson SP & Bartek J (2009) The DNA-damage response in human biology and disease. *Nature* 461: 1071-1078
- Janssens S, Tinel A, Lippens S & Tschopp J (2005) PIDD Mediates NF- κ B Activation in Response to DNA Damage. *Cell* 123: 1079-1092
- Jeyapalan JC, Ferreira M, Sedivy JM & Herbig U (2007) Accumulation of senescent cells in mitotic tissue of aging primates. *Mechanisms of Ageing and Development* 128: 36-44
- Kaneko YS, Watanabe N, Morisaki H, Akita H, Fujimoto A, Tominaga K, Terasawa M, Tachibana A, Ikeda K, Nakanishi M & Kaneko Y (1999) Cell-cycle-dependent and ATM-independent expression of human Chk1 kinase. *Oncogene* 18: 3673-3681
- Kanneganti T-D, Lamkanfi M & Núñez G (2007) Intracellular NOD-like Receptors in Host Defense and Disease. *Immunity* 27: 549-559
- Karen KA & Hearing P (2011) Adenovirus Core Protein VII Protects the Viral Genome from a DNA Damage Response at Early Times after Infection. *J. Virol.*: JVI.02540-10
- Karpova AY, Trost M, Murray JM, Cantley LC & Howley PM (2002) Interferon regulatory factor-3 is an in vivo target of DNA-PK. *Proc Natl Acad Sci U S A.* 99: 2818-2823
- Kastan M, Onyekwere O, Sidransky D, Vogelstein B & Craig R (1991) Participation of p53 protein in the cellular-response to DNA damage. *Cancer Research* 51: 6304-6311
- Kawanishi S, Hiraku Y, Pinlaor S & Ma N (2006) Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. *Biological Chemistry* 387: 365-372
- Khanna. KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998) ATM associates with and phosphorylates p53: mapping the region of interaction. *Nat Genet* 20: 398-400
- Kim TY, Lee K-H, Chang S, Chung C, Lee H-W, Yim J & Kim TK (2003) Oncogenic Potential of a Dominant Negative Mutant of Interferon Regulatory Factor 3. *Journal of Biological Chemistry* 278: 15272-15278

- Kim T, Kim TY, Song Y-H, Min IM, Yim J & Kim TK (1999) Activation of Interferon Regulatory Factor 3 in Response to DNA-damaging Agents. *J. Biol. Chem.* 274: 30686-30689
- Kim T-K, Lee J-S, Jung J-E, Oh S-Y, Kwak S, Jin X, Lee S-Y, Lee J-B, Chung YG, Choi YK, You S & Kim H (2006) Interferon regulatory factor 3 activates p53-dependent cell growth inhibition. *Cancer Letters* 242: 215-221
- Kirshner JR, Karpova AY, Kops M & Howley PM (2005) Identification of TRAIL as an Interferon Regulatory Factor 3 Transcriptional Target. *J. Virol.* 79: 9320-9324
- Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L & Cho KR (1993) Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. *Proceedings of the National Academy of Sciences of the United States of America* 90: 3988-3992
- Kos FJ (1998) Regulation of adaptive immunity by natural killer cells. *Immunol Res* 17: 303-312
- Kortlever RM & Bernards R (2006) Senescence, Wound Healing, and Cancer: the PAI-1 Connection. *cc* 5: 2697-2703
- Krmpotic A, Hasan M, Loewendorf A, Saulig T, Halenius A, Lenac T, Polic B, Bubic I, Kriegeskorte A, Pernjak-Pugel E, Messerle M, Hengel H, Busch DH, Koszinowski UH & Jonjic S (2005) NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. *The Journal of Experimental Medicine* 201: 211-220
- Kuilman T, Michaloglou C, Vredeveld LCW, Douma S, Doorn R van, Desmet CJ, Aarden LA, Mooi WJ & Peeper DS (2008) Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network. *Cell* Vol 133: 1019-1031
- Kumar KP, McBride KM, Weaver BK, Dingwall C & Reich NC (2000) Regulated Nuclear-Cytoplasmic Localization of Interferon Regulatory Factor 3, a Subunit of Double-Stranded RNA-Activated Factor 1. *Mol Cell Biol* 20: 4159-4168
- Kunkel TA (2004) DNA Replication Fidelity. *Journal of Biological Chemistry* 279: 16895-16898
- Kunsch C, Lang R, Rosen C & Shannon M (1994) Synergistic transcriptional activation of the IL-8 gene by NF-kappa B p65 (RelA) and NF-IL-6. *The Journal of Immunology* 153: 153-164
- Kurimasa A, Kumano S, Boubnov NV, Story MD, Tung C-S, Peterson SR & Chen DJ (1999) Requirement for the Kinase Activity of Human DNA-Dependent Protein Kinase Catalytic Subunit in DNA Strand Break Rejoining. *Mol Cell Biol* 19: 3877-3884
- Lakshmikanth T, Burke S, Ali TH, Kimpfler S, Ursini F, Ruggeri L, Capanni M, Umansky V, Paschen A, Sucker A, Pende D, Groh V, Biassoni R, Höglund P, Kato M, Shibuya K, Schadendorf D, Anichini A, Ferrone S, Velardi A, et al (2009) NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. *J Clin Invest* 119: 1251-1263
- Lau A, Swinbank KM, Ahmed PS, Taylor DL, Jackson SP, Smith GCM & O'Connor MJ (2005) Suppression of HIV-1 infection by a small molecule inhibitor of the ATM kinase. *Nat Cell Biol* 7: 493-500
- Lemon SM, Hutt LM, Shaw JE, Li J-LH & Pagano JS (1977) Replication of EBV in epithelial cells during infectious mononucleosis. *Nature* 268: 268-270

- Lenac T, Budt M, Arapovic J, Hasan M, Zimmermann A, Simic H, Krmpotic A, Messerle M, Ruzsics Z, Koszinowski UH, Hengel H & Jonjic S (2006) The herpesviral Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. *The Journal of Experimental Medicine* 203: 1843 -1850
- Leslie K, Gao S, Berishaj M, Podsypanina K, Ho H, Ivashkiv L & Bromberg J (2010) Differential interleukin-6/Stat3 signaling as a function of cellular context mediates Ras-induced transformation. *Breast Cancer Research* 12: R80
- Li N, Banin S, Ouyang H, Li GC, Courtois G, Shiloh Y, Karin M & Rotman G (2001) ATM Is Required for I κ B Kinase (IKK) Activation in Response to DNA Double Strand Breaks. *Journal of Biological Chemistry* 276: 8898-8903
- Li Q, Tang L, Roberts PC, Kraniak JM, Fridman AL, Kulaeva OI, Tehrani OS & Tainsky MA (2008) Interferon Regulatory Factors IRF5 and IRF7 Inhibit Growth and Induce Senescence in Immortal Li-Fraumeni Fibroblasts. *Molecular Cancer Research* 6: 770 - 784
- Libermann TA & Baltimore D (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol. Cell. Biol.* 10: 2327-2334
- Lilley CE, Schwartz RA & Weitzman MD (2007) Using or abusing: viruses and the cellular DNA damage response. *Trends in Microbiology* 15: 119-126
- Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* 362: 709-715
- Liu Q, Guntuku S, Cui X-S, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA & Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. *Genes & Development* 14: 1448 -1459
- Lodoen M, Ogasawara K, Hamerman JA, Arase H, Houchins JP, Mocarski ES & Lanier LL (2003) NKG2D-mediated Natural Killer Cell Protection Against Cytomegalovirus Is Impaired by Viral gp40 Modulation of Retinoic Acid Early Inducible 1 Gene Molecules. *The Journal of Experimental Medicine* 197: 1245 -1253
- Lodoen MB, Abenes G, Umamoto S, Houchins JP, Liu F & Lanier LL (2004) The Cytomegalovirus m155 Gene Product Subverts Natural Killer Cell Antiviral Protection by Disruption of H60-NKG2D Interactions. *The Journal of Experimental Medicine* 200: 1075 -1081
- Lowe SW & Ruley HE (1993a) Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes & Development* 7: 535 -545
- Lowe SW, Schmitt EM, Smith SW, Osborne BA & Jacks T (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362: 847-849
- Lukas C, Bartkova J, Latella L, Falck J, Mailand N, Schroeder T, Sehested M, Lukas J & Bartek J (2001) DNA Damage-activated Kinase Chk2 Is Independent of Proliferation or Differentiation Yet Correlates with Tissue Biology. *Cancer Research* 61: 4990 -4993
- Matsuoka S, Huang M & Elledge SJ (1998) Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase. *Science* 282: 1893 -1897
- Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP & Elledge SJ (2007) ATM and

- ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. *Science* 316: 1160-1166
- McGowan CH (2002) Checking in on Cds1 (Chk2): A checkpoint kinase and tumor suppressor. *BioEssays* 24: 502-511
- McMichael A (1998) T Cell Responses and Viral Escape. *Cell* 93: 673-676
- Meylan E, Dooley AL, Feldser DM, Shen L, Turk E, Ouyang C & Jacks T (2009) Requirement for NF- κ B signaling in a mouse model of lung adenocarcinoma. *Nature* 462: 104-107
- Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P & Moll UM (2003) p53 has a direct apoptogenic role at the mitochondria. *Molecular Cell* 11: 577-590
- Mori T, Anazawa Y, Iizumi M, Fukuda S, Nakamura Y & Arakawa H (2002) Identification of the interferon regulatory factor 5 gene (IRF-5) as a direct target for p53. *Oncogene* 21: 2914-8
- Nakai-Murakami C, Shimura M, Kinomoto M, Takizawa Y, Tokunaga K, Taguchi T, Hoshino S, Miyagawa K, Sata T, Kurumizaka H, Yuo A & Ishizaka Y (online) HIV-1 Vpr induces ATM-dependent cellular signal with enhanced homologous recombination. *Oncogene* 26: 477-486
- Nikitin PA, Yan CM, Forte E, Bocedi A, Tourigny JP, White RE, Allday MJ, Patel A, Dave SS, Kim W, Hu K, Guo J, Tainter D, Rusyn E & Luftig MA (2010) An ATM/Chk2-Mediated DNA Damage-Responsive Signaling Pathway Suppresses Epstein-Barr Virus Transformation of Primary Human B Cells. *Cell Host & Microbe* 8: 510-522
- Norgauer J, Metzner B & Schraufstatter I (1996) Expression and growth-promoting function of the IL-8 receptor beta in human melanoma cells. *The Journal of Immunology* 156: 1132 -1137
- Nyberg KA, Michelson RJ, Putnam CW & Weinert TA (2002) Toward Maintaining the Genome: DNA Damage and Replication Checkpoints. *Annu. Rev. Genet.* 36: 617-656
- Ou Y-H, Chung P-H, Sun T-P & Shieh S-Y (2005) p53 C-Terminal Phosphorylation by CHK1 and CHK2 Participates in the Regulation of DNA-Damage-induced C-Terminal Acetylation. *Mol Biol Cell* 16: 1684-1695
- Pahl HL (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18: 6853-6866
- Pamment J, Ramsay E, Kelleher M, Dornan D & Ball KL (2002) Regulation of the IRF-1 tumour modifier during the response to genotoxic stress involves an ATM-dependent signalling pathway. *Oncogene* 21: 7776-85
- Pappworth IY, Wang EC & Rowe M (2007) The Switch from Latent to Productive Infection in Epstein-Barr Virus-Infected B Cells Is Associated with Sensitization to NK Cell Killing. *J. Virol.* 81: 474-482
- Park HU, Jeong S-J, Jeong J-H, Chung JH & Brady JN (2005) Human T-cell leukemia virus type 1 Tax attenuates γ -irradiation-induced apoptosis through physical interaction with Chk2. *Oncogene* 25: 438-447
- Park HU, Jeong J-H, Chung JH & Brady JN (2004) Human T-cell leukemia virus type 1 Tax interacts with Chk1 and attenuates DNA-damage induced G2 arrest mediated by Chk1. *Oncogene* 23: 4966-4974
- Parmar S & Plataniias LC (2003) Interferons: mechanisms of action and clinical applications. *Curr Opin Oncol* 15: 431-439

- Pende D, Rivera P, Marcenaro S, Chang C-C, Biassoni R, Conte R, Kubin M, Cosman D, Ferrone S, Moretta L & Moretta A (2002) Major Histocompatibility Complex Class I-related Chain A and UL16-Binding Protein Expression on Tumor Cell Lines of Different Histotypes. *Cancer Research* 62: 6178 -6186
- Peraldi M-N, Berrou J, Dulphy N, Seidowsky A, Haas P, Boissel N, Metivier F, Randoux C, Kossari N, Guérin A, Geffroy S, Delavaud G, Marin-Esteban V, Glotz D, Charron D & Toubert A (2009) Oxidative Stress Mediates a Reduced Expression of the Activating Receptor NKG2D in NK Cells from End-Stage Renal Disease Patients. *The Journal of Immunology* 182: 1696 -1705
- Perkins EJ, Nair A, Cowley DO, Van Dyke T, Chang Y & Ramsden DA (2002) Sensing of intermediates in V(D)J recombination by ATM. *Genes & Development* 16: 159 -164
- Pober JS, Gimbrone MA Jr, Lapierre LA, Mendrick DL, Fiers W, Rothlein R & Springer TA (1986) Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J. Immunol* 137: 1893-1896
- Raulet DH (2003) Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 3: 781-790
- Richard J, Sindhu S, Pham TNQ, Belzile J-P & Cohen ÉA (2010) HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. *Blood* 115: 1354 -1363
- Rodier F, Coppe J-P, Patil CK, Hoeijmakers WAM, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR & Campisi J (2009) Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* 11: 973-979
- Rohner A, Langenkamp U, Siegler U, Kalberer CP & Wodnar-Filipowicz A (2007) Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemia cells to natural killer cell-mediated lysis. *Leukemia Research* 31: 1393-1402
- Rölle A, Mousavi-Jazi M, Eriksson M, Odeberg J, Söderberg-Nauclér C, Cosman D, Kärre K & Cerboni C (2003) Effects of Human Cytomegalovirus Infection on Ligands for the Activating NKG2D Receptor of NK Cells: Up-Regulation of UL16-Binding Protein (ULBP)1 and ULBP2 Is Counteracted by the Viral UL16 Protein. *The Journal of Immunology* 171: 902 -908
- Roshal M, Kim B, Zhu Y, Nghiem P & Planelles V (2003) Activation of the ATR-mediated DNA Damage Response by the HIV-1 Viral Protein R. *Journal of Biological Chemistry* 278: 25879 -25886
- Rothlein R, Dustin M, Marlin S & Springer T (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *The Journal of Immunology* 137: 1270 -1274
- Scheidereit C (2006) IκB kinase complexes: gateways to NF-κB activation and transcription. *Oncogene* 25: 6685-6705
- Sen R & Baltimore D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47: 921-928
- Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL & Phillips JH (1996) DNAM-1, A Novel

- Adhesion Molecule Involved in the Cytolytic Function of T Lymphocytes. *Immunity* 4: 573-581
- Shieh S-Y, Ikeda M, Taya Y & Prives C (1997) DNA Damage-Induced Phosphorylation of p53 Alleviates Inhibition by MDM2. *Cell* 91: 325-334
- Shieh S-Y, Ahn J, Tamai K, Taya Y & Prives C (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes & Development* 14: 289-300
- Shiloh Y (2003) ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 3: 155-168
- Simmons D, Makgoba MW & Seed B (1988) ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature* 331: 624-627
- Simon MM, Aragane Y, Schwarz A, Luger TA & Schwarz T (1994) UVB Light Induces Nuclear Factor κ B (NF κ B) Activity Independently from Chromosomal DNA Damage in Cell-Free Cytosolic Extracts. *J Invest Dermatol* 102: 422-427
- Skalka AM & Katz RA (2005) Retroviral DNA integration and the DNA damage response. *Cell Death Differ* 12: 971-978
- Sligh JE, Ballantyne CM, Rich SS, Hawkins HK, Smith CW, Bradley A & Beaudet AL (1993) Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proceedings of the National Academy of Sciences* 90: 8529-8533
- Sørensen CS, Syljuåsen RG, Falck J, Schroeder T, Rønnstrand L, Khanna KK, Zhou B-B, Bartek J & Lukas J (2003) Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. *Cancer Cell* 3: 247-258
- Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Galleonardo V, Cippitelli M, Fionda C, Petrucci MT, Guarini A, Foa R & Santoni A (2009) ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* 113: 3503-3511
- Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, Levine Z, Beiman M, Dassa L, Achdout H, Stern-Ginossar N, Tsukerman P, Jonjic S & Mandelboim O (2009) The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proceedings of the National Academy of Sciences* 106: 17858-17863
- Stang MT, Armstrong MJ, Watson GA, Sung KY, Liu Y, Ren B & Yim JH (2007) Interferon regulatory factor-1-induced apoptosis mediated by a ligand-independent fas-associated death domain pathway in breast cancer cells. *Oncogene* 26: 6420-6430
- Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, Horwitz E, Prokocimer Z, Prichard M, Hahn G, Goldman-Wohl D, Greenfield C, Yagel S, Hengel H, Altuvia Y, Margalit H & Mandelboim O (2007) Host Immune System Gene Targeting by a Viral miRNA. *Science* 317: 376-381
- Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Löbrich M & Jeggo PA (2004) ATM and DNA-PK Function Redundantly to Phosphorylate H2AX after Exposure to Ionizing Radiation. *Cancer Research* 64: 2390-2396

- Swift LP, Rephaeli A, Nudelman A, Phillips DR & Cutts SM (2006) Doxorubicin-DNA Adducts Induce a Non-Topoisomerase II-Mediated Form of Cell Death. *Cancer Research* 66: 4863-4871
- Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM & Hayakawa Y (2005) NKG2D function protects the host from tumor initiation. *J. Exp. Med* 202: 583-588
- Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M & Nakayama K-ichi (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1 $-/-$ mice. *Genes & Development* 14: 1439-1447
- Takai H, Naka K, Okada Y, Watanabe M, Harada N, Saito S, Anderson CW, Appella E, Nakanishi M, Suzuki H, Nagashima K, Sawa H, Ikeda K & Motoyama N (2002) Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *EMBO J* 21: 5195-5205
- Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K & Taniguchi T (2003) Integration of interferon-[alpha]/[beta] signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 424: 516-523
- Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S-ichi, Honda K, Ohba Y, Mak TW & Taniguchi T (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434: 243-249
- Tamura T, Ishihara M, Lamphier MS, Tanaka N, Oishi I, Aizawa S, Matsuyama T, Mak TW, Taki S & Taniguchi T (1995) An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* 376: 596-599
- Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW & Taniguchi T (1994) Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 77: 829-839
- Tanaka N, Sato M, Lamphier MS, Nozawa H, Oda E, Noguchi S, Schreiber RD, Tsujimoto Y & Taniguchi T (1998) Type I interferons are essential mediators of apoptotic death in virally infected cells. *Genes Cells* 3: 29-37
- Tanaka N, Kawakami T & Taniguchi T (1993) Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol Cell Biol.* 13: 4531-4538
- Tanaka N, Ishihara M, Lamphier MS, Nozawa H, Matsuyama T, Mak TW, Aizawa S, Tokino T, Oren M & Taniguchi T (1996) Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. *Nature* 382: 816-818
- Taniguchi T & Takaoka A (2002) The interferon- α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Current Opinion in Immunology* 14: 111-116
- Taniguchi T, Ogasawara K, Takaoka A & Tanaka N (2001) IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* 19: 623-655
- Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh S-Y, Taya Y, Prives C & Abraham RT (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev* 13: 152-157
- Tomasec P, Wang ECY, Davison AJ, Vojtesek B, Armstrong M, Griffin C, McSharry BP, Morris RJ, Llewellyn-Lacey S, Rickards C, Nomoto A, Sinzger C & Wilkinson GWG

- (2005) Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. *Nat Immunol* 6: 181-188
- Turpin E, Luke K, Jones J, Tumpey T, Konan K & Schultz-Cherry S (2005) Influenza Virus Infection Increases p53 Activity: Role of p53 in Cell Death and Viral Replication. *J. Virol.* 79: 8802-8811
- Upreti M, Koonce NA, Hennings L, Chambers TC & Griffin RJ (2010) Pegylated IFN- α sensitizes melanoma cells to chemotherapy and causes premature senescence in endothelial cells by IRF-1-mediated signaling. *Cell Death Dis* 1: e67
- Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD & Leibson PJ (2006) NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol* 7: 524-532
- Unger T, Juven-Gershon T, Moallem E, Berger M, Vogt Sionov R, Lozano G, Oren M & Haupt Y (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J* 18: 1805-1814
- Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA & Apte RN (2003) IL-1 is required for tumor invasiveness and angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 100: 2645-2650
- Vousden KH & Lu X (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer* 2: 594-604
- Wade Harper J, Adami GR, Wei N, Keyomarsi K & Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75: 805-816
- Walker JR, Corpina RA & Goldberg J (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 412: 607-614
- Wang BX, Rahbar R & Fish EN (2011) Interferon: Current Status and Future Prospects in Cancer Therapy. *J Interferon Cytokine Res* ahead of print. doi:10.1089/jir.2010.0158.
- Wang C-Y, Mayo MW & Baldwin AS (1996) TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF- κ B. *Science* 274: 784-787
- Wang C-Y, Mayo MW, Korneluk RG, Goeddel DV & Baldwin AS (1998) NF- κ B Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation. *Science* 281: 1680-1683
- Wang C-Y, Guttridge DC, Mayo MW & Baldwin AS (1999) NF- κ B Induces Expression of the Bcl-2 Homologue A1/Bfl-1 To Preferentially Suppress Chemotherapy-Induced Apoptosis. *Mol. Cell. Biol.* 19: 5923-5929
- Ward J, Davis Z, DeHart J, Zimmerman E, Bosque A, Brunetta E, Mavilio D, Planelles V & Barker E (2009) HIV-1 Vpr Triggers Natural Killer Cell-Mediated Lysis of Infected Cells through Activation of the ATR-Mediated DNA Damage Response. *PLoS Pathog* 5: e1000613
- Weaver BK, Ando O, Kumar KP & Reich NC (2001) Apoptosis is promoted by the dsRNA-activated factor (DRAF1) during viral infection independent of the action of interferon or p53. *FASEB J* 15: 501-515
- Weaver BK, Kumar KP & Reich NC (1998) Interferon Regulatory Factor 3 and CREB-Binding Protein/p300 Are Subunits of Double-Stranded RNA-Activated Transcription Factor DRAF1. *Mol. Cell. Biol.* 18: 1359-1368

- Wei L-H, Kuo M-L, Chen C-A, Chou C-H, Lai K-B, Lee C-N & Hsieh C-Y (2003) Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene* 22: 1517-1527
- Wen C, He X, Ma H, Hou N, Wei C, Song T, Zhang Y, Sun L, Ma Q & Zhong H (2008) Hepatitis C Virus Infection Downregulates the Ligands of the Activating Receptor NKG2D. *Cell Mol Immunol* 5: 475-478
- Werness B, Levine A & Howley P (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248: 76-79
- Wold MS (1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem* 66: 61-92
- Wu GS, Burns TF, McDonald ER, Jiang W, Meng R, Krantz ID, Kao G, Gan D-D, Zhou J-Y, Muschel R, Hamilton SR, Spinner NB, Markowitz S, Wu G & El-Deiry WS (1997) KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 17: 141-143
- Wu Z-H, Shi Y, Tibbetts RS & Miyamoto S (2006) Molecular Linkage Between the Kinase ATM and NF- κ B Signaling in Response to Genotoxic Stimuli. *Science* 311: 1141-1146
- Wu Z-H, Wong ET, Shi Y, Niu J, Chen Z, Miyamoto S & Tergaonkar V (2010) ATM- and NEMO-Dependent ELKS Ubiquitination Coordinates TAK1-Mediated IKK Activation in Response to Genotoxic Stress. *Molecular Cell* 40: 75-86
- Yajima H, Lee K-J & Chen BPC (2006) ATR-Dependent Phosphorylation of DNA-Dependent Protein Kinase Catalytic Subunit in Response to UV-Induced Replication Stress. *Mol Cell Biol* 26: 7520-7528
- Yang J, Yu Y, Hamrick HE & Duerksen-Hughes PJ (2003) ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. *Carcinogenesis* 24: 1571-1580
- Yanai H, Chen H-min, Inuzuka T, Kondo S, Mak TW, Takaoka A, Honda K & Taniguchi T (2007) Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. *Proc Natl Acad Sci U S A*. 104: 3402-3407
- Yoneyama M & Fujita T (2007) Function of RIG-I-like Receptors in Antiviral Innate Immunity. *J. Biol. Chem.* 282: 15315-15318
- Yu X, Harden K, C Gonzalez L, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D & Grogan JL (2009) The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10: 48-57
- Yuan X-wei, Zhu X-feng, Huang X-fang, Sheng P-yi, He A-shan, Yang Z-bo, Deng R, Feng G-kan & Liao W-ming (2007) Interferon-[alpha] enhances sensitivity of human osteosarcoma U2OS cells to doxorubicin by p53-dependent apoptosis. *Acta Pharmacol Sin* 28: 1835-1841
- Zhao H & Piwnica-Worms H (2001) ATR-Mediated Checkpoint Pathways Regulate Phosphorylation and Activation of Human Chk1. *Mol. Cell. Biol.* 21: 4129-4139
- Zhao H, Watkins JL & Piwnica-Worms H (2002) Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proceedings of the National Academy of Sciences* 99: 14795-14800
- Zhou B-BS & Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433-439

Zou L & Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 300: 1542-1548

Non-Steroidal Anti-Inflammatory Drugs, DNA Repair and Cancer

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1. Introduction

Colorectal cancer is the third most common cancer in women and fourth in men, with respect to incidence, and 529,000 deaths occurred worldwide in 2002 (Parkin *et al.*, 2005). Approximately 2 - 5% of cases of colorectal cancer are due to a genetic predisposition of which the most common is hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is an autosomal dominant disorder with high penetrance and exhibits allelic and locus heterogeneity (Aarnio *et al.*, 1995; de la Chapelle, 2004; Dunlop *et al.*, 1997). In HNPCC there are heterozygous germline mutations in the DNA mismatch repair (MMR) genes MutS homologue 2 (*MSH2*), MutL homologue 1 (*MLH1*), MutS homologue 6 (*MSH6*), post-meiotic segregation increased 2 (*PMS2*) and post-meiotic segregation increased 1 (*PMS1*) (Bocker *et al.*, 1999; Buermeyer *et al.*, 1999; Jiricny, 1998; Jiricny & Marra, 2003; Lucci-Cordisco *et al.*, 2003; Mitchell *et al.*, 2002; Narayan & Roy, 2003; Nicolaidis *et al.*, 1998; Plaschke *et al.*, 2004; Zabkiewicz & Clarke, 2004). Germline mutations in *hMLH1* and *hMSH2* are the most common with abnormalities in these genes found in more than 90% of HNPCC mutation carriers (Abdel-Rahman *et al.*, 2006; de la Chapelle, 2004; Hampel *et al.*, 2005; Lagerstedt Robinson *et al.*, 2007). The phenomenon of transmission of an epimutation in *hMLH1* has also been reported (Hitchins *et al.*, 2007).

The DNA MMR system plays an essential role in identifying and correcting any replication errors and any additional errors which arise through physical or chemical damage. These errors may be base-base mismatches, short insertions/deletions and heteroduplexes, which can occur during DNA replication and recombination (Jiricny, 1998; Jiricny & Marra, 2003). The DNA MMR system therefore maintains genomic integrity and stability and in essence provides a tumour suppressor function. Deficiencies in DNA MMR lead to the accumulation of mutations in repetitive nucleotide regions, a phenomenon termed microsatellite instability (MSI) (Parsons *et al.*, 1993; Parsons *et al.*, 1995; Thibodeau *et al.*, 1993; Thibodeau *et al.*, 1998). Microsatellites are classically defined as simple tandem nucleotide sequence repeats of 1 - 6 base pairs in the genome (Hancock, 1999). Changes in the number of the repeat units due to defective DNA MMR are potentially cancer causing (Riccio *et al.*, 1999; Yamamoto *et al.*, 1998). The MSI phenotype or replication error positive (RER+) phenotype can be considered as an almost canonical feature of DNA MMR deficiency (Kinzler & Vogelstein, 1996; Parsons *et al.*, 1993). This MSI phenotype is observed in approximately 15% of all human colorectal cancer, gastric and endometrial carcinomas (Lothe *et al.*, 1993; Seruca *et al.*, 1995; Shibata, 1999; Umar *et al.*, 1994). Somatic inactivation of DNA MMR largely

arises as a consequence of epigenetic silencing of *hMLH1* (through hypermethylation of promoter CpG islands) rather than via classic mutational inactivation (Herman *et al.*, 1998; Jacinto & Esteller, 2007; Jones & Laird, 1999; Peltomaki, 2001; H. Yamamoto *et al.*, 1998). Genes particularly prone to MSI include *Bax*, *TGF- β receptor II*, *hMSH3* and *hMSH6* (Yamamoto *et al.*, 1998); other susceptible genes include the DNA glycosylase *MBD4* (Bader *et al.*, 2000; Bader *et al.*, 1999; Riccio *et al.*, 1999) and the epidermal growth factor receptor (EGFR) (Woerner *et al.*, 2010). Additionally, as a consequence of MSI, inactivation of proteins in the Wnt signalling pathway (eg TCF-4) has been reported (Shimizu *et al.*, 2002). Clinically defined MSI is where at least two of the loci tested in a panel out of five exhibit instability (Boland *et al.*, 1998). Mutation frequencies in cells defective in DNA MMR can be increased 100-1000 fold (Parsons *et al.*, 1993; Shibata, 1999).

The protective role of the DNA MMR system in suppressing the mutator phenotype in a range of common cancers is thus well established. The hypothesis that aspirin may potentially modulate this pathway to prevent carcinogenesis (Goel *et al.*, 2003) is of central interest, and has so far received relatively little attention and merits further investigation. The aim of this chapter will be to review the evidence that non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, celecoxib, sulindac and so on, affect DNA repair mechanisms and pathways and to further examine the consequences of this in relation to cancer development and progression. As NSAIDs are considered to be one of the most widely used over-the-counter drugs, we will also discuss the potential effect of NSAID use on cancer treatment. Aspirin and other NSAIDs may have the capacity to perturb DNA repair pathways and this may have important implications for the patient response to chemotherapeutic agents. It is also worth noting that inflammation – the ‘seventh hallmark’ of cancer (Colotta *et al.*, 2009) – can possibly repress (by epigenetic mechanisms) DNA mismatch repair.

2. Cancer and NSAIDs

From evidence adduced from epidemiological studies and clinical trials, it has been proposed that regular ingestion of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can promote colorectal tumour regression and reduce the relative risk of developing colorectal cancer (CRC) in the general population and in genetically susceptible individuals (for example see; (Baron *et al.*, 2003; Chan *et al.*, 2009; Cuzick *et al.*, 2009; Giovannucci, 1999; Imperiale, 2003; Logan *et al.*, 1993; Paganini-Hill, 1993; Sandler *et al.*, 2003; Thun *et al.*, 2002). NSAID use is also associated with a reduced risk of oesophageal adenocarcinoma particularly in patients with high risk molecular abnormalities, for example, with 17p LOH, 9p LOH, and DNA content abnormalities (Galipeau *et al.*, 2007). Recent meta-analyses of randomised clinical trials have strengthened the contention that aspirin has protective effects against CRC (Din *et al.*, 2010) and non-CRC related adenocarcinomas, including oesophageal and lung cancer (Rothwell *et al.*, 2011). Although such studies have now provided substantial evidence that regular use of aspirin based medication can reduce the risk of colorectal cancer (Bosetti *et al.*, 2002; Muscat *et al.*, 1994; Thun *et al.*, 1991) the molecular basis for the protective effect of aspirin *vis-à-vis* CRC and other cancers is rather controversial. A substantial number of theories are now in circulation. Whilst much of the focus in the recent past has understandably been on the intrinsic anti-inflammatory nature of the compounds in use, see for example, (Giovannucci, 1999; Keller & Giardiello, 2003), there

are a number of intriguing findings which suggest that NSAIDs can impact upon genetic stability and it is these aspects which we wish to highlight in this chapter. However, the optimism that arises from the findings that NSAIDs may offer protection against cancer should be tempered: NSAID use can result in serious side effects (gastrointestinal disturbances and cardiovascular events) in susceptible individuals (Cuzick *et al.*, 2009). This has led to considerable debate amongst clinicians over recent years as to whether NSAIDs should be prescribed as chemopreventative agents, in particular, to those individuals at high risk of developing colorectal cancer, such as HNPCC or Familial Adenomatous Polyposis (FAP) patients.

Animal studies confirm that NSAIDs can protect against the development of colorectal neoplasia (Corpet & Pierre, 2003, 2005). For example, aspirin has been shown to suppress spontaneous intestinal adenoma formation and reduce incidence and volume of colon tumour induced by the carcinogen 1,2-dimethylhydrazine in rat models (Barnes & Lee, 1999). Continuous administration of a clinically relevant aspirin dosage was crucial in these studies in comparison to other studies where aspirin was administered at the start of carcinogenesis or one week after carcinogen exposure (Craven & DeRubertis, 1992). Taken together with findings from epidemiological studies in humans, there is thus the suggestion that long term, continuous usage of aspirin is required to gain any beneficial chemopreventative effects. Several studies carried out in the 1990s demonstrated inhibition of carcinogen induced tumour development by NSAIDs including aspirin and sulindac in rats (Rao *et al.*, 1995; Reddy *et al.*, 1993) and also in a murine model of FAP (Barnes & Lee, 1998; Chiu *et al.*, 1997; Jacoby *et al.*, 1996; Mahmoud *et al.*, 1998; Oshima *et al.*, 1996). Familial adenomatous polyposis (FAP) is a colorectal cancer syndrome inherited in humans in an autosomal dominant manner caused by an absence of a functional caretaker APC protein (Narayan & Roy, 2003). Lifetime administration of aspirin to a mouse model with germline defects in both the APC and Msh2 genes (APC^{Min/+}, Msh2^{-/-}) suppresses intestinal and mammary neoplasia formation (Sansom *et al.*, 2001).

Epidemiological studies have identified environmental and dietary factors which alter the risk of developing colorectal cancer. Protective dietary factors include NSAIDs, fruit, vegetables, and folic acid and possibly calcium, whilst red and processed meat ingestion, alcohol use and obesity are perceived to increase risk (Forte *et al.*, 2008; Key, 2011; La Vecchia *et al.*, 2001; Ryan-Harshman & Aldoori, 2007; Scheier, 2001; Serrano *et al.*, 2004). An assessment of chemopreventative measures, such as NSAID and micronutrient intake, for the general population and individuals with an increased risk for colorectal cancer based on family history, has recently been published (Cooper *et al.*, 2010).

2.1 Inflammation and cancer

Debate has arisen with regards to the molecular mechanism of action of aspirin and other NSAIDs in reducing the incidence of certain cancers. Based on the anti-inflammatory effects of these agents, one obvious explanation is that inflammation can drive cancer development. Indeed, inflammation is becoming increasingly recognised as being critical to cancer formation, and building on the framework proposed by Hannahan and Weinberg (Hannahan & Weinberg, 2000), it has been proposed that inflammation should be considered the seventh hallmark of genetic instability (Colotta *et al.*, 2009). It has been estimated that one in four cancers are linked to infection and chronic inflammation (Hussain & Harris, 2007). In an inflammatory microenvironment mutation frequency is increased (Bielas *et al.*, 2006). There is strong evidence for an increased risk of cancer in individuals with chronic

inflammatory states (inflammatory bowel disease, gastroesophageal reflux disease, asthma) with or without attendant bacterial or viral infection (eg *Helicobacter pylori*, hepatitis) (Grivennikov & Karin, 2010; Schetter *et al.*, 2010; Xie & Itzkowitz, 2008). The molecular drivers of cancer formation resulting from the interaction of pre-cancerous cells with activated immune cells and the surrounding stroma are complex. The host response to infection, injury and wound repair through production of reactive oxygen (ROS) and nitrogen oxide (RNOS) species, and pro-inflammatory cytokines (eg TNF- α , IL-1, IL-6) and chemokines (eg IL-8) (Wang *et al.*, 2009) is of central importance. Such 'micro-cytokine' storms can stimulate activation of transcription factors (eg Nuclear Factor- κ B, AP-1, STAT3) fundamentally altering the expression profile of a cell and promoting cell survival, proliferation, angiogenesis, motility and invasion. It is clear that for individual transcription factors their activation can be context (eg tissue) dependent and they may not always promote tumour cell formation, and may indeed exhibit tumour suppressor activity (eg NF- κ B in skin cancer) (Chaturvedi *et al.*, 2011; Ditsworth & Zong, 2004).

Microsatellite instability has been observed in non-neoplastic tissue in patients with chronic inflammatory conditions prior to the presence of dysplastic tissue, indicating that defects in DNA MMR can be an early event in inflammation-associated cancers (Brentnall *et al.*, 1995; Park *et al.*, 1998). There are tantalising findings which hint at the molecular basis for this MSI. Oxidative stress, in the guise of H₂O₂, increases frameshift mutations (Gasche *et al.*, 2001), and can inactivate the DNA MMR system (Chang *et al.*, 2002). Moreover, in a p53 and p21 dependent fashion, activated neutrophils induced replication errors and a G2/M arrest in colonic epithelial cells (Campregher *et al.*, 2008). In inflammatory bowel disease neoplasia, hypermethylation of the *hMLH1* gene and reduced hMLH1 protein expression occurs frequently (Fleisher *et al.*, 2000). In an animal model of colorectal cancer, inflammation and hypoxia were found to epigenetically silence hMLH1 expression: down-regulated expression of this DNA mismatch repair gene occurred as a consequence of decreased acetylation, which was reversible when the animals were treated with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) (Edwards *et al.*, 2009). Decreased expression of hMLH1 can alter hPMS2 stability and consequently, genetic integrity. Moreover, hMLH1 and hPMS2 can have a role in activating cell-cycle checkpoints and promoting apoptosis (Cejka *et al.*, 2003; Davis *et al.*, 1998; Ding *et al.*, 2009; McDaid *et al.*, 2009; Sansom *et al.*, 2003; Yanamadala & Ljungman, 2003; Zhang *et al.*, 1999). Thus, a functional DNA mismatch repair system prohibits expansion of cells containing DNA damage (Carethers *et al.*, 1996; Papouli *et al.*, 2004). Dysregulated hMLH1/hPMS2 expression could conceivably impact on the survival of cells containing damaged DNA and promote cancer progression. hMLH1 expression has been reported to be altered by tobacco usage and inflammatory state in the epithelium of the oral mucosa (Fernandes *et al.*, 2007). Increased tissue specific hMLH1 hypermethylation has been observed in the progression of oesophageal cancer (Vasavi *et al.*, 2006); the authors also reported that patients with gastroesophageal reflux disease (GERD) exhibited a very significant degree of hMLH1 hypermethylation prompting the suggestion that reflux can promote hypermethylation. The relationship between acid reflux and inflammation in GERD has been recently reviewed (Orlando, 2010). The phenomenon of elevated microsatellite instability at selected nucleotide repeats (EMAST) is also seen in 60% of sporadic colon cancers, is more common in individuals of African-American origin and has been linked to reduced expression of the hMSH3 protein. Moreover, EMAST is also more prevalent in rectal cancer with immune cell infiltration (Devaraj *et al.*, 2010; Lee *et al.*, 2010). As MSI in CRC can result in products with potentially increased immunogenicity, it is

possible that this further stimulates inflammation (Banerjea *et al.*, 2004). Tumour infiltrating lymphocytes in CRC with MSI are activated and cytotoxic (Phillips *et al.*, 2004). In a model of experimental colon carcinogenesis in rats, long-term, low-dose administration of aspirin significantly reduced cytokine and matrix metalloproteinase release (Bousserouel *et al.*, 2010). Based on the above findings, it would appear to be reasonable to suppose that research into the epigenetic modifying effects of NSAIDs, and inflammation itself (Maekawa & Watanabe, 2007), particularly with respect to alterations in DNA repair protein expression and MSI, should be a focus in the future.

Oxidative stress occurs as a consequence of the activation of the immune system, where oxidative bursts have a role in protecting the host against microbial invaders. Reactive oxide and nitrogen oxide species (ROS and RNOS) thus produced can ultimately cause DNA damage such as abasic sites, oxidised bases, DNA-intrastrand adducts, strand breaks, as well as RNA alkylation, and protein damage (Hussain *et al.*, 2003). Reaction with lipids produces extremely reactive peroxidation products, including malondialdehyde (MDA) and *trans*-4-hydroxynonenal (4-HNE). 4-HNE can form etheno adducts in DNA and has been found to preferentially form adducts in codon 249 of the human p53 gene (Federico *et al.*, 2007; Hu *et al.*, 2002). Mutations in the tumour suppressor p53 are found in inflamed tissue in ulcerative colitis (UC) patients (Hussain *et al.*, 2000). Accelerated telomere shortening, and DNA damage - as assessed by analysis of phosphorylated histone H2AX (γ H2AX; a measure of DSBs) occurs in the bowel of UC patients (Risques *et al.*, 2008). ROS and RNOS - whilst generally perceived as having a negative impact on cell function - do have important roles as secondary messengers (Valko *et al.*, 2006). However, if the cellular defence mechanisms - anti-oxidant enzymes such as manganese superoxide dismutase and glutathione peroxidase, detoxification, and DNA repair systems - are overwhelmed, these reactive species are potentially mutagenic (Ferguson, 2010). The bystander effects from released cytokines and reactive signalling species may also occur some distance from the initial trauma. Redon *et al.* have shown that increased levels of DNA damage (measuring γ H2AX levels) can occur systemically in mice implanted with a non-metastatic tumour (Redon *et al.*, 2010). Animal model studies confirm the role of RNOS and the inflammatory process in contributing to cancer development (Hussain *et al.*, 2003; Itzkowitz & Yio, 2004).

The base excision repair (BER) system is critically important for dealing with oxidative damage to DNA, such as removal of 8-oxo-G lesions (David *et al.*, 2007; Lindahl & Wood, 1999; McCullough *et al.*, 1999; Wood *et al.*, 2001). Increased expression of the BER proteins AAG (a 3-methyladenine DNA glycosylase) and APE1 (apurinic endonuclease1; Ref-1) is seen in inflamed tissue from UC patients. Paradoxically a positive correlation of MSI was noted with overexpression of AAG, and for MSI-high tissues with increased APE1 expression. In model systems this adaptive response was confirmed to positively correlate with an increase in MSI in human cells and frameshift mutations in *S. cerevisiae* (Hofseth *et al.*, 2003). Dysregulated expression of BER proteins can generate a mutator phenotype (Glassner *et al.*, 1998) (as cited in Hofseth, 2003). In a mouse model of UC, expression of the Mutyh BER protein, which can recognise 8-oxoG:A mispairs and oxidised adenines was actually found to influence the inflammatory response to dextran sulphate sodium induced oxidative stress (Casorelli *et al.* 2010). The biomediator signalling molecule nitric oxide (NO \cdot) formed during inflammation has the capacity to inhibit *in vitro* and *in vivo* the formamidopyrimidine-DNA glycosylase, which can recognise abasic sites and 8-oxoguanine lesions (Wink & Laval, 1994). Thus, oxidative stress can compromise genetic stability.

RNOS, and inflammatory cytokines and chemokines can activate the transcription factors activator protein-1 (AP-1), HIF-1 and NF- κ B, the latter resulting in transcription of target genes including COX-2 and TNF- α (for example, see Olson & van der Vliet, 2011; Valko *et al.*, 2006). DNA double strand breaks can also activate NF- κ B (Rakoff-Nahoum, 2006), thus potentially creating a feedback loop where the inflammatory response is perpetuated. Persistent DNA damage can also trigger secretion of cytokines such as IL-6 (Rodier *et al.*, 2009). There is evidence that NF- κ B and cyclooxygenase activation and HIF-1 signalling are interlinked (Jung *et al.*, 2003; Qiao *et al.*, 2010). Hypoxia can induce MSI (Kondo *et al.*, 2001): in human sporadic colon cancers HIF-1 α over-expression is associated with loss of hMSH2 expression, and *in vitro* experiments confirm that in a p53 dependent manner HIF-1 α can repress hMSH2 and hMSH6 (Koshiji *et al.*, 2005; To *et al.*, 2005). Recently HIF-1 α has been suggested to regulate expression of an inhibitor of apoptosis protein, Survivin (Wu *et al.*, 2010), which is notably present in CRC (Chen *et al.*, 2004). There is increasing evidence that microRNAs (short, non coding RNAs that regulate translation) are mediators of the inflammatory process (Schetter *et al.*, 2010). Two micro-RNAs, miR-210 and miR-373 are up regulated in an HIF-1 α dependent manner in hypoxic cells: forced expression of mir-210 reduced expression of homologous recombination factor RAD52, whilst reduced mir-373 expression was found to suppress RAD52 and RAD23B (Crosby *et al.*, 2009). Over-expression of another micro-RNA, miR-155 has been reported in colorectal cancer, and can regulate DNA MMR protein expression (Valeri *et al.*, 2010). Inflammation can up-regulate miR-155 expression and increase mutation frequency two to threefold in spontaneous hypoxanthine phosphoribosyltransferase gene mutation assays (Tili *et al.*, 2011). MiR-155 expression is associated with a poor prognosis in lung cancer (Yanaihara *et al.*, 2006). The cyclooxygenase family of enzymes (COX-1: constitutively expressed; COX-2: induced in inflammation; COX-3: splice variant of COX-1) catalyse the conversion of arachidonic acid into prostanoids (prostaglandins and thromboxanes). Cyclooxygenase expression is increased in CRC (Kutcher *et al.*, 1996). Prostaglandins promote epidermal growth factor receptor (EGFR) transactivation, increased cell proliferation, motility, invasion and angiogenesis (eg by altering vascular endothelial growth factor expression) and inhibit apoptosis, for example, by increasing Bcl-2 expression (Ghosh *et al.*, 2010; Pai *et al.*, 2003; Sheng *et al.*, 1998; Wang & DuBois, 2008). To summarise: inflammation and hypoxia can result in genetic instability and suppression of apoptosis.

2.2 Mechanistic aspects of NSAID cytotoxicity

The mechanism by which NSAIDs protect the host from colorectal cancer development has been under investigation for decades, and as a consequence a plethora of hypotheses (some of which may be competing) have been proposed to explain this phenomenon. In addition to the evidence alluded to above of epidemiological studies and animal models examining the protective effect of NSAIDs, there also exists a substantive literature reporting that NSAIDs exhibit a degree of specific toxicity *in vitro* to colorectal cancer cell lines. Because of the intrinsic anti-inflammatory activity of NSAIDs a significant number of researchers have focused on this aspect as a protective mechanism. For example, aspirin can acetylate the cyclo-oxygenases (COX) significantly reducing arachidonic acid metabolism and prostaglandin production, thereby reducing inflammation (Elwood *et al.*, 2009). Expression of the inducible COX, cyclooxygenase-2 is notably elevated in colorectal malignancies and in other cancers (Ferrandez *et al.*, 2003; Kutcher *et al.*, 1996; Soslow *et al.*, 2000), and this over-

expression has been actively implicated in the metastatic potential of tumours (Jang *et al.*, 2009; Tsujii *et al.*, 1997). These effects, however, may not be restricted only to colorectal cancer: a case control study has found that use of selective and non-selective COX-2 inhibitors (celecoxib, rofecoxib and aspirin and ibuprofen) has utility in the chemoprevention of lung cancer (Harris *et al.*, 2007).

NSAIDs do not only affect cyclooxygenase activity: exposure to these drug can significantly alter gene expression and thus NSAIDs can be reasonably described as having pleiotropic effects, some of which may be relatively compound dependent. Chronic NSAID use can suppress CpG island hypermethylation of tumour suppressor genes [p14(Arf), p16(INK4a), E-cadherin] in the human gastric mucosa (Tahara *et al.*, 2009). Furthermore, NSAID toxicity may not be absolutely dependent on inhibition of COX activity: the proliferation of COX-2 negative cell lines can also be inhibited by NSAIDs (Lai *et al.*, 2008; Richter *et al.*, 2001) and the chemical precursor of aspirin - salicylate - which has weak anti-COX activity, can itself be anti-inflammatory (Amann & Peskar, 2002) and pro-apoptotic to CRC cells (Elder *et al.*, 1996). Additionally, whilst the NSAID sulindac sulfide and its sulfone derivative can both inhibit the HT-29 CRC cell line growth, sulindac sulfone is "devoid of prostaglandin inhibitory activity" (Piazza *et al.*, 1995). Additionally, aberrant crypt foci formation in a chemically induced rat model of CRC were suppressed by treatment with sulindac sulfone (Charalambous & O'Brien, 1996). Supporting these findings, a mechanism for NSAID toxicity (anti-proliferative and inducing apoptosis) - testing sulindac sulfide and piroxicam - toward a CRC line lacking cyclooxygenase activity (HCT-15) and thus independent of prostaglandin inhibition, has also been reported (Hanif *et al.*, 1996). Sulindac metabolites (sulphide and sulfone; see Fig.1) can inhibit the activation and expression of the EGF receptor (Pangburn *et al.*, 2005), with the down-regulated activity mediated by lysosomal and proteasomal degradation (Pangburn *et al.*, 2010). Rigas and others have cogently proposed that the anti-neoplastic activities of NSAIDs can be categorised as either being COX-dependent or COX-independent (Keller & Giardiello, 2003; Shiff & Rigas, 1999). This concept has been reviewed in some detail in (Ferrandez *et al.*, 2003). Smith *et al* examined the effects of NS-398 (a selective COX inhibitor), indomethacin (a non-selective COX inhibitor) and aspirin on the HT29Fu, HCA-7, SW480 and HCT116 CRC cell lines with respect to effects on cell proliferation, cell cycle arrest, apoptosis induction, β -catenin and COX-1 and COX-2 protein production. They concluded that NSAIDs act via COX-dependent and COX-independent mechanisms (Smith *et al.*, 2000). Indeed, acetylsalicylic acid regulates MMP-2 activity and inhibits colorectal invasion of B16F0 melanoma cells (Tsai *et al.*, 2009). Intriguingly, although medium conditioned by cultured colorectal cancer cell lines is capable of inducing endothelial cell (EC) tube formation (as a model for angiogenesis); aspirin and salicylate (both at 1mM) can reduce the ability of the conditioned medium from treated DLD-1, HT-29 and HCT116 CRC cell lines to promote EC tube formation (Shtivelband *et al.*, 2003).

Aspirin is principally metabolised to salicylate *in vivo* (Law *et al.*, 2000; Paterson & Lawrence, 2001), with plasma salicylate concentrations of 0.95-1.9 mM achievable in patients receiving aspirin as an anti-inflammatory agent (Amann & Peskar, 2002; Urios *et al.*, 2007; Yin *et al.*, 1998). The effect of significantly higher concentrations than that achievable physiologically has been used by a number of investigators in a number of *in vitro* studies and one must interpret cautiously data produced from these analyses. To facilitate interpretation, we have incorporated the concentrations utilised in the relevant publications. We should also point out that whilst vegetables and fruits have been considered to be a

natural source of salicylate, where it functions as a plant signalling molecule (Paterson & Lawrence, 2001; Schenk *et al.*, 2000, and refs therein), it has been reported that SA may be an endogenous compound, with SA found in the blood of carnivorous animals, for example in the burrowing owl (Paterson *et al.*, 1998; Paterson *et al* 2008).

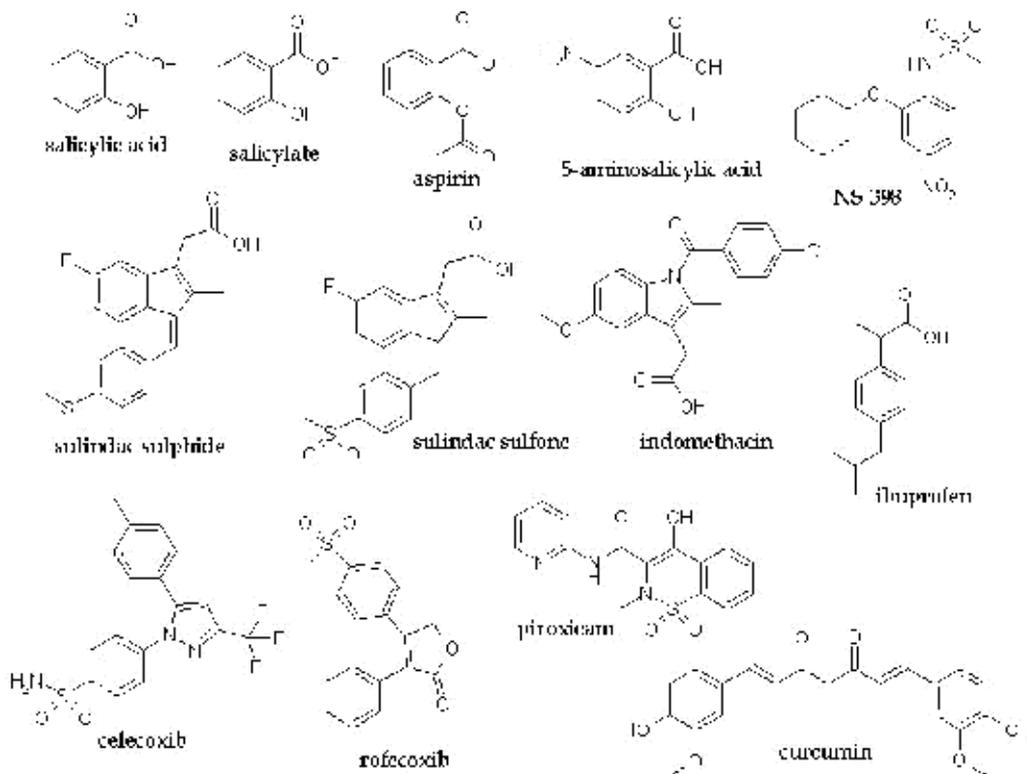


Fig. 1. The non-steroidal anti-inflammatory drugs (NSAIDs) referred to in this chapter can be sub-categorised into five groups. 1. Salicylates (salicylic acid/salicylate anion, aspirin, 5-aminosalicylic acid). 2 Acetic acid derivatives (indomethacin, sulindac sulphide, sulindac sulphone) 3. Propionic acid derivatives (ibuprofen). 4. Enolic acid derivatives or oxicams (piroxicam). 5. Selective COX-2 inhibitors (celecoxib, rofecoxib, NS-398). Curcumin has a dimeric structure with two identical phenolic moieties suggestive of salicylate-like character.

2.2.1 COX independent effects of NSAIDs

A substantial number of hypotheses regarding NSAID cytotoxicity not directly involving inhibition of COX activity exist (reviewed in: Goke, 2002; Watson, 2006). Much of the data is obtained from examining the effect of NSAIDs on cultured cell lines. For example, salicylate can cause cell cycle arrest: in a range of adenoma and carcinoma derived cell lines, incubation with 5 mM resulted in a decrease of cells in the S-phase with cells accumulating in the G₀-G₁ phase (Elder *et al.*, 1996). Activating mutations stabilizing the Wnt pathway and β -catenin are common in CRC (Behrens, 2005; Bienz & Clevers, 2000). In four CRC cell lines (SW948, SW480, HCT116, LoVo), aspirin (5 mM) and indomethacin (400 μ M) downregulated cyclin D1 expression, and the authors proposed this was a consequence of reduced

transcriptional activity of the β -catenin/TCF complex (Dihlmann *et al.*, 2001). Bos *et al* using reporter assays also showed that aspirin (up to 5 mM) can down-regulate APC- β -catenin-TCF4 signalling in the SW480 CRC cell line and increase the phosphorylation of protein phosphatase 2A (PP2A), resulting in decreased PP2A enzymatic activity. This alteration is stated to be essential for the observed effect on the Wnt/ β -catenin pathway activity (Bos *et al.*, 2006). Mutations in the APC or β -catenin genes may also result in increased peroxisome proliferator-activated receptor δ (PPAR δ) activity; indeed, sulindac sulfide and indomethacin have been reported to suppress (by direct inhibition) in a dose-dependent fashion, PPAR δ activity (He *et al.*, 1999). However, it should be noted that regulation of PPAR δ by the APC/ β -catenin/TCF4 pathway and the effect of NSAIDs on PPAR function is a field not without some controversy; for example, Foreman *et al* recently reported not finding decreased PPAR β/δ expression following NSAID treatment in a number of human CRC cell lines (Foreman *et al.*, 2009).

2.2.2 NSAIDS, signalling pathways and apoptosis

Other transcriptional pathways affected by NSAID use include NF- κ B signalling, which is critically involved in regulating immunity, inflammation and apoptosis and in cancer development (Nakano, 2004; Olivier *et al.*, 2006; Staudt, 2010). Aspirin and sodium salicylate can antagonise the NF- κ B pathway (Kopp & Ghosh, 1994), via inhibition - by direct, but not covalent binding- of I κ B kinase-B (IKK β kinase) (Yin *et al.*, 1998). Yamamoto *et al* extended this hypothesis by reporting that sulindac, sulindac sulfide and sulindac sulfone (but not indomethacin) also inhibits the NF- κ B pathway by decreasing IKK β kinase activity (Yamamoto *et al.*, 1999). In HT-29 CRC cells, nitric oxide (NO)-donating aspirin inhibited NF- κ B transcription more potently than aspirin as assessed by electrophoretic mobility shift assays (Williams *et al.*, 2003).

Zerbini *et al* reported that NF- κ B can mediate the repression (assessed by RT-PCR analysis) of the growth arrest and DNA damage inducible (*GADD*) 45 α and *GADD45* γ genes and that this repression is both necessary and sufficient for cell survival, and that *GADD45* α and γ activity can contribute towards apoptosis of prostate cancer cells (assessed by transfection studies in DU145 and PC-3) (Zerbini *et al.*, 2004). The melanoma differentiation associated gene 8 (*mda-7/IL-24*) was induced by NSAIDs and affected growth arrest and apoptosis *in vitro*, in a manner dependent on *GADD45* α and γ expression affecting the p38/JNK pathway (Sarkar *et al.*, 2002; Zerbini *et al.*, 2006). However, it has been suggested that recombinant IL-24 lacks apoptosis inducing properties to melanoma cells (Kreis *et al.*, 2007). In a recent review, Sifakas and Richardson have suggested that the *GADD* family of proteins can be considered to be molecular targets for anti-tumour agents; indeed, commonly used NSAIDs can up-regulate *GADD45* α expression (Rosemary Sifakas & Richardson, 2009). *GADD45* α overexpression in NIH 3T3 cells has been shown to promote global DNA demethylation and loss of expression has been shown to induce DNA hypermethylation (including in *hMLH1*). Such findings led Barreto *et al* to conclude that *GADD45* α can relieve epigenetic silencing by promoting DNA repair (Barreto *et al.*, 2007). It is noteworthy that *GADD45* γ can be inactivated by epigenetic mechanisms in multiple tumours (Ying *et al.*, 2005).

In a review of the role of the NF- κ B pathway in inflammation and cancer, the authors suggested that, '...constitutive NF- κ B activation is likely involved in the enhanced growth properties seen in a variety of cancers' (Yamamoto & Gaynor, 2001). Specifically targeting

the NF- κ B pathway may thus have utility in cancer treatment (Olivier *et al.*, 2006): for example, attempts are ongoing to identify clinically useful and novel IKK- β inhibitors (for example see (Lauria *et al.*, 2010)), and inhibition of NF- κ B (by siRNA) can enhance the chemosensitivity of HCT116 CRC cells to the DNA topoisomerase inhibitor, irinotecan (Guo *et al.*, 2004). In marked contrast however, Stark *et al.*, have suggested that aspirin can activate NF- κ B signalling *in vitro* (SW480, HRT-18, HCT116, CT26 cell lines) and has the capacity to induce apoptosis in *in vivo* (xenografts and APC^{Min+/-}) models of CRC (Stark *et al.*, 2001; Stark *et al.*, 2007). Moreover, these effects are cell type specific to aspirin (for CRC cells): the induction of apoptosis was independent of COX-2 expression, APC and β -catenin mutation status, and DNA mismatch repair proficiency (Din *et al.*, 2004).

A number of other hypotheses have been invoked for the observed cytotoxicity of NSAIDs to CRC cell lines. Cytosolic phospholipase A₂ (cPLA₂) expression is decreased in NIH 3T3 cells treated with either aspirin or sulindac (Yuan *et al.*, 2000) and in CRC cell lines (SW480, Colo320 and HT-29) when treated with low mM (2.5-10) concentrations of aspirin (Yu *et al.*, 2003). This observation is intriguing given that cPLA₂ expression has been proposed to participate in intestinal tumorigenesis (eg (Lim *et al.*, 2010) and refs therein). Law *et al* have indicated that (high mM concentrations of) salicylate (in Balb/MK cells) can inhibit the activity and phosphorylation of the mitogen activated protein kinase, p70^{s6k} independent of p38 MAPK, with a concomitant reduction in DNA synthesis, cell proliferation and in expression of proliferation associated proteins such as c-MYC, cyclin D1, and PCNA and led the authors to conclude that salicylate may act via the mTOR pathway (Law *et al.*, 2000). In contrast, it has been suggested that salicylate-induced apoptosis in HCT116 CRC cells occurs through activation of p38 MAPK and p53; however, the concentration tested was - in our opinion - high (10mM), and thus casts doubt on the utility of the findings (Lee *et al.*, 2003). NO-donating aspirin has also been reported to activate p38 and JNK MAP kinase pathways in HT-29 colorectal cancer cells (Hundley & Rigas, 2006). Using subtractive hybridization, Baek *et al* identified an increase in the expression of the NSAID activated gene (NAG-1) - a member of the TGF- β superfamily, with pro-apoptotic properties - in indomethacin treated HCT116 cells. A range of NSAIDs including sulindac sulfide and aspirin also increased NAG-1 expression (Baek *et al.*, 2001). NCX-4040 (*para*-NO-aspirin), an NO donating aspirin derivative can also induce NAG-1 expression and it was confirmed that NAG-1 has a pro-apoptotic role (Tesei *et al.*, 2008, and refs therein). Another nitro-derivative of aspirin, NCX-4016 inhibited EGFR and STAT3 signalling in cisplatin-resistant human ovarian cancer cells (Selvendiran *et al.*, 2008). NSAIDs can also up-regulate 15-lipoxygenase-1 (15-LOX-1) in CRC cells: NS-398 and sulindac sulfone both induced expression in the DLD-1 (COX-1 and COX-2 negative) cell line; significantly, inhibiting 15-LOX-1 blocked the induction of apoptosis (Shureiqi *et al.*, 2000).

With respect to characterisation of the pathway by which NSAID toxicity occurs, reports have been rather contradictory: *in vitro* studies have suggested that whilst aspirin and other NSAIDs can cause cell cycle arrest and inhibit CRC proliferation, this may, (Din *et al.*, 2004; Elder *et al.*, 1996; Piazza *et al.*, 1995; Yu *et al.*, 2002; Yu *et al.*, 2003) or may not (Shiff *et al.*, 1996; Smith *et al.*, 2000) occur with the induction of apoptosis, or may occur as consequence a combination of activation of both apoptotic and necrotic 'pathways' (Lai *et al.*, 2008). Notwithstanding the apparently contradictory reports, an absence of Bax expression in CRC cells has been found to abolish the apoptotic response to NSAIDs (Zhang *et al.*, 2000). These authors reported finding Bax mutations in indomethacin resistant cells. Bax and BCL-2

expression can be up and down-regulated respectively, in a dose-dependent fashion, (up to 10 mM) in SW480 cells incubated with aspirin (Lai *et al.*, 2008). BCL-2 expression can be reduced by aspirin in SW480 cells (Yu *et al.*, 2002) and can also suppress apoptosis in CRC cells (Jiang & Milner, 2003). Pretreating LNCap (human prostate) and CX-01 (colorectal carcinoma) cell lines with aspirin was found to enhance the capacity of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to initiate apoptosis, an effect - according to the authors - related to down regulation of BCL-2 gene expression resulting from the inhibition of NF- κ B (of which BCL-2 is a known target) (K.M.Kim *et al.*, 2005). HeLa cells incubated with aspirin undergo apoptosis as assessed by cleavage of procaspase 3, PARP and PKC- δ , and annexin V staining. Moreover, the caspase inhibitor zVAD-fmk suppressed cell death, Bax was noted to translocate from the cytosol to mitochondria, and cytochrome c release from mitochondria was seen using time-lapse confocal microscopy (Zimmermann *et al.*, 2000). Sulindac sulfide (up to 500 μ M tested) can inhibit growth of NIH3T3 and SAOS cells: an inhibition of ras mediated proliferation and transformation was noted (Herrmann *et al.*, 1998). Hughes *et al.*, (2003) showed that NSAID treatment of colorectal cancer cell lines caused a decrease in intracellular polyamine content and was cytotoxic. Polyamines are growth factors, and involved in protein synthesis. An increase in intracellular polyamine concentration is observed in the early stages of carcinogenesis. When polyamines were re-introduced to NSAID treated cells, apoptosis was inhibited suggesting that the polyamine pathway is affected by NSAID treatment in CRC cell, and that modulation of this pathway may explain the chemoprotective effects of NSAIDs (Hughes *et al.*, 2003). Dikshit *et al.*, reported that aspirin disrupted proteasome and mitochondrial function in mouse Neuro 2a cells (Dikshit *et al.*, 2006). Hardwick *et al.*, (2004) analysed changes to gene expression employing DNA microarray in the colorectal cancer cell line HT-29 upon aspirin treatment, and found a significant increase in *Rac1* gene and protein expression in a time and concentration dependant manner. *Rac1* is involved in intestinal epithelial cell differentiation (Stappenbeck & Gordon, 2000) proliferation, motility and resistance to apoptosis (Parri & Chiarugi, 2010). Spitz *et al* report that aspirin and salicylate can inhibit purified phosphofructokinase (PFK) in a dose-dependent manner: as cancer cells utilise glycolysis for energy production in which PFK is the rate limiting step, the authors suggest that these NSAIDs may be pro-apoptotic through disturbing the glycolytic pathway (Spitz *et al.*, 2009). We have recently identified novel derivatives of di-aspirin (bis-carboxyphenol succinate) to have potential as anti-colorectal cancer agents (Deb *et al.*, 2011).

2.3 NSAIDS and DNA repair

A relatively unexplored area of research is that the anti-tumour mechanism of action of aspirin may be via 'interaction' with the DNA repair systems. This is of key interest particularly in the context of hereditary and sporadic colorectal cancer where DNA repair defects can be a causative factor. It has been shown that the MSI mutator phenotype is suppressed by aspirin. For example, treatment of the colorectal cancer cell line HCT116 - which is DNA MMR deficient with a defect in *hMLH1* - reduced the MSI phenotype and induced apoptosis (Ruschoff *et al.*, 1998). It is speculated that aspirin exposure resulted in genetic selection against cells with MSI and that MSI unstable cells were weeded out by apoptosis (Ruschoff *et al.*, 1998). This observation is important as MSI resulting from a genetic mutation in *hMLH1*, accounts for 5 - 10% of colonic tumours. Nitric oxide releasing NSAIDS (NO-NSAIDS) suppressed MSI in DNA mismatch repair deficient colorectal cancer cells (McIlhatton *et al.*, 2007), and the authors suggested that since these NO containing

derivatives of aspirin are more effective than aspirin itself, they should be considered for use in chemopreventive trials in patients at high risk of developing CRC. Recently, in a mouse model of HNPCC, McIlhatton *et al.*, reported that aspirin and low dose NO-aspirin increased life span. However, though the intestinal tumours in aspirin treated animals showed less microsatellite instability, low dose NO-aspirin had 'minimal effect on MSI status' and high dose NO-aspirin decreased life span and increased MSI (McIlhatton *et al.*, 2011). In addition to the phenomenon of aspirin selecting for microsatellite stability in colorectal cancer cells (Ruschoff *et al.*, 1998), aspirin *in vitro* can inhibit CRC growth and increase the level of the DNA MMR proteins hMLH1, hMSH2, hMSH6 and hPMS2 in DNA MMR proficient cells (Goel *et al.*, 2003). Increased DNA MMR levels could conceivably facilitate programmed cell death. This is a highly significant observation as defects in DNA MMR proteins are ultimately responsible for HNPCC and this study speculates in particular on the involvement of MLH1 function in the chemoprotective effect of aspirin. In addition to this, recent evidence has shown that although MLH1 expression is decreased in cases of sporadic colorectal adenoma, MLH1 expression was found to be increased in cases of sporadic colorectal adenomas with regular aspirin use (Sidelnikov *et al.*, 2009). Aspirin is not alone in having the capacity to select for microsatellite stability: the NSAID mesalazine (5-aminosalicylic acid), used to treat patients with inflammatory bowel disease (IBD), experimentally reduced frameshift mutations in cultured CRC cells (HCT116 and HCT116+chr 3) independent of DNA mismatch repair proficiency (Gasche *et al.*, 2005). *In vitro*, mesalazine inhibits the growth of HCT116 and HT-29 cells, a result reported a consequence of the compound inhibiting CDC25A, a cell cycle protein (Stolfi *et al.*, 2008). Studies have also implicated cell-cycle arrest as a cellular response to aspirin exposure (Lai *et al.*, 2008; Ricchi *et al.*, 1997). Decreased transcription of CCNB1 (Cyclin B1) which regulates cell-cycle progression at the G₂/M phase by sulindac treatment has been observed (Iizaka *et al.*, 2002). To summarise: NSAIDs can select for microsatellite stability and cause cell-cycle arrest.

2.3.1 NSAIDs and DNA repair protein expression

Microarray analysis has been extensively utilised to examine alterations in gene expression in response to NSAID exposure in a number of colon cancer cell lines (Germann *et al.*, 2003; Hardwick *et al.*, 2004; Huang *et al.*, 2006; Iizaka *et al.*, 2002; Yin *et al.*, 2006). Hardwick *et al.*, (2004) reported that cell-cycle related genes and NF- κ B were repressed upon aspirin treatment. Iizaka *et al.*, (2002) reported an increase in *GADD45 α* upon treatment with the NSAID sulindac. However, although, microarray analysis has been previously carried out to determine the effects of aspirin on colorectal cancer (Hardwick *et al.*, 2004; Iizaka *et al.*, 2002) these studies have not looked specifically at DNA damage signalling pathways. In addition, Hardwick *et al.*, (2004) and also Yin *et al.*, (2006) have shown that notably different gene expression patterns are seen when different concentrations of NSAID are used. Both studies showed that at low concentrations (1 mM) aspirin elicited activation of a different set of genes in the colorectal cancer cell line HT-29, compared to cells incubated with 5 mM aspirin. There is a lack of consistency in the literature regarding dosages utilised for both clinical and experimental studies. Intriguingly, clinical trials have shown that low dose aspirin (81mg) is more protective against colorectal cancer than 'high' dose aspirin (325mg) (Baron *et al.*, 2003). The variation in experimental design, especially with regards to what dose is clinically relevant makes it problematic when deciding on appropriate drug concentrations for experimental work.

In addition to the report by Iizaka *et al.*, (2002), a number of other studies have also shown NSAID regulation of growth arrest and DNA damage (GADD) gene expression. Germann *et al.* (2003) analysed gene expression of CC531 colorectal cancer cells treated with 4.5mM Butyrate or 3mM aspirin and showed that *GADD153* is up-regulated upon aspirin treatment. *GADD153* expression has also been shown to be induced by celecoxib treatment in cervical cancer cells (Kim *et al.*, 2007). Microarray analysis of the colorectal cancer cell line HT-29 treated with 1mM and 5mM aspirin showed an up-regulation of *GADD45 α* (Yin *et al.*, 2006). Further to this, microarray analysis also shows *GADD45* gene expression to be up-regulated by NS-398 (Huang *et al.*, 2006) and celecoxib (Fatima *et al.*, 2008) in colorectal cancer cells and by ibuprofen in human gastric adenocarcinoma cell line (Bonelli *et al.*, 2010). Suppression of *GADD45 α* expression confers resistance to sulindac and indomethacin induced gastric mucosal injury and apoptosis (Chiou *et al.*, 2010).

Other genes involved in DNA repair have also been shown to be regulated by NSAIDs. High concentrations of NS-398, ibuprofen, and RNAi mediated inhibition of COX-2 in human prostate carcinoma cells affected genes involved in DNA replication, recombination and repair (John-Aryankalayil *et al.*, 2009). *PCNA* gene expression has been shown to be up-regulated by ibuprofen treatment of a human gastric adenocarcinoma cell line (Bonelli *et al.*, 2010), and also in the human hepatocellular carcinoma cell line HepG2 treated with 25mM vanillin (Cheng *et al.*, 2007). In contrast, down-regulation of *PCNA* gene expression has been reported in colorectal cancer cell lines treated with celecoxib suggesting that these compounds may modulate cell cycle regulation in these model (Fatima *et al.*, 2008). Interestingly, *FADD* gene expression is down-regulated by NS-398 but up-regulated by indomethacin in the colorectal cancer cell line Caco-2 (Huang *et al.*, 2006). Sulindac has been reported to down-regulate *XRCC5*, *ERCC5* and *UNG* gene expression in colorectal cancer cell lines (Iizaka *et al.*, 2002). Celecoxib up-regulated *ATM*, *MAP3K2*, *CDKN1A* and *Bax* gene expression in the colorectal cancer cell line HCA-7; in contrast, *Bax* gene expression was down-regulated in the HCT116 cell line upon celecoxib treatment (Fatima *et al.*, 2008) suggesting that there may be variation in response from cell line to cell line to NSAID exposure.

We have recently reported finding that *XRCC3* protein expression in SW480 cells was increased upon 1 mM aspirin treatment for 48 hours (Dibra *et al.*, 2010). The altered expression of *XRCC3* upon aspirin exposure may have implications to the sensitivity of cells to chemotherapeutic agents. Indeed, previous research in the breast cancer cell line MCF7 demonstrated that over-expression of *XRCC3* induced cisplatin resistance (Xu *et al.*, 2005). Studies have shown that in contrast to *XRCC3* over-expression and cisplatin resistance, *XRCC3* deficient HCT116 cells have increased sensitivity to cisplatin and also mitomycin C (Yoshihara *et al.*, 2004). Depletion of *XRCC3* by siRNA in MCF7 cells inhibited cell proliferation, leading to accumulation of DNA breaks and triggering activation of p53-dependant cell death (Loignon *et al.*, 2007). Although some studies have shown no association between polymorphisms in *XRCC3* and colorectal cancer risk (Mort *et al.*, 2003; Yeh *et al.*, 2005; Tranah *et al.*, 2004; Skjelbred *et al.*, 2006) some studies have (Improta *et al.*, 2008), and in *XRCC3* polymorphisms have also been associated with breast and lung cancer susceptibility (Smith *et al.*, 2003; Jacobsen *et al.*, 2004). One essential point to note is that there is a lack of information in current literature about the effects of NSAIDs on normal human colonocytes. The information that we have relates to cell lines/*in vitro* studies and murine models. There is a need to understand the effects of these compounds on normal, healthy colon cells and how these effects prevent carcinogenesis in these cells. With the majority of the

models used in studies already established as cancerous, it is difficult to separate the effects of these compounds on normal cells rather than cells which are already cancerous and subject to pathway dysregulation. In addition to NSAIDs, flavonoids have also been associated with anti – carcinogenic properties. It is interesting to note that a recent study has shown that these compounds, which occur naturally in fruits and vegetables, not only have a protective effect against oxidative DNA damage but also increase repair activity *in vitro* (Ramos *et al.*, 2010). To summarise: NSAIDs can alter the expression of DNA repair, and pro- and anti-apoptotic proteins.

2.3.2 Curcumin and DNA repair

Curcumin is a naturally occurring turmeric derivative with anti-inflammatory properties and apoptotic, anti-proliferative, anti-oxidant and anti-angiogenic effects (Shehzad *et al.*, 2010). *In vivo* studies have shown that curcumin decreased intestinal polyp formation in the APC^{min/+} mouse model (Murphy *et al.*, 2010). Curcumin is considered an attractive compound for chemopreventative use and there are at present clinical trials ongoing testing the effectiveness of the compound against different types of cancer (Shehzad *et al.*, 2010). However, as with aspirin, a known mechanism of action of curcumin is yet to be elucidated with a range of molecular targets proposed (as reviewed in Shehzad *et al.*, 2010 and Ravindran *et al.*, 2009). As discussed in a recent review (Burgos-Moron *et al.*, 2010), although curcumin is widely regarded as a potential chemopreventative drug its safety and efficiency is yet to be fully elucidated and results from studies, clinical or otherwise, should be interpreted with this in mind. Curcumin has been shown to affect DNA damage and repair. Curcumin has been found to induce DNA damage in mouse-rat hybrid retina ganglion cells (Lu *et al.*, 2009). The study reported that curcumin decreased expression of the DNA repair genes *ATR*, *ATM*, *BRCA1*, *DNA-PK* and *MGMT*. Curcumin has also been seen to cause DNA damage, as tested in the Comet Assay, in gastric mucosa cells and human peripheral blood lymphocytes (Blasiak *et al.*, 1999). Curcumin has been shown to induce DNA single strand breaks (Scott & Loo, 2004) and induce the expression of the pro-apoptotic gene *GADD153* in HCT116 colonocytes. It is suggested that the up-regulation of *GADD153* is a direct response to DNA damage caused by curcumin, ultimately resulting in the induction of apoptosis (Scott & Loo, 2004). Microarray analysis of human lung cancer cells after curcumin treatment saw an upregulation of *GADD45* and *GADD153* gene expression (Saha *et al.*, 2010) and microarray analysis has also shown an up-regulation of *GADD45* by curcumin treatment in a human breast cancer cell line (Ramachandran *et al.*, 2005). It has recently been proposed that DNA MMR may play a role in the cellular response to curcumin (Jiang *et al.*, 2010): DNA MMR proficient cells showed a greater accumulation of double strand breaks (DSB) upon curcumin treatment compared to MMR deficient cells suggesting that DSB formation induced by curcumin is primarily a DNA MMR-dependent process; further to this, curcumin was reported to activate *ATM/Chk1* and cause cell-cycle arrest and apoptosis in human pancreatic cancer cells. DNA MMR proficient cells showed activation of *Chk1* and induction of the G(2)-M cell cycle checkpoint (Jiang *et al.*, 2010) suggesting that the curcumin induced checkpoint response may be a DNA MMR dependent mechanism. Interestingly, microarray analysis of gene expression of invasive lung adenocarcinoma (CL1-5) cells exposed to curcumin found an induction in *MLH1* gene expression and reduction in *MMP* expression (Chen *et al.*, 2004). Curcumin is also reported to affect the Fanconi anemia (FA)/BRCA pathway. Curcumin sensitises ovarian and breast cancer cells with a functional FA/BRCA pathway to cisplatin

(Chirnomas *et al.*, 2006). This is a clinically significant finding as resistance to chemotherapeutic drugs such as cisplatin is common and monoketone analogs of curcumin are now being developed as a new class of FA pathway inhibitors (Landais *et al.*, 2009). Recently, curcumin inhibition of the FA/BRCA pathway has also been suggested to be a mechanism for the reversal of multiple resistance in a multiple myeloma cell line (Xiao *et al.*, 2010). In a recent phase IIa clinical trial report, curcumin reduced aberrant crypt foci formation (Carroll *et al.*, 2011).

2.3.3 Interaction of NSAIDs with anti-cancer treatments

The capacity for specific cyclooxygenase inhibitors and NSAIDs to enhance the cellular response to chemotherapeutic and radiotherapeutic agents has been examined. Additive, synergistic and antagonistic effects have been reported. For example, the effect of a selective COX-2 inhibitor SC-236 (4-[5-(4-chlorophenyl)-3-trifluoromethyl]-1H-pyrazol-1-yl) benzene sulphamide) was tested on cells derived from a murine sarcoma in the absence and presence of γ -ray irradiation, and a clonogenic cell survival assay confirmed that the compound significantly enhanced cell radiosensitivity (Raju *et al.*, 2002). In the human hepatocellular carcinoma cell line HepG2, indomethacin and SC-236 enhanced doxorubicin toxicity reportedly via inhibiting P-glycoprotein and the multidrug resistance-associated protein 1 (MRP1) (Ye *et al.*, 2011). Exogenous prostaglandin E2 addition failed to reverse the cellular accumulation and retention of doxorubicin and the authors concluded that the action of the drugs was via a COX-independent mechanism. Synergistic cell death characterised as being apoptotic based on Bax expression, DNA fragmentation and TUNEL assay, was observed in HT-29 cells co-treated with aspirin and 5-fluorouracil (Ashktorab *et al.*, 2005). In marked contrast to the above findings, an antagonistic activity of celecoxib and SC-236 to cytotoxicity mediated by cisplatin to human esophageal squamous cell carcinoma cells has been observed; mechanistic analysis indicated that the compounds decrease cisplatin accumulation and DNA platination, in a COX-2 independent manner (Yu *et al.*, 2011). As previously intimated, there is evidence that NSAIDs can affect double strand break repair pathways. It is known that the homologous recombination protein Rad51 is overexpressed in chemo-radioresistant carcinomas. A study by Ko *et al.*, (2009) showed that celecoxib enhanced gefitinib induced cytotoxicity in NSCLC cells: combined celecoxib/gefitinib treatment resulted in the reduction of Rad51 protein levels. Degradation of Rad51 occurred via a 26S proteasome-dependent pathway. Celecoxib has been shown to inhibit growth of head and neck carcinoma cells and enhance radiosensitivity in a dose-dependent manner: celecoxib downregulated Ku70 protein expression and inhibited DNA-PKcs kinase activity which is known to be involved in DSB repair (Raju *et al.*, 2005). Further to this, sodium salicylate has been shown to inhibit the kinase activity of ATM and DNA-PK which suppresses their DNA damage response (Fan *et al.*, 2010). A recent study in prostate cancer demonstrated that treatment of the prostate cancer cell line PC-3 with NO-sulindac increased the rate of single strand DNA breaks and that there was slower repair of these lesions (Stewart *et al.*, 2011). We thus suggest that caution is exercised in situations where patients are prescribed NSAIDs when undergoing chemotherapy or radiotherapy as unexpected additive or antagonistic reactions may arise, and thus potentially compromise treatment effectiveness. However, there is also the promise that NSAIDs (either known or novel) may significantly synergise and enhance responses to chemotherapeutic, biologic, or radiotherapeutic modalities.

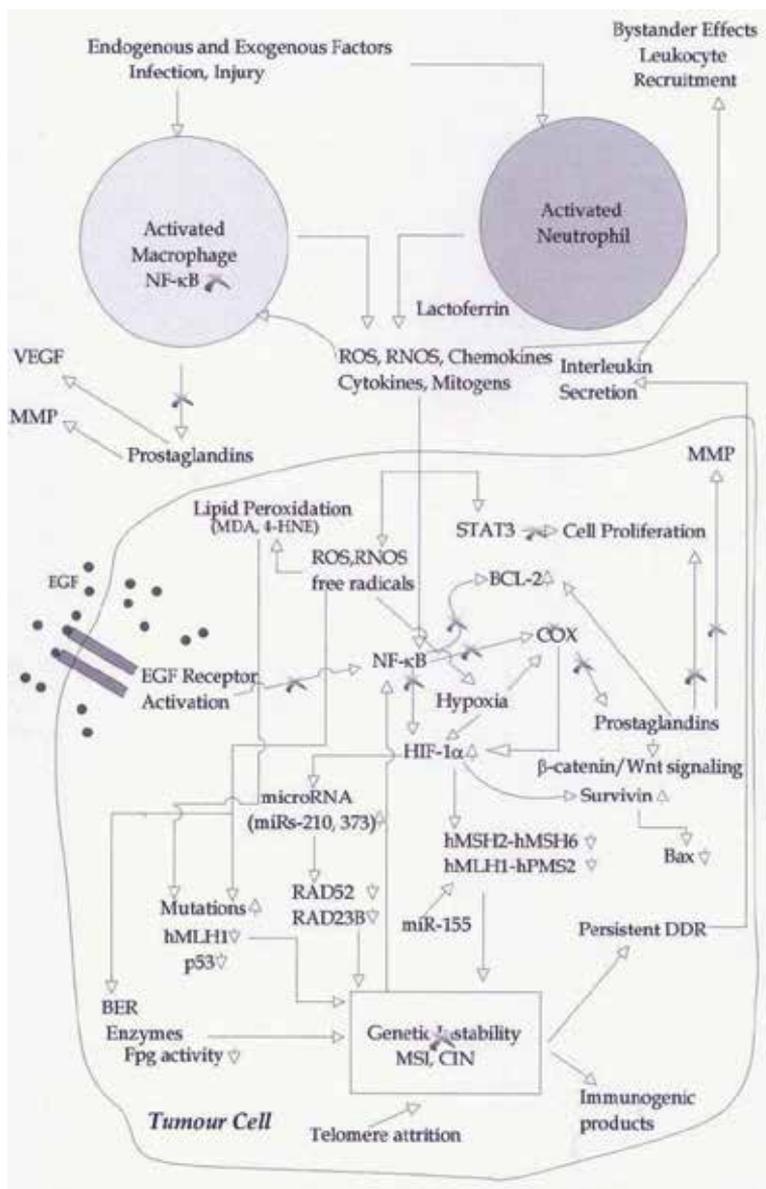


Fig. 2. A schematic illustration (composite) of the possible interconnections of the molecules and activities cited in the text. Where NSAIDs can potentially act is illustrated with X. Abbreviations: BER, base excision repair; CIN, chromosomal instability; COX, cyclooxygenase; DDR, DNA damage response; EGF, epidermal growth factor; Fpg, formamidopyrimidine-DNA glycosylase; 4-HNE, trans-4-hydroxynonenal; HIF-1 α , hypoxia inducible factor-1 α ; MDA, malondialdehyde; MIN, microsatellite instability; MMP, matrix metalloproteinase; miR, micro RNA; NF- κ B, nuclear factor kappa B; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor. For reasons of clarity, chemokine and cytokine receptor interactions are not shown.

3. Conclusion

The accumulated findings from a significant body of research indicates that NSAIDs – including aspirin- act via multiple mechanisms in reducing the morbidity and mortality of cancer, and that aspirin and other NSAIDs reduce inflammation via COX-dependent *and* COX-independent pathways. NSAIDs can select for DNA mismatch repair competency and microsatellite stable cells thus inhibiting at least one well recognised pathway of colorectal cancer progression; furthermore, *in vitro* data suggest that NSAIDs exhibit ‘direct’ and relatively specific and rapid toxicity to colorectal cancer cells, with evidence suggesting that this may (but almost certainly not exclusively) involve DNA repair pathways. There is a paucity of information regarding the effect of NSAIDs on gene expression in non-transformed cell lines, including colonocytes and stromal cells and stem cells. Epidemiological evidence strongly indicates that NSAIDs are particularly protective against adenocarcinoma formation. Given the potential as chemopreventative agents, significant effort should be directed into producing novel NSAID derivatives that do not produce the adverse gastrointestinal and cardiovascular effects but retain the multiple and potent protective actions that are involved in suppressing adenocarcinoma formation.

We feel that the following questions need to be addressed:

- a. What is the mechanism by which aspirin selects for microsatellite stability?
- b. What is the mechanism by which aspirin and other NSAIDs show their relatively specific toxicity to colorectal cancer cells?
- c. Does long term usage of aspirin and other NSAIDs preferentially reprogram the colonic epithelium via epigenetic mechanisms *eg* altering histone deacetylase and DNA methyltransferase activities?
- d. How do NSAIDs affect gene expression in non-transformed cells, including colonic stem cells? Do NSAIDs affect epithelial miRNA profiles?

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5. References

- Aarnio, M., Mecklin, J. P., Aaltonen, L. A., Nystrom-Lahti, M., & Jarvinen, H. J. (1995). Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. *Int J Cancer*, 64, pp.430-433.
- Abdel-Rahman, W. M., Mecklin, J. P., & Peltomaki, P. (2006). The genetics of HNPCC: Application to diagnosis and screening. *Crit Rev Oncol Hematol*, 58, pp.208-220.
- Amann, R., & Peskar, B. A. (2002). Anti-inflammatory effects of aspirin and sodium salicylate. *Eur J Pharmacol*, 447, pp.1-9.
- Ashktorab, H., Dawkins, F. W., Mohamed, R., Larbi, D., & Smoot, D. T. (2005). Apoptosis induced by aspirin and 5-fluorouracil in human colonic adenocarcinoma cells. *Dig Dis Sci*, 50, pp.1025-1032.
- Bader, S., Walker, M., & Harrison, D. (2000). Most microsatellite unstable sporadic colorectal carcinomas carry mbd4 mutations. *Br J Cancer*, 83, pp.1646-1649.

- Bader, S., Walker, M., Hendrich, B., Bird, A., Bird, C., Hooper, M., *et al.* (1999). Somatic frameshift mutations in the MBD4 gene of sporadic colon cancers with mismatch repair deficiency. *Oncogene*, 18, pp.8044-8047.
- Baek, S. J., Kim, K. S., Nixon, J. B., Wilson, L. C., & Eling, T. E. (2001). Cyclooxygenase inhibitors regulate the expression of a tgf-beta superfamily member that has proapoptotic and antitumorigenic activities. *Mol Pharmacol*, 59, pp.901-908.
- Banerjea, A., Ahmed, S., Hands, R. E., Huang, F., Han, X., Shaw, P. M., *et al.* (2004). Colorectal cancers with microsatellite instability display mRNA expression signatures characteristic of increased immunogenicity. *Mol Cancer*, 3, pp.21.
- Barnes, C. J., & Lee, M. (1998). Chemoprevention of spontaneous intestinal adenomas in the adenomatous polyposis coli min mouse model with aspirin. *Gastroenterology*, 114, pp.873-877.
- Barnes, C. J., & Lee, M. (1999). Determination of an optimal dosing regimen for aspirin chemoprevention of 1,2-dimethylhydrazine-induced colon tumours in rats. *Br J Cancer*, 79, pp.1646-1650.
- Baron, J. A., Cole, B. F., Sandler, R. S., Haile, R. W., Ahnen, D., Bresalier, R., *et al.* (2003). A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med*, 348, pp.891-899.
- Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S. K., Handa, V., *et al.* (2007). Gadd45 α promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature*, 445, pp.671-675.
- Behrens, J. (2005). The role of the wnt signalling pathway in colorectal tumorigenesis. *Biochem Soc Trans*, 33, pp.672-675.
- Bielas, J. H., Loeb, K. R., Rubin, B. P., True, L. D., & Loeb, L. A. (2006). Human cancers express a mutator phenotype. *Proc Natl Acad Sci U S A*, 103, pp.18238-18242.
- Bienz, M., & Clevers, H. (2000). Linking colorectal cancer to wnt signaling. *Cell*, 103, pp.311-320.
- Blasiak, J., Trzeciak, A., and Kowalik, J. (1999) Curcumin damages DNA in human gastric mucosa cells and lymphocytes. *J Environ Pathol Toxicol Oncol*. 18, pp.271-6.
- Bocker, T., Ruschoff, J., & Fishel, R. (1999). Molecular diagnostics of cancer predisposition: Hereditary non- polyposis colorectal carcinoma and mismatch repair defects. *Biochim Biophys Acta*, 1423, 3. pp.O1-O10.
- Boland, C. R., Thibodeau, S. N., Hamilton, S. R., Sidransky, D., Eshleman, J. R., Burt, R. W., *et al.* (1998). A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: Development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*, 58, pp.5248-5257.
- Bonelli, P., Tuccillo, F. M., Calemme, R., Pezzetti, F., Borrelli, A., Martinelli, R., *et al.* (2010). Changes in the gene expression profile of gastric cancer cells in response to ibuprofen: A gene pathway analysis. *Pharmacogenomics J*, June 15.
- Bos, C. L., Kodach, L. L., van den Brink, G. R., Diks, S. H., van Santen, M. M., Richel, D. J., *et al.* (2006). Effect of aspirin on the wnt/beta-catenin pathway is mediated via protein phosphatase 2a. *Oncogene*, 25, pp.6447-6456.
- Bosetti, C., Gallus, S., & La Vecchia, C. (2002). Aspirin and cancer risk: An update to 2001. *Eur J Cancer Prev*, 11, pp.535-542.
- Bousserouel, S., Gosse, F., Bouhadjar, M., Soler, L., Marescaux, J., & Raul, F. (2010). Long-term administration of aspirin inhibits tumour formation and triggers anti-

- neoplastic molecular changes in a pre-clinical model of colon carcinogenesis. *Oncol Rep*, 23, pp.511-517.
- Brentnall, T. A., Chen, R., Lee, J. G., Kimmey, M. B., Bronner, M. P., Haggitt, R. C., *et al.* (1995). Microsatellite instability and k-ras mutations associated with pancreatic adenocarcinoma and pancreatitis. *Cancer Res*, 55, pp.4264-4267.
- Buermeyer, A. B., Wilson-Van Patten, C., Baker, S. M., & Liskay, R. M. (1999). The human MLH1 cDNA complements DNA mismatch repair defects in mlh1-deficient mouse embryonic fibroblasts. *Cancer Res*, 59, pp.538-541.
- Burgos-Morón, E., Calderón-Montaño, J. M., Salvador, J., Robles, A., and López-Lázaro, M. (2010) The dark side of curcumin. *Int J Cancer*. 126, pp.1771-5.
- Campregher, C., Luciani, M. G., & Gasche, C. (2008). Activated neutrophils induce an hMSH2-dependent G2/M checkpoint arrest and replication errors at a (CA)13-repeat in colon epithelial cells. *Gut*, 57, pp.780-787.
- Carethers, J. M., Hawn, M. T., Chauhan, D. P., Luce, M. C., Marra, G., Koi, M., *et al.* (1996). Competency in mismatch repair prohibits clonal expansion of cancer cells treated with n-methyl-n'-nitro-n-nitrosoguanidine. *J Clin Invest*, 98, pp.199-206.
- Carroll, R. E., Benya, R. V., Turgeon, D. K., Vareed, S., Neuman, M., Rodriguez, L., *et al.* (2011). Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. *Cancer Prev Res (Phila)*, 4, pp.354-364.
- Casorelli, I., Pannellini, T., De Luca, G., Degan, P., Chiera, F., Iavarone, I., *et al.* The Mutyh base excision repair gene influences the inflammatory response in a mouse model of ulcerative colitis. *PLoS ONE*, 5, e12070.
- Cejka, P., Stojic, L., Mojas, N., Russell, A. M., Heinimann, K., Cannavo, E., *et al.* (2003). Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1. *EMBO J*, 22, pp.2245-2254.
- Chan, A. T., Ogino, S., & Fuchs, C. S. (2009). Aspirin use and survival after diagnosis of colorectal cancer. *Jama*, 302, pp.649-658.
- Chang, C. L., Marra, G., Chauhan, D. P., Ha, H. T., Chang, D. K., Ricciardiello, L., *et al.* (2002). Oxidative stress inactivates the human DNA mismatch repair system. *Am J Physiol Cell Physiol*, 283, pp.C148-154.
- Charalambous, D., & O'Brien, P. E. (1996). Inhibition of colon cancer precursors in the rat by sulindac sulphone is not dependent on inhibition of prostaglandin synthesis. *J Gastroenterol Hepatol*, 11, pp.307-310.
- Chaturvedi, M. M., Sung, B., Yadav, V. R., Kannappan, R., & Aggarwal, B. B. (2011). NF- κ B addiction and its role in cancer: 'one size does not fit all'. *Oncogene*, 30, pp.1615-1630.
- Chen, H. W., Yu, S. L., Chen, J. J., Li, H. N., Lin, Y. C., Yao, P. L., *et al.* (2004). Anti-invasive gene expression profile of curcumin in lung adenocarcinoma based on a high throughput microarray analysis. *Mol Pharmacol*, 65, pp.99-110.
- Chen, W. C., Liu, Q., Fu, J. X., & Kang, S. Y. (2004). Expression of survivin and its significance in colorectal cancer. *World J Gastroenterol*, 10, 2886-2889.
- Cheng, W. Y., Hsiang, C. Y., Bau, D. T., Chen, J. C., Shen, W. S., Li, C. C., *et al.* (2007). Microarray analysis of vanillin-regulated gene expression profile in human hepatocarcinoma cells. *Pharmacol Res*, 56, pp.474-482.
- Chiou, S. K., Hodges, A., & Hoa, N. (2010). Suppression of growth arrest and DNA damage-inducible 45 α expression confers resistance to sulindac and indomethacin-induced gastric mucosal injury. *J Pharmacol Exp Ther*, 334, pp.693-702.

- Chirnomas, D., Taniguchi, T., de la Vega, M., Vaidya, A. P., Vasserman, M., Hartman, A. R., Kennedy, R., Foster, R., Mahoney, J., Seiden, M. V., and D'Andrea, A. D. (2006) Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. *Mol Cancer Ther.* 5, pp.952-61.
- Chiu, C. H., McEntee, M. F., & Whelan, J. (1997). Sulindac causes rapid regression of preexisting tumors in min/+ mice independent of prostaglandin biosynthesis. *Cancer Res*, 57, pp.4267-4273.
- Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis*, 30, pp.1073-1081.
- Cooper, K., Squires, H., Carroll, C., Papaioannou, D., Booth, A., Logan, R. F., *et al.* (2010). Chemoprevention of colorectal cancer: Systematic review and economic evaluation. *Health Technol Assess*, 14, pp.1-206.
- Corpet, D. E., & Pierre, F. (2003). Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. *Cancer Epidemiol Biomarkers Prev*, 12, pp.391-400.
- Corpet, D. E., & Pierre, F. (2005). How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men. *Eur J Cancer*, 41, pp.1911-1922.
- Craven, P. A., & DeRubertis, F. R. (1992). Effects of aspirin on 1,2-dimethylhydrazine-induced colonic carcinogenesis. *Carcinogenesis*, 13, pp.541-546.
- Crosby, M. E., Kulshreshtha, R., Ivan, M., & Glazer, P. M. (2009). MicroRNA regulation of DNA repair gene expression in hypoxic stress. *Cancer Res*, 69, pp.1221-1229.
- Cuzick, J., Otto, F., Baron, J. A., Brown, P. H., Burn, J., Greenwald, P., *et al.* (2009). Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: An international consensus statement. *Lancet Oncol*, 10, pp.501-507.
- David, S. S., O'Shea, V. L., & Kundu, S. (2007). Base-excision repair of oxidative DNA damage. *Nature*, 447, pp.941-950.
- Davis, T. W., Wilson-Van, P. C., Meyers, M., Kunugi, K. A., Cuthill, S., Reznikoff, C., *et al.* (1998). Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. *Cancer Res*, 58, 767-778.
- de la Chapelle, A. (2004). Genetic predisposition to colorectal cancer. *Nat Rev Cancer*, 4, pp.769-780.
- Deb, J., Dibra, H., Shan, S., Rajan, S., Manneh, J., Kankipati, C.S., Perry, C.J., & Nicholl, I.D. (2011). Activity of aspirin analogues and vanillin in a human colorectal cancer cell line. *Oncol Rep* (in press).
- Devaraj, B., Lee, A., Cabrera, B. L., Miyai, K., Luo, L., Ramamoorthy, S., *et al.* (2010). Relationship of emast and microsatellite instability among patients with rectal cancer. *J Gastrointest Surg*, 14, pp.1521-1528.
- Dibra, H.K., Brown, J.E., Hooley, P. & Nicholl, I.D. (2010). Aspirin and alterations in DNA repair proteins in the SW480 colorectal cancer cell line. *Oncol Rep*, 24:37-46.
- Dihlmann, S., Siermann, A., & von Knebel Doeberitz, M. (2001). The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate beta-catenin/tcf-4 signaling. *Oncogene*, 20, 645-653.
- Dikshit, P., Chatterjee, M., Goswami, A., Mishra, A., & Jana, N. R. (2006). Aspirin induces apoptosis through the inhibition of proteasome function. *J Biol Chem*, 281, pp.29228-29235.

- Din, F. V., Dunlop, M. G., & Stark, L. A. (2004). Evidence for colorectal cancer cell specificity of aspirin effects on NF κ B signalling and apoptosis. *Br J Cancer*, 91, 381-388.
- Din, F. V., Theodoratou, E., Farrington, S. M., Tenesa, A., Barnetson, R. A., Cetnarskyj, R., *et al.* (2010). Effect of aspirin and nsaids on risk and survival from colorectal cancer. *Gut*, 59, 1670-1679.
- Ding, X., Mohd, A. B., Huang, Z., Baba, T., Bernardini, M. Q., Lyerly, H. K., *et al.* (2009). Mlh1 expression sensitises ovarian cancer cells to cell death mediated by xiap inhibition. *Br J Cancer*, 101, pp.269-277.
- Ditsworth, D., & Zong, W. X. (2004). NF- κ B: Key mediator of inflammation-associated cancer. *Cancer Biol Ther*, 3, 1214-1216.
- Dunlop, M. G., Farrington, S. M., Carothers, A. D., Wyllie, A. H., Sharp, L., Burn, J., *et al.* (1997). Cancer risk associated with germline DNA mismatch repair gene mutations. *Hum Mol Genet*, 6, pp.105-110.
- Edwards, R. A., Witherspoon, M., Wang, K., Afrasiabi, K., Pham, T., Birnbaumer, L., *et al.* (2009). Epigenetic repression of DNA mismatch repair by inflammation and hypoxia in inflammatory bowel disease-associated colorectal cancer. *Cancer Res*, 69, pp.6423-6429.
- Elder, D. J., Hague, A., Hicks, D. J., & Paraskeva, C. (1996). Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: Enhanced apoptosis in carcinoma and in vitro-transformed adenoma relative to adenoma relative to adenoma cell lines. *Cancer Res*, 56, pp.2273-2276.
- Elwood, P. C., Gallagher, A. M., Duthie, G. G., Mur, L. A., & Morgan, G. (2009). Aspirin, salicylates, and cancer. *Lancet*, 373, 1301-1309.
- Fan, J. R., Huang, T. H., Wen, C. Y., Shen, T. L., & Li, T. K. (2010). Sodium salicylate acts through direct inhibition of phosphoinositide 3-kinase-like kinases to modulate topoisomerase-mediated DNA damage responses. *Eur J Pharmacol*, 638, pp.13-20.
- Fatima, N., Yi, M., Ajaz, S., Stephens, R. M., Stauffer, S., Greenwald, P., *et al.* (2008). Altered gene expression profiles define pathways in colorectal cancer cell lines affected by celecoxib. *Cancer Epidemiol Biomarkers Prev*, 17, 3051-3061.
- Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F., & Loguercio, C. (2007). Chronic inflammation and oxidative stress in human carcinogenesis. *Int J Cancer*, 121, pp.2381-2386.
- Ferguson, L. R. (2010). Chronic inflammation and mutagenesis. *Mutat Res*, 690, 3-11.
- Fernandes, A. M., De Souza, V. R., Springer, C. R., Cardoso, S. V., Loyola, A. M., Mesquita, R. A., *et al.* (2007). Tobacco and inflammation effects in immunoexpression of hMSH2 and hMLH1 in epithelium of oral mucosa. *Anticancer Res*, 27, pp.2433-2437.
- Ferrandez, A., Prescott, S., & Burt, R. W. (2003). Cox-2 and colorectal cancer. *Curr Pharm Des*, 9, pp.2229-2251.
- Fleisher, A. S., Esteller, M., Harpaz, N., Leytin, A., Rashid, A., Xu, Y., *et al.* (2000). Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1. *Cancer Res*, 60, 4864-4868.
- Foreman, J. E., Sorg, J. M., McGinnis, K. S., Rigas, B., Williams, J. L., Clapper, M. L., *et al.* (2009). Regulation of peroxisome proliferator-activated receptor-beta/delta by the apc/beta-catenin pathway and nonsteroidal antiinflammatory drugs. *Mol Carcinog*, 48, pp.942-952.

- Forte, A., De Sanctis, R., Leonetti, G., Manfredelli, S., Urbano, V., & Bezzi, M. (2008). Dietary chemoprevention of colorectal cancer. *Ann Ital Chir*, 79, pp.261-267.
- Galipeau, P. C., Li, X., Blount, P. L., Maley, C. C., Sanchez, C. A., Odze, R. D., et al. (2007). NSAIDs modulate CDKN2A, TP53, and DNA content risk for progression to esophageal adenocarcinoma. *PLoS Med*, 4, e67.
- Gasche, C., Chang, C. L., Rhees, J., Goel, A., & Boland, C. R. (2001). Oxidative stress increases frameshift mutations in human colorectal cancer cells. *Cancer Res*, 61, pp.7444-7448.
- Gasche, C., Goel, A., Natarajan, L., & Boland, C. R. (2005). Mesalazine improves replication fidelity in cultured colorectal cells. *Cancer Res*, 65, 3993-3997.
- Germann, A., Dihlmann, S., Hergenbahn, M., Doeberitz, M. K., & Koesters, R. (2003). Expression profiling of cc531 colon carcinoma cells reveals similar regulation of beta-catenin target genes by both butyrate and aspirin. *Int J Cancer*, 106, pp. 187-197.
- Ghosh, N., Chaki, R., Mandal, V., & Mandal, S. C. (2010). Cox-2 as a target for cancer chemotherapy. *Pharmacol Rep*, 62, pp.233-244.
- Giovannucci, E. (1999). The prevention of colorectal cancer by aspirin use. *Biomed Pharmacother*, 53, pp.303-308.
- Glassner, B. J., Rasmussen, L. J., Najarian, M. T., Posnick, L. M., & Samson, L. D. (1998). Generation of a strong mutator phenotype in yeast by imbalanced base excision repair. *Proc Natl Acad Sci U S A*, 95, pp.9997-10002.
- Goel, A., Chang, D. K., Ricciardiello, L., Gasche, C., & Boland, C. R. (2003). A novel mechanism for aspirin-mediated growth inhibition of human colon cancer cells. *Clin Cancer Res*, 9, pp.383-390.
- Goke, M. N. (2002). Cox-2-independent antiproliferative action of acetylsalicylic acid in human colon cancer cells. *Eur J Clin Invest*, 32, pp.793-794.
- Grivennikov, S. I., & Karin, M. (2010). Inflammation and oncogenesis: A vicious connection. *Curr Opin Genet Dev*, 20, 65-71.
- Guo, J., Verma, U. N., Gaynor, R. B., Frenkel, E. P., & Becerra, C. R. (2004). Enhanced chemosensitivity to irinotecan by rna interference-mediated down-regulation of the nuclear factor-kappa p65 subunit. *Clin Cancer Res*, 10, pp.3333-3341.
- Hampel, H., Frankel, W. L., Martin, E., Arnold, M., Khanduja, K., Kuebler, P., et al. (2005). Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). *N Engl J Med*, 352, pp.1851-1860.
- Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, 100, 57-70.
- Hancock, J. M. 1999. Microsatellites and other simple sequences: Genomic context and mutational mechanisms. In D. B. Goldstein & C. Schlotterer (Eds) *Microsatellites evolution and applications* pp. 1-9). Oxford: Oxford University Press. ISBN 978-0198504078.
- Hanif, R., Pittas, A., Feng, Y., Koutsos, M. I., Qiao, L., Staiano-Coico, L., et al. (1996). Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol*, 52, pp.237-245.
- Hardwick, J. C., van Santen, M., van den Brink, G. R., van Deventer, S. J., & Peppelenbosch, M. P. (2004). DNA array analysis of the effects of aspirin on colon cancer cells: Involvement of rac1. *Carcinogenesis*, 25, 1293-1298.

- Harris, R. E., Beebe-Donk, J., & Alshafie, G. A. (2007). Reduced risk of human lung cancer by selective cyclooxygenase 2 (cox-2) blockade: Results of a case control study. *Int J Biol Sci*, 3, pp.328-334.
- He, T. C., Chan, T. A., Vogelstein, B., & Kinzler, K. W. (1999). PPAR δ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell*, 99, pp.335-345.
- Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., et al. (1998). Incidence and functional consequences of hmlh1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A*, 95, pp.6870-6875.
- Herrmann, C., Block, C., Geisen, C., Haas, K., Weber, C., Winde, G., et al. (1998). Sulindac sulfide inhibits ras signaling. *Oncogene*, 17, pp.1769-1776.
- Hitchins, M. P., Wong, J. J., Suthers, G., Suter, C. M., Martin, D. I., Hawkins, N. J., et al. (2007). Inheritance of a cancer-associated MLH1 germ-line epimutation. *N Engl J Med*, 356, pp.697-705.
- Hofseth, L. J., Khan, M. A., Ambrose, M., Nikolayeva, O., Xu-Welliver, M., Kartalou, M., et al. (2003). The adaptive imbalance in base excision-repair enzymes generates microsatellite instability in chronic inflammation. *J Clin Invest*, 112, pp.1887-1894.
- Hu, W., Feng, Z., Eveleigh, J., Iyer, G., Pan, J., Amin, S., et al. (2002). The major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma. *Carcinogenesis*, 23, pp.1781-1789.
- Huang, R. H., Chai, J., & Tarnawski, A. S. (2006). Identification of specific genes and pathways involved in nsaid-induced apoptosis of human colon cancer cells. *World J Gastroenterol*, 12, pp.6446-6452.
- Hughes, A., Smith, N. I., & Wallace, H. M. (2003). Polyamines reverse non-steroidal anti-inflammatory drug-induced toxicity in human colorectal cancer cells. *Biochem J*, 374, pp.481-488.
- Hundley, T. R., & Rigas, B. (2006). Nitric oxide-donating aspirin inhibits colon cancer cell growth via mitogen-activated protein kinase activation. *J Pharmacol Exp Ther*, 316, pp.25-34.
- Hussain, S. P., Amstad, P., Raja, K., Ambs, S., Nagashima, M., Bennett, W. P., et al. (2000). Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: A cancer-prone chronic inflammatory disease. *Cancer Res*, 60, pp.3333-3337.
- Hussain, S. P., & Harris, C. C. (2007). Inflammation and cancer: An ancient link with novel potentials. *Int J Cancer*, 121, pp.2373-2380.
- Hussain, S. P., Hofseth, L. J., & Harris, C. C. (2003). Radical causes of cancer. *Nat Rev Cancer*, 3, pp.276-285.
- Iizaka, M., Furukawa, Y., Tsunoda, T., Akashi, H., Ogawa, M., & Nakamura, Y. (2002). Expression profile analysis of colon cancer cells in response to sulindac or aspirin. *Biochem Biophys Res Commun.*, 292, pp.498-512.
- Imperiale, T. F. (2003). Aspirin and the prevention of colorectal cancer. *N Engl J Med*, 348, 879-880.
- Improta, G., Sgambato, A., Bianchino, G., Zupa, A., Grieco, V., La Torre, G., Traficante, A. and Cittadini, A. (2008) Polymorphisms of the DNA repair genes XRCC1 and XRCC3 and risk of lung and colorectal cancer: a case-control study in a Southern Italian population. *Anticancer Res*, 28, pp.2941-2946.

- Itzkowitz, S. H., & Yio, X. (2004). Inflammation and cancer iv. Colorectal cancer in inflammatory bowel disease: The role of inflammation. *Am J Physiol Gastrointest Liver Physiol*, 287, G7-17.
- Jacinto, F. V., & Esteller, M. (2007). Mutator pathways unleashed by epigenetic silencing in human cancer. *Mutagenesis*, 22, pp.247-253.
- Jacobsen, N. R., Raaschou-Nielsen, O., Nexø, B., Wallin, H., Overvad, K., Tjønneland, A. and Vogel, U. (2004) XRCC3 polymorphisms and risk of lung cancer. *Cancer Lett*, 213, pp.67-72.
- Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovic, K., Tutsch, K., Cole, C. E., et al. (1996). Chemoprevention of spontaneous intestinal adenomas in the *apc* min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer Res*, 56, pp.710-714.
- Jang, T. J., Jeon, K. H., & Jung, K. H. (2009). Cyclooxygenase-2 expression is related to the epithelial-to-mesenchymal transition in human colon cancers. *Yonsei Med J*, 50, pp.818-824.
- Jiang, M., & Milner, J. (2003). Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. *Genes Dev*, 17, 832-837.
- Jiang, Z., Jin, S., Yalowich, J. C., Brown, K. D, and Rajasekaran, B. (2010) The mismatch repair system modulates curcumin sensitivity through induction of DNA strand breaks and activation of G2-M checkpoint. *Mol Cancer Ther*. 9, pp.558-68.
- Jiricny, J. (1998). Replication errors: Cha(lle)nging the genome. *EMBO J*, 17, pp.6427-6436.
- Jiricny, J., & Marra, G. (2003). DNA repair defects in colon cancer. *Curr Opin Genet Dev.*, 13, pp.61-69.
- John-Aryankalayil, M., Palayoor, S. T., Cerna, D., Falduto, M. T., Magnuson, S. R., & Coleman, C. N. (2009). NS-398, ibuprofen, and cyclooxygenase-2 RNA interference produce significantly different gene expression profiles in prostate cancer cells. *Mol Cancer Ther*, 8, pp.261-273.
- Jones, P. A., & Laird, P. W. (1999). Cancer epigenetics comes of age. *Nat Gene*, 21, 163-167.
- Jung, Y. J., Isaacs, J. S., Lee, S., Trepel, J., & Neckers, L. (2003). Il-1 β -mediated up-regulation of HIF-1 α via an NF κ B/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *Faseb J*, 17, pp.2115-2117.
- Keller, J. J., & Giardiello, F. M. (2003). Chemoprevention strategies using NSAIDs and COX-2 inhibitors. *Cancer Biol Ther*, 2, S140-149.
- Key, T. J. (2011). Fruit and vegetables and cancer risk. *Br J Cancer*, 104, pp.6-11.
- Kim, K. M., Song, J. J., An, J. Y., Kwon, Y. T., & Lee, Y. J. (2005). Pretreatment of acetylsalicylic acid promotes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by down-regulating BCL-2 gene expression. *J Biol Chem*, 280, pp.41047-41056.
- Kim, S. H., Hwang, C. I., Juhn, Y. S., Lee, J. H., Park, W. Y., & Song, Y. S. (2007). GADD153 mediates celecoxib-induced apoptosis in cervical cancer cells. *Carcinogenesis*, 28, pp.223-231.
- Kinzler, K. W., & Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell*, 87, pp.159-170.
- Ko, J. C., Wang, L. H., Jhan, J. Y., Ciou, S. C., Hong, J. H., Lin, S. T., et al. (2009). The role of celecoxib in Rad51 expression and cell survival affected by gefitinib in human non-small cell lung cancer cells. *Lung Cancer*, 65, pp.290-298.

- Kondo, A., Safaei, R., Mishima, M., Niedner, H., Lin, X., & Howell, S. B. (2001). Hypoxia-induced enrichment and mutagenesis of cells that have lost DNA mismatch repair. *Cancer Res*, 61, pp.7603-7607.
- Kopp, E., & Ghosh, S. (1994). Inhibition of NF- κ B by sodium salicylate and aspirin. *Science*, 265, 956-959.
- Koshiji, M., To, K. K., Hammer, S., Kumamoto, K., Harris, A. L., Modrich, P., et al. (2005). HIF-1 α induces genetic instability by transcriptionally downregulating mutsalpha expression. *Mol Cell*, 17, pp.793-803.
- Kreis, S., Philippidou, D., Margue, C., Rolvering, C., Haan, C., Dumoutier, L., et al. (2007). Recombinant interleukin-24 lacks apoptosis-inducing properties in melanoma cells. *PLoS ONE*, 2, e1300.
- Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., et al. (1996). Prostaglandin h synthase 2 is expressed abnormally in human colon cancer: Evidence for a transcriptional effect. *Proc Natl Acad Sci U S A*, 93, pp.4816-4820.
- La Vecchia, C., Altieri, A., & Tavani, A. (2001). Vegetables, fruit, antioxidants and cancer: A review of Italian studies. *Eur J Nutr*, 40, pp.261-267.
- Lagerstedt Robinson, K., Liu, T., Vandrovcova, J., Halvarsson, B., Clendenning, M., Frebourg, T., et al. (2007). Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. *J Natl Cancer Inst*, 99, pp.291-299.
- Lai, M. Y., Huang, J. A., Liang, Z. H., Jiang, H. X., & Tang, G. D. (2008). Mechanisms underlying aspirin-mediated growth inhibition and apoptosis induction of cyclooxygenase-2 negative colon cancer cell line SW480. *World J Gastroenterol*, 14, pp.4227-4233.
- Lauria, A., Ippolito, M., Fazzari, M., Tutone, M., Di Blasi, F., Mingoia, F., et al. (2010). Ikk- β inhibitors: An analysis of drug-receptor interaction by using molecular docking and pharmacophore 3d-qsar approaches. *J Mol Graph Model*, 29, pp.72-81.
- Law, B. K., Waltner-Law, M. E., Entingh, A. J., Chytil, A., Aakre, M. E., Norgaard, P., et al. (2000). Salicylate-induced growth arrest is associated with inhibition of p70^{s6k} and down-regulation of c-Myc, Cyclin D1, Cyclin A, and Proliferating Cell Nuclear Antigen. *J Biol Chem*, 275, pp.38261-38267.
- Lee, E. J., Park, H. G., & Kang, H. S. (2003). Sodium salicylate induces apoptosis in HCT116 colorectal cancer cells through activation of p38MAPK. *Int J Oncol*, 23, pp.503-508.
- Lee, S. Y., Chung, H., Devaraj, B., Iwaizumi, M., Han, H. S., Hwang, D. Y., et al. (2010). Microsatellite alterations at selected tetranucleotide repeats are associated with morphologies of colorectal neoplasias. *Gastroenterology*, 139, pp.1519-1525.
- Lim, S. C., Cho, H., Lee, T. B., Choi, C. H., Min, Y. D., Kim, S. S., et al. (2010). Impacts of cytosolic phospholipase A2, 15-prostaglandin dehydrogenase, and cyclooxygenase-2 expressions on tumor progression in colorectal cancer. *Yonsei Med J*, 51, pp.692-699.
- Lindahl, T., & Wood, R. D. (1999). Quality control by DNA repair. *Science*, 286, 1897-1905.
- Logan, R. F., Little, J., Hawtin, P. G., & Hardcastle, J. D. (1993). Effect of aspirin and non-steroidal anti-inflammatory drugs on colorectal adenomas: Case-control study of subjects participating in the nottingham faecal occult blood screening programme. *BMJ*, 307, 285-289.
- Loignon, M., Amrein, L., Dunn, M., & Aloyz, R. (2007). Xrcc3 depletion induces spontaneous DNA breaks and p53-dependent cell death. *Cell Cycle*, 6, pp.606-611.

- Lothe, R. A., Peltomaki, P., Meling, G. I., Aaltonen, L. A., Nystrom-Lahti, M., Pylkkanen, L., *et al.* (1993). Genomic instability in colorectal cancer: Relationship to clinicopathological variables and family history. *Cancer Res*, 53, pp.5849-5852.
- Lu, H. F., Lai, K. C., Hsu, S. C., Lin, H. J., Yang, M. D., Chen, Y. L., Fan, M.J., Yang, J.S., Cheng, P.Y., Kuo, C. L., and Chung, J. G. (2009) Curcumin induces apoptosis through FAS and FADD, in caspase-3-dependent and -independent pathways in the N18 mouse-rat hybrid retina ganglion cells. *Oncol Rep.* 22, pp.97-104.
- Lucci-Cordisco, E., Zito, I., Gensini, F., & Genuardi, M. (2003). Hereditary nonpolyposis colorectal cancer and related conditions. *Am J Med Genet*, 122A, pp.325-334.
- Maekawa, M., & Watanabe, Y. (2007). Epigenetics: Relations to disease and laboratory findings. *Curr Med Chem*, 14, pp.2642-2653.
- Mahmoud, N. N., Boolbol, S. K., Dannenberg, A. J., Mestre, J. R., Bilinski, R. T., Martucci, C., *et al.* (1998). The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. *Carcinogenesis*, 19, pp.87-91.
- McCullough, A. K., Dodson, M. L., & Lloyd, R. S. (1999). Initiation of base excision repair: Glycosylase mechanisms and structures. *Annu Rev Biochem* 68, pp.255-285.
- McDaid, J. R., Loughery, J., Dunne, P., Boyer, J. C., Downes, C. S., Farber, R. A., *et al.* (2009). Mlh1 mediates parp-dependent cell death in response to the methylating agent n-methyl-n-nitrosourea. *Br J Cancer*, 101, pp.441-451.
- McIlhatton, M. A., Tyler, J., Burkholder, S., Ruschoff, J., Rigas, B., Kopelovich, L., *et al.* (2007). Nitric oxide-donating aspirin derivatives suppress microsatellite instability in mismatch repair-deficient and hereditary nonpolyposis colorectal cancer cells. *Cancer Res*, 67, pp.10966-10975.
- McIlhatton, M. A., Tyler, J., Kerepesi, L. A., Bocker Edmonston, T., Kucherlapati, M. H., Edelman, W., *et al.* (2011). Aspirin and low dose nitric oxide-donating aspirin increase life span in a lynch syndrome mouse model. *Cancer Prev Res (Phila)*, 4, pp.684-693.
- Landais, I., Hiddingh, S., McCarroll, M., Yang, C., Sun, A., Turker, M. S., Snyder, J.P, and Hoatlin, M. E. (2009) Monoketone analogs of curcumin, a new class of Fanconi anemia pathway inhibitors. *Mol Cancer.*, pp.133.
- Mitchell, R. J., Farrington, S. M., Dunlop, M. G., & Campbell, H. (2002). Mismatch repair genes hmlh1 and hmsh2 and colorectal cancer: A huge review. *Am J Epidemiol*, 156, pp.885-902.
- Mort, R., Mo, L., McEwan, C. and Melton, D. W. (2003) Lack of involvement of nucleotide excision repair gene polymorphisms in colorectal cancer. *Br J Cancer*, 89, pp.333-337.
- Murphy, E. A., Davis, J. M., McClellan, J. L., Gordon, B. T, and Carmichael, M. D. (2011) Curcumin's effect on intestinal inflammation and tumorigenesis in the ApcMin/+ mouse. *J Interferon Cytokine Res.* 31, pp.219-26.
- Muscat, J. E., Stellman, S. D., & Wynder, E. L. (1994). Nonsteroidal antiinflammatory drugs and colorectal cancer. *Cancer*, 74, pp.1847-1854.
- Nakano, H. (2004). Signaling crosstalk between NF- κ B and jnk. *Trends Immunol*, 25, pp.402-405.
- Narayan, S., & Roy, D. (2003). Role of apc and DNA mismatch repair genes in the development of colorectal cancers. *Mol Cancer* 2, pp.41.

- Nicolaides, N. C., Littman, S. J., Modrich, P., Kinzler, K. W., & Vogelstein, B. (1998). A naturally occurring hpm2 mutation can confer a dominant negative mutator phenotype. *Mol Cell Biol*, 18, pp.1635-1641.
- Olivier, S., Robe, P., & Bours, V. (2006). Can NF- κ B be a target for novel and efficient anti-cancer agents? *Biochem Pharmacol*, 72, pp.1054-1068.
- Olson, N., & van der Vliet, A. (2011). Interactions between nitric oxide and hypoxia-inducible factor signaling pathways in inflammatory disease. *Nitric Oxide*. In Press.
- Orlando, R. C. (2010). The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms. *Best Pract Res Clin Gastroenterol*, 24, pp.873-882.
- Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., et al. (1996). Suppression of intestinal polyposis in APC^{A716} knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 87, pp.803-809.
- Paganini-Hill, A. (1993). Aspirin and colorectal cancer. *BMJ*, 307, pp.278-279.
- Pai, R., Nakamura, T., Moon, W. S., & Tarnawski, A. S. (2003). Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *Faseb J*, 17, pp.1640-1647.
- Pangburn, H. A., Ahnen, D. J., & Rice, P. L. (2010). Sulindac metabolites induce proteosomal and lysosomal degradation of the epidermal growth factor receptor. *Cancer Prev Res (Phila)*, 3, pp.560-572.
- Pangburn, H. A., Kraus, H., Ahnen, D. J., & Rice, P. L. (2005). Sulindac metabolites inhibit epidermal growth factor receptor activation and expression. *J Carcinog*, 4, pp.16.
- Papouli, E., Cejka, P., & Jiricny, J. (2004). Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. *Cancer Res*, 64, pp.3391-3394.
- Park, W. S., Pham, T., Wang, C., Pack, S., Mueller, E., Mueller, J., et al. (1998). Loss of heterozygosity and microsatellite instability in non-neoplastic mucosa from patients with chronic ulcerative colitis. *Int J Mol Med*, 2, pp.221-224.
- Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, 55, pp.74-108.
- Parri, M., & Chiarugi, P. (2010). Rac and rho gtpases in cancer cell motility control. *Cell Commun Signal*, 8, pp.23.
- Parsons, R., Li, G. M., Longley, M. J., Fang, W. H., Papadopoulos, N., Jen, J., et al. (1993). Hypermutability and mismatch repair deficiency in rer+ tumor cells. *Cell*, 75, pp.1227-1236.
- Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K., Markowitz, S. D., Kinzler, K. W., et al. (1995). Microsatellite instability and mutations of the transforming growth factor beta type ii receptor gene in colorectal cancer. *Cancer Res*, 55, pp.5548-5550.
- Paterson, J. R., Baxter, G., Dreyer, J.S., Halket, J.M., Flynn, R., Lawrence, J.R. (2008). Salicylic acid sans aspirin in animals and man: Persistence in fasting and biosynthesis from benzoic acid. *J Agric Food Chem*, 56, pp.11648-11652.
- Paterson, J. R., Blacklock, C., Campbell, G., Wiles, D., & Lawrence, J. R. (1998). The identification of salicylates as normal constituents of serum: A link between diet and health? *J Clin Pathol*, 51, pp.502-505.
- Paterson, J. R., & Lawrence, J. R. (2001). Salicylic acid: A link between aspirin, diet and the prevention of colorectal cancer. *Qjm*, 94, pp.445-448.
- Peltomaki, P. (2001). Deficient DNA mismatch repair: A common etiologic factor for colon cancer. *Hum Mol Genet*, 10, pp.735-740.

- Phillips, S. M., Banerjea, A., Feakins, R., Li, S. R., Bustin, S. A., & Dorudi, S. (2004). Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite instability are activated and cytotoxic. *Br J Surg*, 91, pp.469-475.
- Piazza, G. A., Rahm, A. L., Krutzsch, M., Sperl, G., Paranka, N. S., Gross, P. H., et al. (1995). Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res*, 55, pp.3110-3116.
- Plaschke, J., Kruger, S., Jeske, B., Theissig, F., Kreuz, F. R., Pistorius, S., et al. (2004). Loss of MSH3 protein expression is frequent in MLH1-deficient colorectal cancer and is associated with disease progression. *Cancer Res*, 64, pp.864-870.
- Qiao, Q., Nozaki, Y., Sakoe, K., Komatsu, N., & Kirito, K. (2010). NF- κ B mediates aberrant activation of hif-1 in malignant lymphoma. *Exp Hematol*, 38, pp.1199-1208.
- Ramos, A. A., Azqueta, A., Pereira-Wilson C. and Collins, A. R. (2010) Polyphenolic compounds from Salvia species protect cellular DNA from oxidation and stimulate DNA repair in cultured human cells. *J Agric Food Chem*, 58, pp.7465-71.
- Raju, U., Ariga, H., Dittmann, K., Nakata, E., Ang, K. K., & Milas, L. (2005). Inhibition of DNA repair as a mechanism of enhanced radioresponse of head and neck carcinoma cells by a selective cyclooxygenase-2 inhibitor, celecoxib. *Int J Radiat Oncol Biol Phys*, 63, pp.520-528.
- Raju, U., Nakata, E., Yang, P., Newman, R. A., Ang, K. K., & Milas, L. (2002). In vitro enhancement of tumor cell radiosensitivity by a selective inhibitor of cyclooxygenase-2 enzyme: Mechanistic considerations. *Int J Radiat Oncol Biol Phys*, 54, pp.886-894.
- Rakoff-Nahoum, S. (2006). Why cancer and inflammation? *Yale J Biol Med*, 79, pp.123-130.
- Ravindran, J., Prasad, S, and Aggarwal, B. B. (2009) Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J*, 11, pp.495-510.
- Ramachandran, C., Rodriguez, S., Ramachandran, R., Raveendran Nair, P. K., Fonseca, H., Khatib, Z., Escalon, E, and Melnick, S. J. (2005) Expression profiles of apoptotic genes induced by curcumin in human breast cancer and mammary epithelial cell lines. *Anticancer Res*, 25, pp.3293-302.
- Rao, C. V., Rivenson, A., Simi, B., Zang, E., Kelloff, G., Steele, V., et al. (1995). Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Res*, 55, pp.1464-1472.
- Reddy, B. S., Rao, C. V., Rivenson, A., & Kelloff, G. (1993). Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in f344 rats. *Carcinogenesis*, 14, pp.1493-1497.
- Redon, C. E., Dickey, J. S., Nakamura, A. J., Kareva, I. G., Naf, D., Nowsheen, S., et al. (2010). Tumors induce complex DNA damage in distant proliferative tissues in vivo. *Proc Natl Acad Sci U S A*, 107, pp.17992-17997.
- Ricchi, P., Pignata, S., Di Popolo, A., Memoli, A., Apicella, A., Zarrilli, R., et al. (1997). Effect of aspirin on cell proliferation and differentiation of colon adenocarcinoma caco-2 cells. *Int J Cancer*, 73, pp.880-884.
- Riccio, A., Aaltonen, L. A., Godwin, A. K., Loukola, A., Percesepe, A., Salovaara, R., et al. (1999). The DNA repair gene mbd4 (med1) is mutated in human carcinomas with microsatellite instability. *Nat Genet*, 23, pp.266-268.
- Richter, M., Weiss, M., Weinberger, I., Furstenberger, G., & Marian, B. (2001). Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors. *Carcinogenesis*, 22, pp.17-25.

- Risques, R. A., Lai, L. A., Brentnall, T. A., Li, L., Feng, Z., Gallaher, J., *et al.* (2008). Ulcerative colitis is a disease of accelerated colon aging: Evidence from telomere attrition and DNA damage. *Gastroenterology*, 135, pp.410-418.
- Rodier, F., Coppe, J. P., Patil, C. K., Hoeijmakers, W. A., Munoz, D. P., Raza, S. R., *et al.* (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol*, 11, pp.973-979.
- Rosemary Sifakos, A., & Richardson, D. R. (2009). Growth arrest and DNA damage-45 alpha (Gadd45 α). *Int J Biochem Cell Biol*, 41, pp.986-989.
- Rothwell, P. M., Fowkes, F. G., Belch, J. F., Ogawa, H., Warlow, C. P., & Meade, T. W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. *Lancet*, 377, 31-41.
- Ruschoff, J., Wallinger, S., Dietmaier, W., Bocker, T., Brockhoff, G., Hofstadter, F., *et al.* (1998). Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proc Natl Acad Sci U S A*, 95, pp.11301-11306.
- Ryan-Harshman, M., & Aldoori, W. (2007). Diet and colorectal cancer: Review of the evidence. *Can Fam Physician*, 53, pp.1913-1920.
- Saha, A., Kuzuhara, T., Echigo, N., Fujii, A., Suganuma, M, and Fujiki, H. (2010). Apoptosis of human lung cancer cells by curcumin mediated through up-regulation of "growth arrest and DNA damage inducible genes 45 and 153". *Biol Pharm Bull*. 33, pp.1291-9.
- Sandler, R. S., Halabi, S., Baron, J. A., Budinger, S., Paskett, E., Keresztes, R., *et al.* (2003). A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med*, 348, pp.883-890.
- Sansom, O. J., Bishop, S. M., Court, H., Dudley, S., Liskay, R. M., & Clarke, A. R. (2003). Apoptosis and mutation in the murine small intestine: Loss of Mlh1- and Pms2-dependent apoptosis leads to increased mutation in vivo. *DNA Repair (Amst)*, 2, pp.1029-1039.
- Sansom, O. J., Stark, L. A., Dunlop, M. G., & Clarke, A. R. (2001). Suppression of intestinal and mammary neoplasia by lifetime administration of aspirin in *apc(min/+)* and *apc(min/+), msh2(-/-)* mice. *Cancer Res*, 61, pp.7060-7064.
- Sarkar, D., Su, Z. Z., Lebedeva, I. V., Sauane, M., Gopalkrishnan, R. V., Valerie, K., *et al.* (2002). *mda-7* (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc Natl Acad Sci U S A*, 99, pp.10054-10059.
- Scheier, L. (2001). Salicylic acid: One more reason to eat your fruits and vegetables. *J Am Diet Assoc*, 101, pp.1406-1408.
- Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C., *et al.* (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc Natl Acad Sci U S A*, 97, 11655-11660.
- Schetter, A. J., Heegaard, N. H., & Harris, C. C. (2010). Inflammation and cancer: Interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis*, 31, pp.37-49.
- Scott, D. W, and Loo, G. (2004) Curcumin-induced GADD153 gene up-regulation in human colon cancer cells. *Carcinogenesis*. 25, pp.2155-64.
- Selvendiran, K., Bratasz, A., Tong, L., Ignarro, L. J., & Kuppusamy, P. (2008). Ncx-4016, a nitro-derivative of aspirin, inhibits egfr and stat3 signaling and modulates bcl-2

- proteins in cisplatin-resistant human ovarian cancer cells and xenografts. *Cell Cycle*, 7, pp.81-88.
- Serrano, D., Lazzeroni, M., & Decensi, A. (2004). Chemoprevention of colorectal cancer: An update. *Tech Coloproctol*, 8 Suppl 2, s248-252.
- Seruca, R., Santos, N. R., David, L., Constancia, M., Barroca, H., Carneiro, F., *et al.* (1995). Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. *Int J Cancer*, 64, pp.32-36.
- Shehzad, A., Wahid, F. and Lee, Y. S. (2010) Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. *Arch Pharm (Weinheim)*, 343, pp.489-99.
- Sheng, H., Shao, J., Morrow, J. D., Beauchamp, R. D., & DuBois, R. N. (1998). Modulation of apoptosis and BCL-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res*, 58, 362-366.
- Shibata, D. 1999. Microsatellite analysis of human tumours. In D. B. S. Goldstein, C. (Ed) *Microsatellites evolution and applications* pp. 266-273). Oxford: Oxford University Press. ISBN 978-0198504078.
- Shiff, S. J., Koutsos, M. I., Qiao, L., & Rigas, B. (1996). Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: Effects on cell cycle and apoptosis. *Exp Cell Res*, 222, pp.179-188.
- Shiff, S. J., & Rigas, B. (1999). The role of cyclooxygenase inhibition in the antineoplastic effects of nonsteroidal antiinflammatory drugs (nsaids). *J Exp Med*, 190, 445-450.
- Shimizu, Y., Ikeda, S., Fujimori, M., Kodama, S., Nakahara, M., Okajima, M., *et al.* (2002). Frequent alterations in the wnt signaling pathway in colorectal cancer with microsatellite instability. *Genes Chromosomes Cancer*, 33, pp.73-81.
- Shtivelband, M. I., Juneja, H. S., Lee, S., & Wu, K. K. (2003). Aspirin and salicylate inhibit colon cancer medium- and vegf-induced endothelial tube formation: Correlation with suppression of cyclooxygenase-2 expression. *J Thromb Haemost*, 1, pp.2225-2233.
- Shureiqi, I., Chen, D., Lotan, R., Yang, P., Newman, R. A., Fischer, S. M., *et al.* (2000). 15-lipoxygenase-1 mediates nonsteroidal anti-inflammatory drug-induced apoptosis independently of cyclooxygenase-2 in colon cancer cells. *Cancer Res*, 60, pp.6846-6850.
- Sidelnikov, E., Bostick, R. M., Flanders, W. D., Long, Q., Cohen, V. L., Dash, C., *et al.* (2009). Mutl-homolog 1 expression and risk of incident, sporadic colorectal adenoma: Search for prospective biomarkers of risk for colorectal cancer. *Cancer Epidemiol Biomarkers Prev*, 18, pp.1599-1609.
- Skjelbred, C. F., Saebo, M., Wallin, H., *et al.* (2006) Polymorphisms of the XRCC1, XRCC3 and XPD genes and risk of colorectal adenoma and carcinoma, in a Norwegian cohort: a case control study. *BMC Cancer*, 6, pp. 67.
- Smith, T. R., Miller, M. S., Lohman, L., Lange, E. M., Case, L. D., Mohrenweiser, H. W. and Hu, J. J. (2003) Polymorphisms of XRCC1 and XRCC3 genes and susceptibility to breast cancer. *Cancer Lett*, 190, pp.183-90.
- Smith, M. L., Hawcroft, G., & Hull, M. A. (2000). The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: Evidence of different mechanisms of action. *Eur J Cancer* 36, pp.664-674.

- Soslow, R. A., Dannenberg, A. J., Rush, D., Woerner, B. M., Khan, K. N., Masferrer, J., *et al.* (2000). Cox-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer*, 89, pp.2637-2645.
- Spitz, G. A., Furtado, C. M., Sola-Penna, M., & Zancan, P. (2009). Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-phosphofructo-1-kinase structure and activity. *Biochem Pharmacol*, 77, pp.46-53.
- Stappenbeck, T. S., & Gordon, J. I. (2000). Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine. *Development*, 127, pp.2629-2642.
- Stark, L. A., Din, F. V., Zwacka, R. M., & Dunlop, M. G. (2001). Aspirin-induced activation of the NF- κ B signaling pathway: A novel mechanism for aspirin-mediated apoptosis in colon cancer cells. *FASEB J*, 15, pp.1273-1275.
- Stark, L. A., Reid, K., Sansom, O. J., Din, F. V., Guichard, S., Mayer, I., *et al.* (2007). Aspirin activates the NF- κ B signalling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. *Carcinogenesis*, 28, pp.968-976.
- Staudt, L. M. (2010). Oncogenic activation of NF- κ B. *Cold Spring Harb Perspect Biol*, 2, (6):a000109.
- Stewart, G. D., Nanda, J., Katz, E., Bowman, K. J., Christie, J. G., Brown, D. J., *et al.* (2011). DNA strand breaks and hypoxia response inhibition mediate the radiosensitisation effect of nitric oxide donors on prostate cancer under varying oxygen conditions. *Biochem Pharmacol*, 81, pp.203-210.
- Stolfi, C., Fina, D., Caruso, R., Caprioli, F., Fantini, M. C., Rizzo, A., *et al.* (2008). Mesalazine negatively regulates CDC25A protein expression and promotes accumulation of colon cancer cells in S phase. *Carcinogenesis*, 29, pp.1258-1266.
- Tahara, T., Shibata, T., Yamashita, H., Nakamura, M., Yoshioka, D., Okubo, M., *et al.* (2009). Chronic nonsteroidal anti-inflammatory drug (nsaid) use suppresses multiple CpG islands hypermethylation (CpG islands hypermethylation) of tumor suppressor genes in the human gastric mucosa. *Cancer Sci*, 100, pp.1192-1197.
- Tranah, G. J., Giovannucci, E., Ma, J., Fuchs, C., Hankinson, S. E. and Hunter, D. J. (2004) XRCC2 and XRCC3 polymorphisms are not associated with risk of colorectal adenoma. *Cancer Epidemiol Biomarkers Prev*, 13, pp.1090-1091.
- Tesei, A., Zoli, W., Fabbri, F., Leonetti, C., Rosetti, M., Bolla, M., *et al.* (2008). Ncx 4040, a non-donating acetylsalicylic acid derivative: Efficacy and mechanisms of action in cancer cells. *Nitric Oxide*, 19, pp.225-236.
- Thibodeau, S. N., Bren, G., & Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science*, 260, 816-819.
- Thibodeau, S. N., French, A. J., Cunningham, J. M., Tester, D., Burgart, L. J., Roche, P. C., *et al.* (1998). Microsatellite instability in colorectal cancer: Different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res*, 58, pp.1713-1718.
- Thun, M. J., Henley, S. J., & Patrono, C. (2002). Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst*, 94, pp.252-266.
- Thun, M. J., Namboodiri, M. M., & Heath, C. W., Jr. (1991). Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med*, 325, 1593-1596.

- Tili, E., Michaille, J. J., Wernicke, D., Alder, H., Costinean, S., Volinia, S., *et al.* (2011). Mutator activity induced by microRNA-155 (mir-155) links inflammation and cancer. *Proc Natl Acad Sci U S A*, 108, pp.4908-4913.
- To, K. K., Koshiji, M., Hammer, S., & Huang, L. E. (2005). Genetic instability: The dark side of the hypoxic response. *Cell Cycle*, 4, pp.881-882.
- Tsai, C. S., Luo, S. F., Ning, C. C., Lin, C. L., Jiang, M. C., & Liao, C. F. (2009). Acetylsalicylic acid regulates mmp-2 activity and inhibits colorectal invasion of murine b16f0 melanoma cells in c57bl/6j mice: Effects of prostaglandin f(2)alpha. *Biomed Pharmacother*, 63, pp.522-527.
- Tsuji, M., Kawano, S., & DuBois, R. N. (1997). Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A*, 94, pp.3336-3340.
- Umar, A., Boyer, J. C., Thomas, D. C., Nguyen, D. C., Risinger, J. I., Boyd, J., *et al.* (1994). Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. *J Biol Chem*, 269, pp.14367-14370.
- Urios, P., Grigorova-Borsos, A. M., & Sternberg, M. (2007). Aspirin inhibits the formation of pentosidine, a cross-linking advanced glycation end product, in collagen. *Diabetes Res Clin Pract*, 77, pp.337-340.
- Valeri, N., Gasparini, P., Fabbri, M., Braconi, C., Veronese, A., Lovat, F., *et al.* (2010). Modulation of mismatch repair and genomic stability by mir-155. *Proc Natl Acad Sci U S A*, 107, pp.6982-6987.
- Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact*, 160, pp.1-40.
- Vasavi, M., Ponnala, S., Gujjari, K., Boddu, P., Bharatula, R. S., Prasad, R., *et al.* (2006). DNA methylation in esophageal diseases including cancer: Special reference to hMLH1 gene promoter status. *Tumori*, 92, pp.155-162.
- Wang, D., & DuBois, R. N. (2008). Pro-inflammatory prostaglandins and progression of colorectal cancer. *Cancer Lett*, 267, pp.197-203.
- Wang, D., Dubois, R. N., & Richmond, A. (2009). The role of chemokines in intestinal inflammation and cancer. *Curr Opin Pharmacol*, 9, pp.688-696.
- Watson, A. J. (2006). An overview of apoptosis and the prevention of colorectal cancer. *Crit Rev Oncol Hematol*, 57, pp.107-121.
- Williams, J. L., Nath, N., Chen, J., Hundley, T. R., Gao, J., Kopelovich, L., *et al.* (2003). Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with Cyclooxygenase-2 induction and β -catenin/T-cell factor signaling, Nuclear Factor- κ B, and NO Synthase 2 inhibition: implications for chemoprevention. *Cancer Res*, 63, pp.7613-7618.
- Wink, D. A., & Laval, J. (1994). The fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. *Carcinogenesis*, 15, pp.2125-2129.
- Woerner, S. M., Yuan, Y. P., Benner, A., Korff, S., von Knebel Doeberitz, M., & Bork, P. (2010). SelTarbase, a database of human mononucleotide-microsatellite mutations and their potential impact to tumorigenesis and immunology. *Nucleic Acids Res*, 38, D682-689.
- Wood, R. D., Mitchell, M., Sgouros, J., & Lindahl, T. (2001). Human DNA repair genes. *Science*, 291, 1284-1289.
- Wu, X. Y., Fu, Z. X., & Wang, X. H. (2010). Effect of hypoxia-inducible factor 1- α on survivin in colorectal cancer. *Mol Med Report*, 3, pp.409-415.

- Xu, Z. Y., Loignon, M., Han, F. Y., Panasci, L. and Aloyz (2005) XRCC3 induces cisplatin resistance by stimulation of Rad51-related recombinational repair, S-phase checkpoint activation, and reduced apoptosis. *J Pharmacol Exp Ther*, 314, pp.495-505.
- Xiao, H., Xiao, Q., Zhang, K., Zuo, X., & Shrestha, U. K. (2010). Reversal of multidrug resistance by curcumin through fa/bra pathway in multiple myeloma cell line molp-2/r. *Ann Hematol*, 89, pp.399-404.
- Xie, J., & Itzkowitz, S. H. (2008). Cancer in inflammatory bowel disease. *World J Gastroenterol*, 14, pp.378-389.
- Yamamoto, H., Sawai, H., Weber, T. K., Rodriguez-Bigas, M. A., & Perucho, M. (1998). Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. *Cancer Res*, 58, pp.997-1003.
- Yamamoto, Y., & Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF- κ B pathway in the treatment of inflammation and cancer. *J Clin Invest*, 107, pp.135-142.
- Yamamoto, Y., Yin, M. J., Lin, K. M., & Gaynor, R. B. (1999). Sulindac inhibits activation of the NF- κ B pathway. *J Biol Chem*, 274, pp.27307-27314.
- Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., et al. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*, 9, pp.189-198.
- Yanamadala, S., & Ljungman, M. (2003). Potential role of MLH1 in the induction of p53 and apoptosis by blocking transcription on damaged DNA templates. *Mol Cancer Res*, 1, pp.747-754.
- Ye, C. G., Wu, W. K., Yeung, J. H., Li, H. T., Li, Z. J., Wong, C. C., et al. (2011). Indomethacin and SC236 enhance the cytotoxicity of doxorubicin in human hepatocellular carcinoma cells via inhibiting P-glycoprotein and MRP1 expression. *Cancer Lett*, 304, pp.90-96.
- Yeh, C. C., Sung, F. C., Tang, R., Chang-Chieh, C. R. and Hsieh, L. L. (2005) Polymorphisms of the XRCC1, XRCC3, & XPD genes, and colorectal cancer risk: a case-control study in Taiwan. *BMC Cancer*, 5, pp.183-193.
- Yin, H., Xu, H., Zhao, Y., Yang, W., Cheng, J., & Zhou, Y. (2006). Cyclooxygenase-independent effects of aspirin on HT-29 human colon cancer cells, revealed by oligonucleotide microarrays. *Biotechnol Lett*, 28, pp.1263-1270.
- Yin, M. J., Yamamoto, Y., & Gaynor, R. B. (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of I κ B kinase- β . *Nature*, 396, pp.77-80.
- Ying, J., Srivastava, G., Hsieh, W. S., Gao, Z., Murray, P., Liao, S. K., et al. (2005). The stress-responsive gene GADD45G is a functional tumor suppressor, with its response to environmental stresses frequently disrupted epigenetically in multiple tumors. *Clin Cancer Res*, 11, pp.6442-6449.
- Yoshihara, T., Ishida, M., Kinomura, A., Katsura, M., Tsuruga, T., Tashiro, S., Asahara, T. and Miyagawa, K. (2004) XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. *EMBO J*, 23, pp.670-680.
- Yu, H. G., Huang, J. A., Yang, Y. N., Huang, H., Luo, H. S., Yu, J. P., et al. (2002). The effects of acetylsalicylic acid on proliferation, apoptosis, and invasion of cyclooxygenase-2 negative colon cancer cells. *Eur J Clin Invest*, 32, pp.838-846.
- Yu, H. G., Huang, J. A., Yang, Y. N., Luo, H. S., Yu, J. P., Meier, J. J., et al. (2003). Inhibition of cytosolic phospholipase A2 mRNA expression: A novel mechanism for acetylsalicylic acid-mediated growth inhibition and apoptosis in colon cancer cells. *Regul Pept*, 114, pp.101-107.

- Yu, L., Chen, M., Li, Z., Wen, J., Fu, J., Guo, D., *et al.* (2011). Celecoxib antagonizes the cytotoxicity of cisplatin in human esophageal squamous cell carcinoma cells by reducing intracellular cisplatin accumulation. *Mol Pharmacol*, 79, pp.608-617.
- Yuan, C. J., Mandal, A. K., Zhang, Z., & Mukherjee, A. B. (2000). Transcriptional regulation of cyclooxygenase-2 gene expression: Novel effects of nonsteroidal anti-inflammatory drugs. *Cancer Res*, 60, 1084-1091.
- Zabkiewicz, J., & Clarke, A. R. (2004). DNA damage-induced apoptosis: Insights from the mouse. *Biochim Biophys Acta*, 1705, pp.17-25.
- Zerbini, L. F., Czibere, A., Wang, Y., Correa, R. G., Otu, H., Joseph, M., *et al.* (2006). A novel pathway involving melanoma differentiation associated gene-7/interleukin-24 mediates nonsteroidal anti-inflammatory drug-induced apoptosis and growth arrest of cancer cells. *Cancer Res*, 66, pp.11922-11931.
- Zerbini, L. F., Wang, Y., Czibere, A., Correa, R. G., Cho, J. Y., Ijiri, K., *et al.* (2004). NF- κ B-mediated repression of growth arrest- and DNA-damage-inducible proteins 45 α and γ is essential for cancer cell survival. *Proc Natl Acad Sci U S A*, 101, pp.13618-13623.
- Zhang, H., Richards, B., Wilson, T., Lloyd, M., Cranston, A., Thorburn, A., *et al.* (1999). Apoptosis induced by overexpression of hMSH2 or hMLH1. *Cancer Res*, 59, pp.3021-3027.
- Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., & Vogelstein, B. (2000). Role of bax in the apoptotic response to anticancer agents. *Science*, 290, pp.989-992.
- Zimmermann, K. C., Waterhouse, N. J., Goldstein, J. C., Schuler, M., & Green, D. R. (2000). Aspirin induces apoptosis through release of cytochrome c from mitochondria. *Neoplasia*, 2, pp.505-513.

Inhibition of DNA Polymerase λ , a DNA Repair Enzyme, and Anti-Inflammation: Chemical Knockout Analysis for DNA Polymerase λ Using Curcumin Derivatives

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1. Introduction

DNA polymerase (i.e., DNA-dependent DNA polymerase [pol], E.C. 2.7.7.7) catalyzes the polymerization of deoxyribonucleotides alongside a DNA strand, which it "reads" and uses as a template (Kornberg & Baker, 1992). The newly polymerized molecule is complementary to the template strand and identical to the template's partner strand. Pol can add free nucleotides only to the 3' end of the newly formed strand, meaning that elongation of the new strand occurs in a 5' to 3' direction.

The human genome encodes at least 14 pols that conduct cellular DNA synthesis (Bebenek & Kunkel, 2004; Hubscher et al., 2002). Eukaryotic cells contain 3 DNA replicative pols (α , δ and ϵ), 1 mitochondrial pol (γ), and at least 10 DNA repair and/or recombination-related pols (β , ζ , η , θ , ι , κ , λ , μ and ν , and REV1) (Friedberg et al., 2000; Takata et al., 2006). Pols have a highly conserved structure, which means that their overall catalytic subunits show little variance among species. Enzymes with conserved structures usually perform important cellular functions, the maintenance of which provides evolutionary advantages. On the basis of sequence homology, eukaryotic pols can be divided into 4 main families, termed A, B, X and Y (Friedberg et al., 2000). Family A includes mitochondrial pol γ , as well as pols θ and ν . Family B includes 3 DNA replicative pols (α , δ and ϵ) and pol ζ . Family X comprises pols β , λ and μ ; and lastly, family Y includes pols η , ι and κ , in addition to REV1.

We have been screening for selective inhibitors of each pol derived from natural products including food materials and nutrients for more than 15 years (Mizushina, 2009; Sakaguchi et al., 2002). In our studies of pol inhibitors, we have found that selective inhibitors of pol λ , which is a DNA repair-related pol, have anti-inflammatory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (Mizushina et al., 2003; Mizushina, 2009). Although tumor promoters such as TPA are classified as compounds that promote tumor formation (Hecker, 1978), they also cause inflammation and are commonly used as artificial inducers of inflammation in order to screen for anti-inflammatory agents (Fujiki & Sugimura, 1987). Tumor promoter-induced inflammation can be distinguished from acute inflammation, which is exudative and accompanied by fibroblast proliferation and granulation. The tumor promoter TPA is frequently used to search for new types of anti-inflammatory compound. TPA not only causes inflammation, but also influences mammalian cell growth (Nakamura et al., 1995), suggesting that the molecular basis of the inflammation stems from pol reactions related to cell proliferation. This relationship, however, needs to be investigated more closely.

In this review, we examine the relationship between pol λ inhibition and anti-inflammation using pol λ -specific inhibitors, such as chemically synthesized curcumin derivatives. On the basis of these results, the pol λ -inhibitory mechanism and anti-inflammation effects of monoacetyl-curcumin, which was the strongest pol λ inhibitor among the compounds tested, is discussed.

2. Effect of curcumin derivatives on the activities of mammalian pols

2.1 Pol assay for inhibitor screening

A pol activity assay to detect pol inhibitors was established by Mizushina et al. (1996a; 1996b; 1997). Purified mammalian pols α , β , γ , δ , ϵ , η , ι , κ and λ , which have high activity, were kind gifts from pol researchers around the world. As shown in Fig. 1, poly(dA)/oligo(dT)₁₈ (A/T = 2/1) and 2'-deoxythymidine 5'-triphosphate (dTTP) were used as the DNA template-primer and nucleotide (dNTP, 2'-deoxynucleotide 5'-triphosphate) substrate, respectively. The candidate inhibitors, which were low molecular weight organic compounds, were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Aliquots (4 μ L) of the sonicated samples were mixed with 16 μ L of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and pre-incubated at 0 °C for 10 min. These inhibitor-enzyme mixtures (8 μ L) were then added to 16 μ L of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min, except for *Taq* pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37 °C under the normal reaction conditions for each enzyme (Mizushina et al., 1996b; 1997).

2.2 Mammalian pol inhibitory effect of curcumin derivatives

As described above, we are searching for natural inhibitors specific to each of the mammalian pols. A phenolic compound produced from a higher plant, a Japanese vegetable

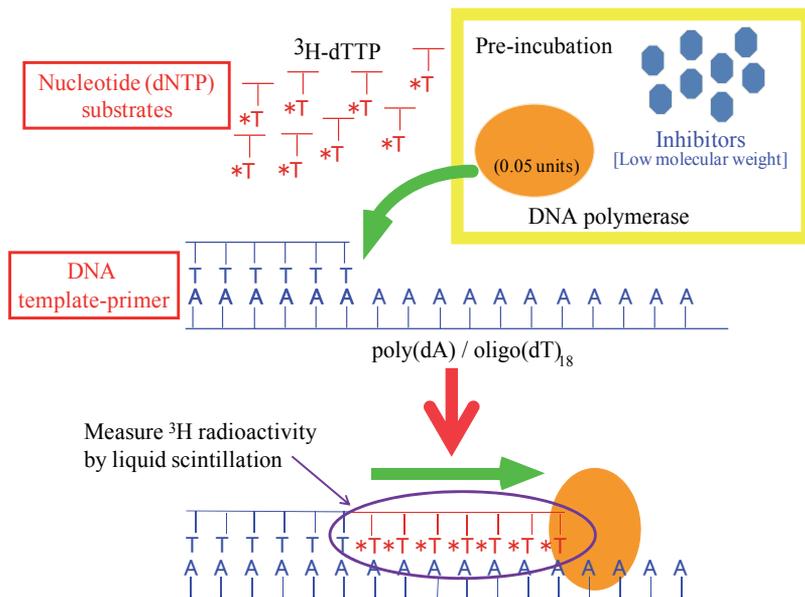


Fig. 1. Pol inhibitor assay scheme

(*Petasites japonicus*) collected from Akita prefecture, Japan, was found to inhibit pol λ activity selectively (Mizushina et al., 2002). The compound was purified and its chemical structure was analyzed, and it was identified as petasiphenol (compound **1** in Fig. 2) (Iriye et al., 1992). The three-dimensional relationship between pol inhibitors and the pol structure was investigated, which suggested that some phenolic compounds might be pol inhibitors. It was therefore tested whether commercial or easily obtainable phenolic compounds might also be pol λ -specific inhibitors. As a result, curcumin (diferuloylmethane, compound **2** in Fig. 2), which is the same type of phenolic compound as petasiphenol, and 13 chemically synthesized derivatives of curcumin (compounds **3–15** in Fig. 2) were prepared, and then tested for their inhibitory effects on mammalian pols.

The inhibition of four mammalian pols, namely calf pol α , human pol γ , human pol κ and human pol λ , by each compound at 10 μ M was investigated. Pols α , γ , κ and λ were used as representatives of the B, A, Y and X families of pols, respectively (Bebenek & Kunkel, 2004; Hubscher et al., 2002; Takata et al., 2006). As shown in Fig. 3, petasiphenol (**1**) and curcumin (**2**) inhibited human pol λ activity. The inhibitory effect on pol λ of compounds **4**, **5**, **13** and **14** was stronger than that of curcumin (**2**). Compounds **6** to **11**, which do not have any enone moieties, did not affect pol λ activity; thus, the enone moiety, which is present in petasiphenol, might be important or essential for pol λ inhibition. As mentioned above, compounds **4**, **5** and **13**, which have one or more acetoxy moieties, strongly inhibited the activity of pol λ , and compound **13** (monoacetyl-curcumin) was the strongest inhibitor among the compounds tested. The one acetoxy moiety at position C4'' in monoacetyl-curcumin (**13**) might stimulate the inhibitory effect on pol λ . On the other hand, at 10 μ M, none of the compounds inhibited the activity of calf pol α , human pol γ or human pol κ .

On the basis of these results, we concentrated on curcumin (**2**), which is a major food component, and monoacetyl-curcumin (**13**), which was the strongest inhibitor of pol λ among the curcumin derivatives tested, in the next part of this study.

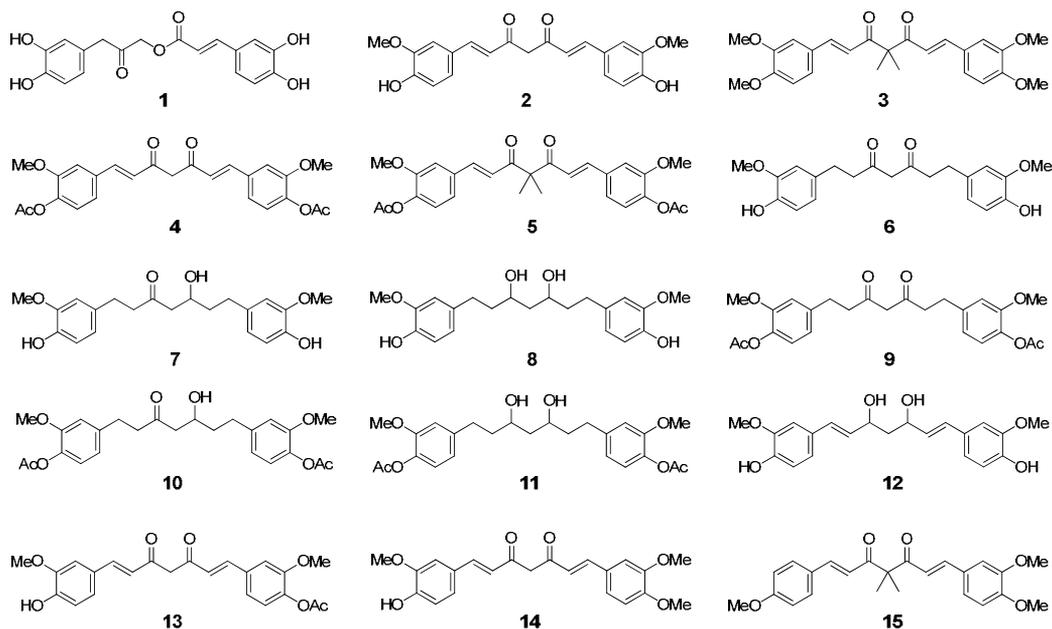


Fig. 2. Structure of curcumin derivatives. Compound 1: petasiphenol; compound 2: curcumin (diferuloylmethane); compound 3: (1*E*,6*E*)-1,7-bis(3',4'-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadien-3,5-dione; compound 4: (1*E*,4*Z*,6*E*)-1,7-bis(4'-acetoxy-3'-methoxyphenyl)-5-hydroxy-1,4,6-heptatrien-3-one (diacetyl-curcumin); compound 5: (1*E*,6*E*)-1,7-bis(4'-acetoxy-3'-methoxyphenyl)-4,4-dimethyl-1,6-heptadien-3,5-dione; compound 6: 1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptadione; compound 7: 1,7-bis(4-hydroxy-3-methoxyphenyl)-5-hydroxy-3-heptanone; compound 8: 1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptadiol; compound 9: 1,7-bis(4-acetoxy-3-methoxyphenyl)-3,5-heptadione; compound 10: 1,7-bis(4-acetoxy-3-methoxyphenyl)-5-hydroxy-3-heptanone; compound 11: 1*E*,6*E*-1,7-bis(4-acetoxy-3-methoxyphenyl)-3,5-dihydroxyheptane; compound 12: 1*E*,6*E*-1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-dihydroxy-1,6-heptadiene; compound 13: (1*E*,4*Z*,6*E*)-7-(4'-acetoxy-3'-methoxyphenyl)-5-hydroxy-1-(4'-hydroxy-3'-methoxyphenyl)hepta-1,4,6-trien-3-one (monoacetyl-curcumin); compound 14: (1*E*,4*Z*,6*E*)-1-(3',4'-dimethoxyphenyl)-5-hydroxy-7-[4'-hydroxy-3'-methoxyphenyl]hepta-1,4,6-trien-3-one (monomethyl-curcumin); compound 15: 1*E*,6*E*-1-3,4-dimethoxyphenyl-4,4-dimethyl-7-4-methoxyphenylhepta-1,6-dien-3,5-diol.

3. Effect of curcumin (2) and monoacetyl-curcumin (13) on the activities of pols and other DNA metabolic enzymes

Curcumin (2) and monoacetyl-curcumin (13) were effective at inhibiting human pol λ activity, and the inhibition was dose-dependent with 50% inhibition observed at a concentration of 7.0 and 3.9 μ M, respectively (Table 1). These compounds had no influence on the activities of not only DNA replicative pols such as calf pol α , human pol δ and human pol ϵ , or mitochondrial DNA replicative pols such as human pol γ , but also DNA repair-related pols such as rat pol β , human pols η , ι and κ . It is interesting that these compounds had no effect on the activity of pol β , because pols β and λ both belong to the

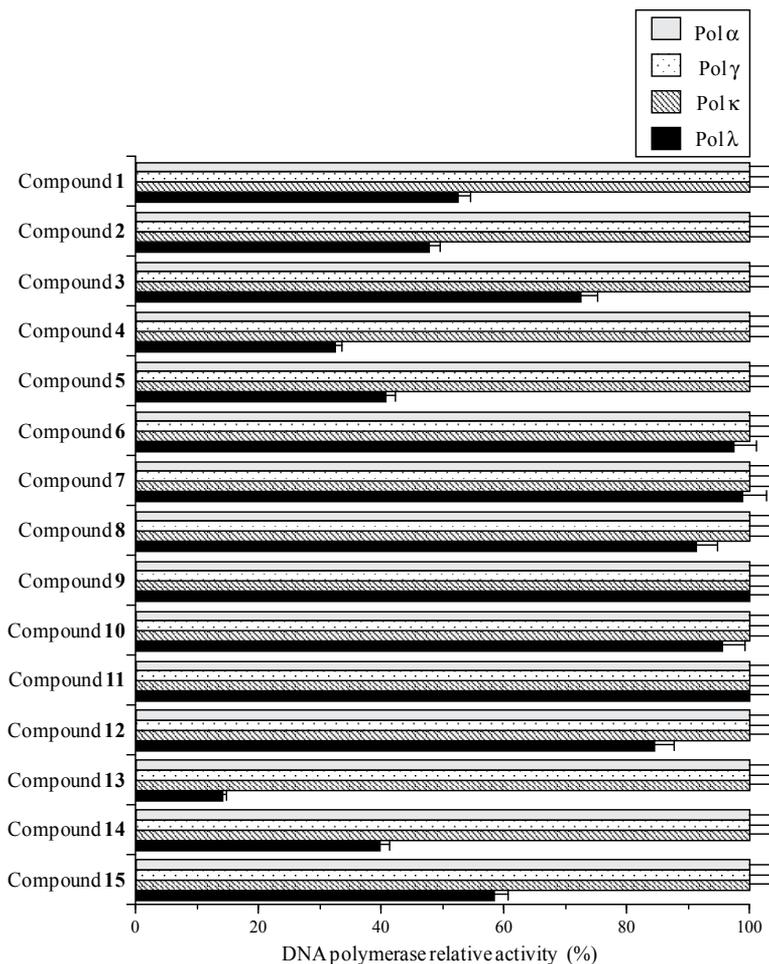


Fig. 3. Inhibitory effect of curcumin derivatives (compounds **1-15**) on the activities of mammalian pols. Each compound (10 μ M) was incubated with calf pol α (B-family pol), human pol γ (A-family pol), human pol κ (Y-family pol) and human pol λ (X-family pol) (0.05 units each). Pol activity in the absence of the compound was taken as 100%, and the relative activity is shown. Data are shown as the mean \pm SE (n=3).

X-family of pols, and the three-dimensional structure of pol β is thought to be highly similar to pol λ (Garcia-Diaz et al., 2004).

Curcumin (**2**) and monoacetyl-curcumin (**13**) also had no inhibitory effect on cherry salmon (fish) pols α and δ , cauliflower (higher plant) pol α , prokaryotic pols such as the Klenow fragment of *E. coli* pol I, *Taq* pol and T4 pol, and other DNA metabolic enzymes such as calf DNA primase of pol α , human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I. Therefore, these phenolic compounds were specific inhibitors of human pol λ among the pols and DNA metabolic enzymes tested. Petasiphenol (**1**) also selectively inhibited the activity of eukaryotic pol λ such as curcumin (**2**) and monoacetyl-curcumin (**13**) (Mizushima et al., 2002).

Enzyme	IC ₅₀ value (μM)	
	Curcumin (2)	Monoacetyl-curcumin (13)
- Mammalian DNA polymerases -		
Calf DNA polymerase α	>100	>100
Rat DNA polymerase β	>100	>100
Human DNA polymerase γ	>100	>100
Human DNA polymerase δ	>100	>100
Human DNA polymerase ε	>100	>100
Human DNA polymerase η	>100	>100
Human DNA polymerase ι	>100	>100
Human DNA polymerase κ	>100	>100
Human DNA polymerase λ	7.0 ± 0.39	3.9 ± 0.25
- Fish DNA polymerases -		
Cherry salmon DNA polymerase α	>100	>100
Cherry salmon DNA polymerase δ	>100	>100
- Plant DNA polymerases -		
Cauliflower DNA polymerase α	>100	>100
- Prokaryotic DNA polymerases -		
<i>E. coli</i> DNA polymerase I	>100	>100
<i>Taq</i> DNA polymerase	>100	>100
T4 DNA polymerase	>100	>100
- Other DNA metabolic enzymes -		
Calf primase of DNA polymerase α	>100	>100
HIV-1 reverse transcriptase	>100	>100
T7 RNA polymerase	>100	>100
T4 polynucleotide kinase	>100	>100
Bovine deoxyribonuclease I	>100	>100

Table 1. IC₅₀ values of curcumin (2) and monoacetyl-curcumin (13) for various pols and other DNA metabolic enzymes. The compounds were incubated with each enzyme (0.05 units). Enzyme activity in the absence of the compound was taken as 100%. Data are shown as the mean ± SE (n=3).

When activated DNA (i.e., bovine deoxyribonuclease I-treated DNA) and dNTP were used as the DNA template-primer and nucleotide substrate instead of synthesized DNA [poly(dA)/oligo(dT)₁₈ (A/T = 2/1)] and dTTP, respectively, the inhibitory effects of these compounds did not change.

4. Effect of curcumin derivatives on TPA-induced anti-inflammatory activity

As mentioned in the Introduction, TPA is known to cause inflammation and is commonly used in screens for anti-inflammatory agents (Fujiki & Sugimura, 1987). Curcumin (2) is known as an anti-TPA-induced inflammatory compound (Ammon & Wahl, 1991), but the other agents (compounds 1 and 3–15) had not previously been tested for anti-TPA-induced inflammatory activity.

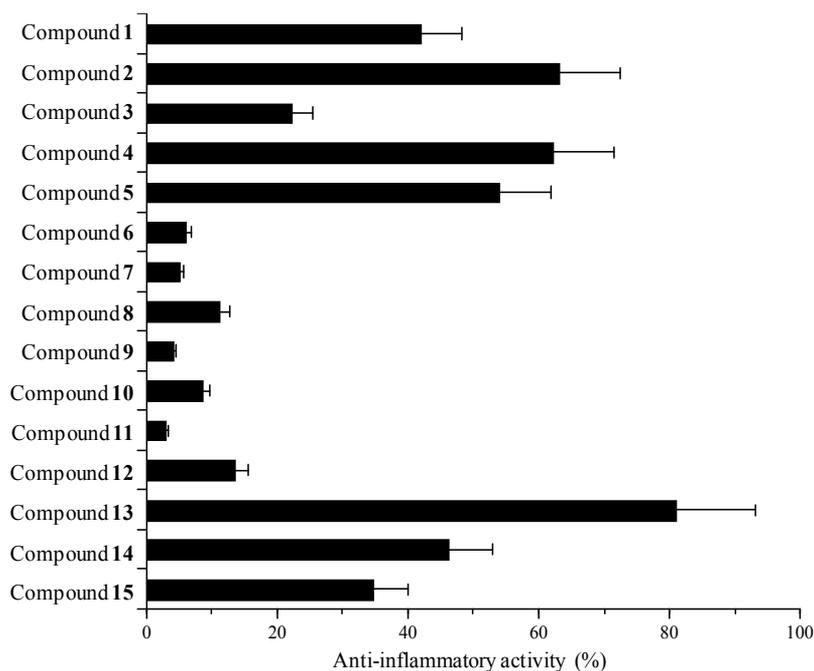


Fig. 4. Anti-inflammatory activity of curcumin derivatives (compounds 1–15) toward TPA-induced edema on mouse ear. Each compound (250 μg) was applied to one of the mouse ears and, after 30 min, TPA (0.5 μg) was applied to both ears. Edema was evaluated after 7 h. The anti-inflammatory activity (%) is expressed as the percentage reduction in edema as compared with the non-treated ear. Data are shown as the mean \pm SE (n=5).

Using an inflammation test in mice, the anti-inflammatory activity of these compounds was examined. The application of TPA (0.5 μg) to a mouse ear induced edema with a 241% increase in the weight of the ear disk at 7 h after application. As expected, curcumin (2) inhibited this inflammation at an applied dose of at least 250 μg (inhibitory effect (IE) = 63%) (Fig. 4). Petasiphenol (1), which was purified from Japanese vegetable (*Petasites japonicus*), was also an anti-inflammatory agent, although its effect was a third weaker than that of curcumin (2). Thus, both petasiphenol (1) and curcumin (2) could be potent inhibitors of inflammation caused by TPA. Interestingly, other curcumin derivatives also caused a marked reduction in TPA-induced inflammation: notably, the anti-inflammatory effect of monoacetyl-curcumin (13) was stronger than that of curcumin (2) with an IE of 81%, indicating that this compound possesses strong anti-inflammatory activity.

5. Structure–activity relationship of curcumin derivatives

Pol λ inhibition had a significant correlation (correlation coefficient = 0.9608) with anti-inflammatory activity, as shown by Fig. 3 and Fig. 4, which led us to speculate that TPA-induced inflammation may involve a process requiring pol λ , which is a DNA repair-related pol. Thus, to confirm whether there is a relationship between pol λ inhibition and anti-inflammation, the inhibitory effects of the curcumin derivatives (compounds 1–15) on the two bio-activities were compared.

Among the fifteen curcumin derivatives tested, including curcumin (**2**) itself (Fig. 1), monoacetyl-curcumin (**13**) was the strongest inhibitor of both pol λ and anti-inflammation. Considering the structure of monoacetyl-curcumin (**13**) (Fig. 5), the essential moieties for these activities might be: <1> two enone moieties, <2> one hydroxyl group at position C4', and <3> one acetoxy group at position C4''. These moieties are specific to monoacetyl-curcumin (**13**); therefore, these moieties are likely to be involved in the activities of both pol λ inhibition and anti-inflammation.

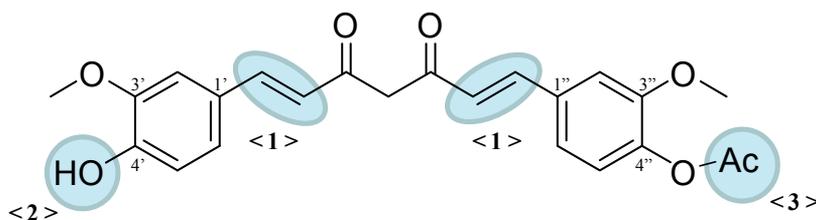


Fig. 5. Chemical structure of monoacetyl-curcumin (**13**). The functional groups likely to be essential for both pol λ inhibitory activity and anti-inflammatory activity in the curcumin derivatives are shown (<1> to <3>).

6. Inhibitory activity of curcumin (**2**) and monoacetyl-curcumin (**13**) against inflammatory responses in cultured cells

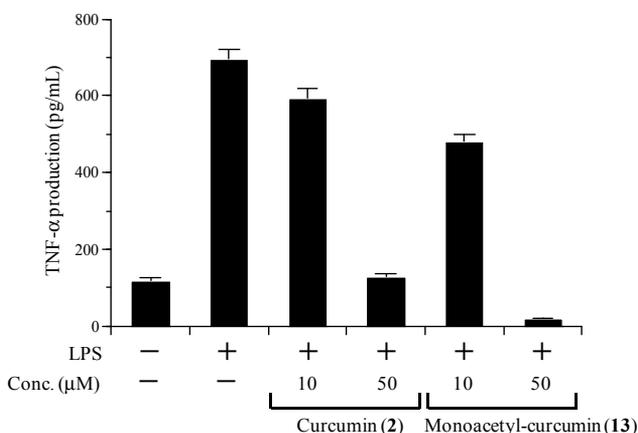
Next, because curcumin (**2**) and monoacetyl-curcumin (**13**) might be chemical knockout agents for DNA repair-related pol λ activity (Table 1), we used these compounds to investigate the anti-inflammatory mechanism of pol λ specific inhibitors in the murine macrophage cell line RAW264.7 treated with lipopolysaccharide (LPS or endotoxin), which stimulates macrophages to release inflammatory cytokines, interleukins (ILs) and tumor necrosis factor (TNF) (Hsu & Wen, 2002).

RAW264.7 cells were seeded on a 12-well plate at 1×10^5 cells/well and incubated for 24 h. The cells were pre-treated with 10 or 50 μM curcumin (**2**) or monoacetyl-curcumin (**13**) for 30 min and then stimulated with 100 ng/mL of LPS. After 30 min or 24 h, the cell culture medium was collected to measure the levels of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), nuclear factor- κB (NF- κB) and I κB . In RAW264.7 cells, cytotoxicity of these compounds at 50 μM was not observed (data not shown).

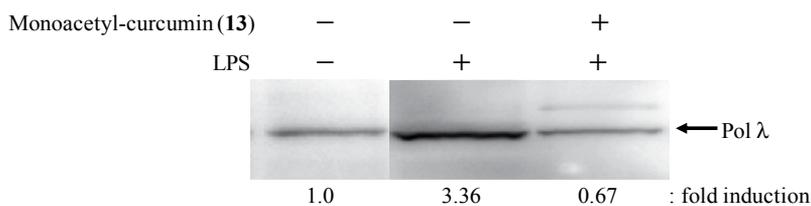
As shown in Fig. 6A, at 50 μM , curcumin (**2**) and monoacetyl-curcumin (**13**) both significantly suppressed LPS-stimulated production of TNF- α , and monoacetyl-curcumin (**13**) showed greater inhibition than curcumin (**2**). At 10 μM , monoacetyl-curcumin (**13**) still showed suppression of TNF- α production, although curcumin (**2**) at 10 μM did not significantly inhibit TNF- α production.

Next, the effect of monoacetyl-curcumin (**13**) on the expression level of pol λ protein in LPS-treated RAW264.7 cells was investigated. Fig. 6B shows that these macrophages underwent a more than 3-fold increase in the expression of pol λ after LPS stimulation, but this increase was suppressed by 50 μM monoacetyl-curcumin (**13**). These results suggest that there is the positive correlation between inflammatory induction by LPS and pol λ expression; thus, not only the DNA polymerization activity but also the protein expression of DNA repair-related pol λ is likely to be important in inflammation.

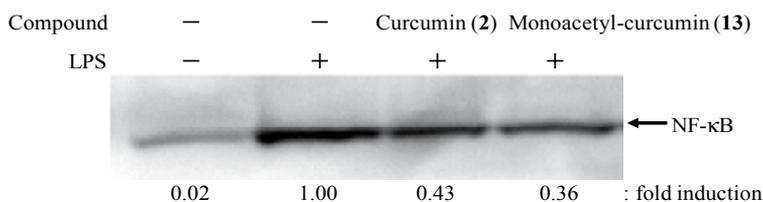
NF- κ B is known to be the rate-controlling factor in inflammatory responses. Therefore, the inhibitory effects of curcumin (**2**) and monoacetyl-curcumin (**13**) on the LPS-induced nuclear translocation of NF- κ B were examined in RAW264.7 cells. At 50 μ M, curcumin (**2**) and monoacetyl-curcumin (**13**) both inhibited NF- κ B nuclear translocation stimulated by 100 ng/mL of LPS, and the effect of monoacetyl-curcumin (**13**) was stronger than that of curcumin (**2**) (Fig. 6C). Stimulation with LPS results in activation of Toll-like receptor 4 and the downstream I κ B kinases (IKKs), which in turn phosphorylate I κ B, leading to degradation of I κ B and translocation of NF- κ B into the nucleus (Hashimoto et al., 2002). Therefore, the suppressive effects of curcumin (**2**) and monoacetyl-curcumin (**13**) on the LPS-induced phosphorylation of I κ B were examined in RAW264.7 cells. By Western blot analysis, it was revealed that, at 50 μ M, both curcumin (**2**) and monoacetyl-curcumin (**13**) significantly inhibited the LPS-induced phosphorylation of I κ B (Fig. 6D). These results demonstrate that monoacetyl-curcumin (**13**), as well as curcumin (**2**), suppresses NF- κ B nuclear translocation by inhibiting the phosphorylation of I κ B.



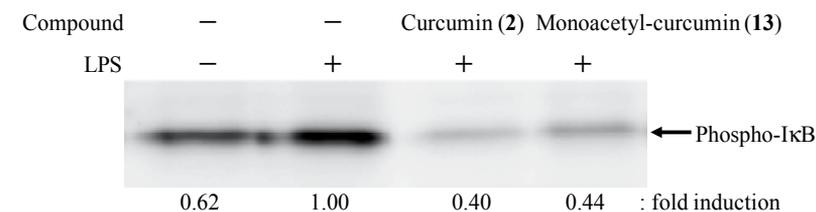
(a)



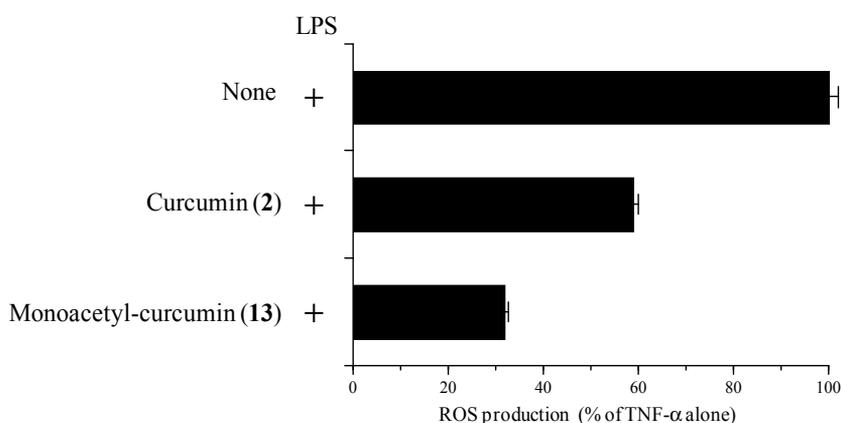
(b)



(c)



(d)



(e)

Fig. 6. Inhibitory activities of curcumin (2) and monoacetyl-curcumin (13) against inflammatory responses in the cultured murine macrophage cell line RAW264.7. (a) RAW264.7 cells were pre-treated with 10 or 50 μ M curcumin (2) or monoacetyl-curcumin (13) for 30 min and then incubated with 100 ng/mL of LPS for 24 h. The TNF- α level in the culture medium was measured by ELISA. Data are shown as the mean \pm SE (n=4). (b) RAW264.7 cells were pre-treated with 50 μ M monoacetyl-curcumin (13), and then incubated with 100 ng/mL of LPS for 30 min. The expression level of pol λ was evaluated by Western blot analysis. The intensity of each band was analyzed, and the values relative to non-treatment with LPS are represented at the lower edge of the image. (c and d) RAW264.7 cells were pre-treated with 50 μ M curcumin (2) or monoacetyl-curcumin (13), and then incubated with 100 ng/mL of LPS for 30 min. Nuclear translocation of NF- κ B p65 (c) and the phosphorylation of I κ B (d) were evaluated by Western blot analysis. The intensity of each band was analyzed, and the values relative to treatment with LPS alone are represented at the lower edge of the image. (e) RAW264.7 cells were pre-treated with 50 μ M curcumin (2) or monoacetyl-curcumin (13) for 30 min, and then treated with 50 ng/mL of TNF- α and 4 μ M DCFH-DA for 30 min. The fluorescent intensity of DCF, which indicates ROS production, was measured as described in a previous report (Corda et al., 2001). Data are shown as the mean \pm SE (n=4).

Anti-oxidative activity has been reported to be linked to anti-inflammatory activity (Rahman et al., 2006). We therefore investigated the anti-oxidative activity of curcumin (**2**) and monoacetyl-curcumin (**13**) against the production of reactive oxygen species (ROS) induced by TNF- α . Measurement of intracellular ROS was performed according to the method of a previous report (Corda et al., 2001). In RAW264.7 cells, at 50 μ M, the two compounds decreased the production of ROS by 50 ng/mL of TNF- α to 59.5% and 32.1%, respectively (Fig. 6E). These results suggest that both compounds possess anti-oxidative activity, but that monoacetyl-curcumin (**13**) has stronger activity than curcumin (**2**).

7. Inhibitory activity of curcumin (**2**) and monoacetyl-curcumin (**13**) against LPS-induced inflammation *in vivo*

To assess their anti-inflammatory effects *in vivo*, the inhibitory activity of curcumin (**2**) and monoacetyl-curcumin (**13**) against LPS-induced acute inflammation was investigated in mice (Fig. 7). As shown in Fig. 7A, treatment with 250 μ g/kg (body weight, BW) of LPS increased the serum TNF- α level, and an oral injection of 100 mg/kg (BW) of monoacetyl-curcumin (**13**) significantly decreased the LPS-induced production of TNF- α to 36%. By contrast, curcumin (**2**) had no effect. Next, the inhibitory effects of these compounds on nuclear translocation of NF- κ B in the liver were examined. Fig. 7B shows that LPS caused translocation of NF- κ B into the nucleus, and monoacetyl-curcumin (**13**) blocked this nuclear translocation. Notably, curcumin (**2**) also inhibited nuclear translocation of NF- κ B even though it did not block TNF- α production.

The serum levels of curcumin (**2**) and monoacetyl-curcumin (**13**) 2 h after oral administration were measured in the mice by liquid-chromatography mass spectrometry. The serum concentrations were below the detection limit and, thus, were less than 0.3 nM for both curcumin (**2**) and monoacetyl-curcumin (**13**) (data not shown). It has been reported that curcumin (**2**) is poorly absorbed in the body (Anand et al., 2007). Thus, a lower concentration of monoacetyl-curcumin (**13**) than of curcumin (**2**) might be able to decrease the serum TNF- α level in mice treated with LPS.

8. Discussion

Inflammatory mediators, such as TPA and LPS, quickly stimulate ROS (Hsu & Wen, 2002), and ROS are known to mediate oxidative DNA damage. As shown in Fig. 8, DNA repair pols such as pol λ induce protein expression and increase DNA polymerization activity to repair the damaged DNA. Furthermore, we consider that pol λ might have a great effect on inflammatory responses, such as TNF- α production, NF- κ B activation, secretion of cytokines [e.g. interferons (IFNs) and interleukins (ILs) etc], tissue damage and cell death. The results summarized in this review suggest that inhibition of DNA repair by pol λ is related to anti-inflammatory pathways, and that pol λ -specific inhibitors such as monoacetyl-curcumin (**13**) might be chemotherapeutic drugs for inflammatory diseases. The detailed molecular mechanism underlying the correlation between DNA repair inhibition by pol λ and anti-inflammatory responses is not yet known; therefore, experiments with small interfering RNA (siRNA) targeting pol λ would help in further analyses.

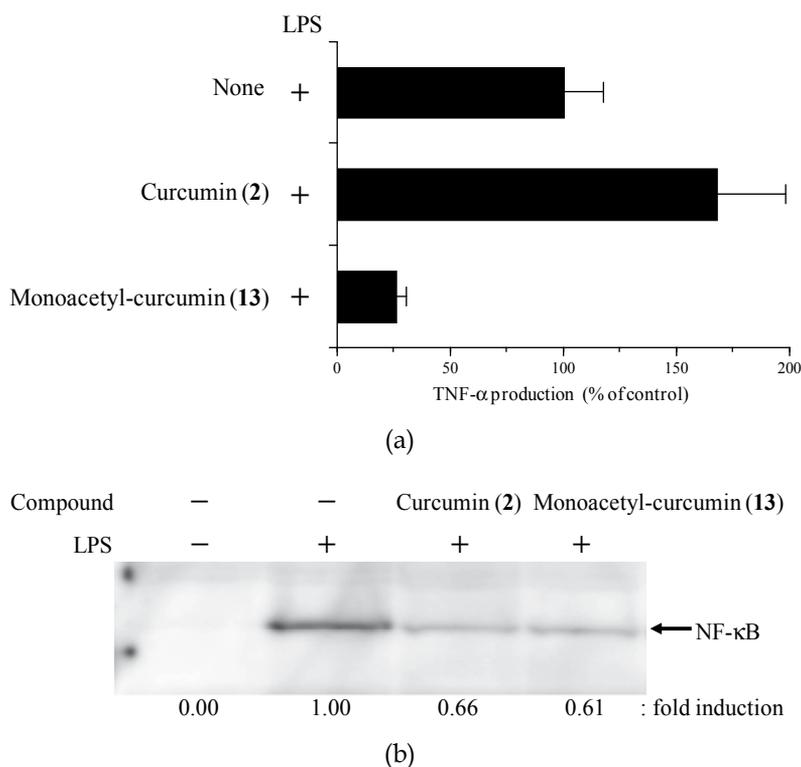


Fig. 7. The inhibitory activity of curcumin (2) and monoacetyl-curcumin (13) against LPS-induced inflammation *in vivo*. Male 8-week-old C57BL/6 mice were given an oral dose of 100 mg/kg (BW) of curcumin (2) or monoacetyl-curcumin (13) dissolved in corn oil or 200 μ L of corn oil as a vehicle control. After 2 h, the mice were intraperitoneally injected with 250 μ g/kg (BW) of LPS dissolved in phosphate-buffered saline (PBS) or 200 μ L of PBS as a vehicle control. After 1 h, the mice were killed. (a) The TNF- α level in serum was measured by ELISA. Data are shown as the mean \pm SE (n=4). The treatment with corn oil and LPS (positive control) was taken as 100% (TNF- α level, 728 pg/mL) and that with corn oil and saline (negative control) as taken as 0% (TNF- α level, 32 pg/mL). (b) NF- κ B p65 in the nuclei of mouse liver cells was detected by Western blotting. The intensity of each band was analyzed, and the values relative to treatment with LPS alone are represented at the lower edge of the image.

As mentioned above, eukaryotic cells reportedly contain 14 pol species belonging to four families (Friedberg et al., 2000; Takata et al., 2006). Among the X family of pols, pol λ has an unclear biochemical function, although it seems to work in a similar way to pol β (Garcia-Diaz et al., 2002). Pol β is involved in the short-patch base excision repair (BER) pathway (Matsumoto & Kim, 1995; Singhal & Wilson, 1993; Sobol et al., 1996), as well as playing an essential role in neural development (Sugo et al., 2000). Recently, pol λ was found to possess 5'-deoxyribose-5-phosphate (dRP) lyase activity, but not apurinic/aprimidinic (AP) lyase activity (Garcia-Diaz et al., 2001). Pol λ is able to substitute for pol β during *in vitro* BER, suggesting that pol λ also participates in BER. Northern blot analysis indicated that transcripts of pol β are abundantly expressed in the

testis, thymus and brain in rats (Hirose et al., 1989), whereas pol λ is efficiently transcribed mostly in the testis (Garcia-Diaz et al., 2000). Bertocci et al. reported that mice in which pol λ expression is knocked down are not only viable and fertile, but also display a normal hyper-mutation pattern (Bertocci et al., 2002).

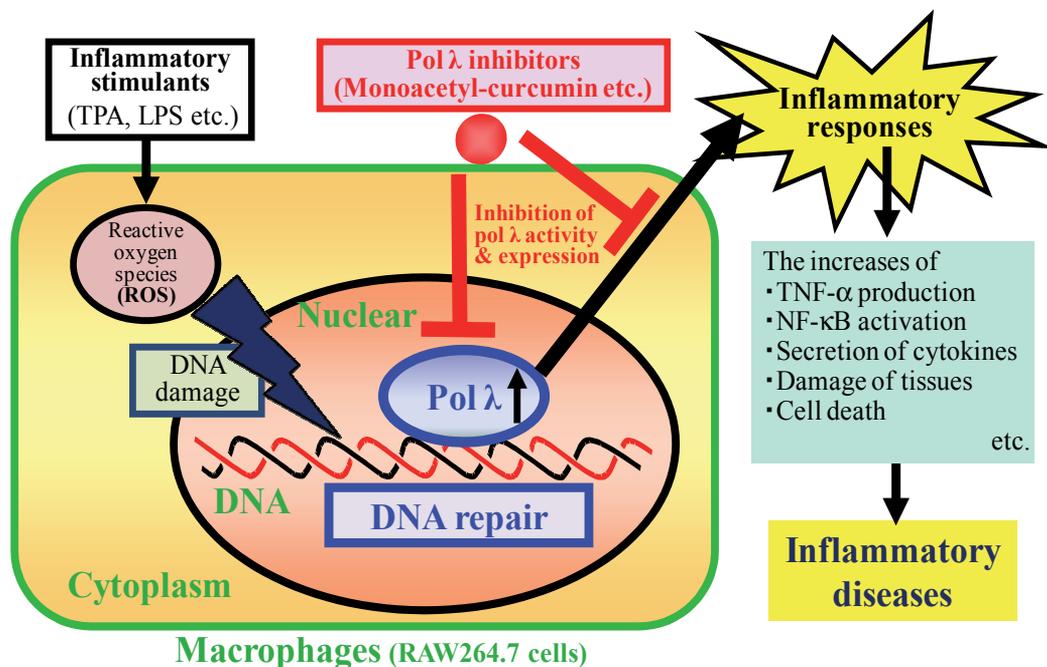


Fig. 8. The relationship between DNA repair by pol λ and inflammation

As well as causing inflammation, TPA influences cell proliferation and has physiological effects on cells because it has tumor promoter activity (Nakamura et al., 1995). Therefore, anti-inflammatory agents are expected to suppress DNA replication/repair/recombination in nuclei in relation to the action of TPA. Because pol λ is a DNA repair-related pol (Garcia-Diaz et al., 2002), our finding - that the molecular target of curcumin derivatives as monoacetyl-curcumin (13) is pol λ - is in good agreement with this expected mechanism of anti-inflammatory agents. As a result, any inhibitor of DNA repair-related pol λ might also be an inflammatory suppressor.

9. Conclusion

This review summarizes data showing that a major anti-inflammatory food compound, curcumin (2), selectively inhibits the activity of pol λ among 9 species of mammalian polys tested. Monoacetyl-curcumin (13) was the strongest inhibitor of pol λ among the 13 chemically synthesized derivatives of curcumin (2), suggesting that monoacetyl-curcumin (13) is a potent candidate for a functional compound. In addition, the inhibitory effects of monoacetyl-curcumin (13) on inflammatory responses in comparison to those of curcumin (2) *in vitro* and *in vivo* were investigated. Monoacetyl-curcumin (13) suppressed NF- κ B activation induced by LPS and TNF- α in RAW264.7 murine macrophages. Moreover,

monoacetyl-curcumin (**13**) exerted inhibitory effects on TNF- α production and NF- κ B activation in an animal model of LPS-induced acute inflammation. These results of the chemical knock out of pol λ by monoacetyl-curcumin (**13**) suggest that the inhibition of pol λ , which is a DNA repair-related pol, is related to anti-inflammatory processes.

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11. References

- Anand, P.; Kunnumakkara, A.B.; Newman, R.A. & Aggarwal, B.B. (2007). Bioavailability of curcumin: problems and promises. *Molecular Pharmacology*, Vol.4, No.6, pp. 807-818, ISSN 0026-895X
- Ammon, H.P. & Wahl, M.A. (1991). Pharmacology of *Curcuma longa*. *Planta Medica*, Vol.57, No.1, pp. 1-7, ISSN 0032-0943
- Bebenek, K. & Kunkel, T.A. (2004). DNA Repair and Replication. In: *Advances in Protein Chemistry*, W. Yang (Ed.), Vol. 69, pp. 137-165, Elsevier: San Diego, CA, USA
- Bertocci, B.; De Smet, A.; Flatter, E.; Dahan, A.; Bories, J.C.; Landreau, C.; Weill, J.C. & Reynaud, C.A. (2002). Cutting edge: DNA polymerases μ and λ are dispensable for Ig gene hypermutation. *The Journal of Immunology*, Vol.168, No.8, pp. 3702-3706, ISSN 0022-1767
- Corde, S.; Laplace, C.; Vicaut, E. & Duranteau, J. (2001). Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor- α is mediated by ceramide. *The American Journal of Respiratory Cell and Molecular Biology*, Vol.24, No.6, pp. 762-768, ISSN 1044-1549
- Friedberg, E.C.; Feaver, W.J. & Gerlach, V.L. (2000). The many faces of DNA polymerases: strategies for mutagenesis and for mutational avoidance. *Proceedings of the National Academy of Sciences USA*, Vol.97, No.11, pp. 5681-5683, ISSN 0027-8424
- Fujiki, H. & Sugimura, T. (1987). *Advances in Cancer Research*, Academic Press Inc., pp. 223-264, London, UK
- Garcia-Diaz, M.; Dominguez, O.; Lopez-Fernandez, L.A.; De Lera, L.T.; Saniger, M.L.; Ruiz, J.F.; Parraga, M.; Garcia-Ortiz, M.J.; Kirchhoff, T.; Del Mazo, J.; Bernad, A. & Blanco, L. (2000). DNA polymerase λ , a novel DNA repair enzyme in human cells. *Journal of Molecular Biology*, Vol.301, No.4, pp. 851-867, ISSN 0022-2836

- Garcia-Diaz, M.; Bebenek, K.; Kunkel, T.A. & Blanco, L. (2001). Identification of an intrinsic 5'-deoxyribose-5-phosphate lyase activity in human DNA polymerase λ : a possible role in base excision repair. *The Journal of Biological Chemistry*, Vol.276, No.37, pp. 34659-34663, ISSN 0021-9258
- Garcia-Diaz, M.; Bebenek, K.; Sabariego, R.; Dominguez, O.; Rodriguez, J.; Kirchhoff, T.; Garcia-Palomero, E.; Picher, A.J.; Juarez, R.; Ruiz, J.F.; Kunkel, T.A. & Blanco, L. (2002). DNA polymerase λ , a novel DNA repair enzyme in human cells. *The Journal of Biological Chemistry*, Vol.277, No.15, pp. 13184-13191, ISSN 0021-9258
- Garcia-Diaz, M.; Bebenek, K.; Krahn, J.M.; Blanco, L.; Kunkel, T.A. & Pedersen, L.C. (2004). A structural solution for the DNA polymerase λ -dependent repair of DNA gaps with minimal homology. *Molecular Cell*, Vol.13, No.4, pp. 561-572, ISSN 1097-2765
- Hashimoto, T.; Nonaka, Y.; Minato, K.; Kawakami, S.; Mizuno, M.; Fukuda, I.; Kanazawa, K. & Ashida, H. (2002). Suppressive effect of polysaccharides from the edible and medicinal mushrooms, *Lentinus edodes* and *Agaricus blazei*, on the expression of cytochrome P450s in mice. *Bioscience, Biotechnology, and Biochemistry*, Vol.66, No.7, pp. 1610-1614, ISSN 0916-8451
- Hecker, E. (1978). *Carcinogenesis*, Raben Press, pp. 11-48, NY, USA
- Hirose, F.; Hotta, Y.; Yamaguchi, M. & Matsukage, A. (1989). Difference in the expression level of DNA polymerase β among mouse tissues: high expression in the pachytene spermatocyte. *Experimental Cell Research*, Vol.181, No.1, pp. 169-180, ISSN 0014-4827
- Hsu, H.Y. & Wen, M.H. (2002). Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *The Journal of Biological Chemistry*, Vol.277, No.25, pp. 22131-22139, ISSN 0021-9258
- Hubscher, U.; Maga, G. & Spadari, S. (2002). Eukaryotic DNA polymerases. *The Annual Review of Biochemistry*, Vol.71, pp.133-163, ISSN 0066-4154
- Iriye, R.; Furukawa, K.; Nishida, R.; Kim, C. & Fukami, H. (1992). Isolation and synthesis of a new bio-antimutagen, petasiphenol, from scapes of *Petasites japonicum*. *Bioscience, Biotechnology, and Biochemistry*, Vol.56, No.11, pp. 1773-1775, ISSN 0916-8451
- Kornberg, A. & Baker, T.A. (1992). Eukaryotic DNA polymerase, In: *DNA replication, Second Edition*, W.D. Freeman & Co. (Ed), Chapter 6, pp. 197-225, New York, USA, ISBN 1-891389-44-0
- Matsumoto, Y. & Kim, K. (1995). Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair. *Science*, Vol.269, No.5224, pp. 699-702, ISSN 0036-8075
- Mizushima, Y.; Tanaka, N.; Yagi, H.; Kurosawa, T.; Onoue, M.; Seto, H.; Horie, T.; Aoyagi, N.; Yamaoka, M.; Matsukage, A.; Yoshida, S. & Sakaguchi, K. (1996a). Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. *Biochimica et Biophysica Acta*, Vol.1308, No.3, pp. 256-262, ISSN 0304-4165
- Mizushima, Y.; Yagi, H.; Tanaka, N.; Kurosawa, T.; Seto, H.; Katsumi, K.; Onoue, M.; Ishida, H.; Iseki, A.; Nara, T.; Morohashi, K.; Horie, T.; Onomura, Y.; Narusawa, M.; Aoyagi, N.; Takami, K.; Yamaoka, M.; Inoue, Y.; Matsukage, A.; Yoshida, S. & Sakaguchi, K. (1996b). Screening of inhibitor of eukaryotic DNA polymerases produced by microorganisms. *The Journal of Antibiotics*, Vol.49, No.5, pp. 491-492, ISSN 0021-8820

- Mizushina, Y.; Yoshida, S.; Matsukage, A. & Sakaguchi, K. (1997). The inhibitory action of fatty acids on DNA polymerase β . *Biochimica et Biophysica Acta*, Vol.1336, No.3, pp. 509-521, ISSN 0304-4165
- Mizushina, Y.; Kamisuki, S.; Kasai, N.; Ishidoh, T.; Shimazaki, N.; Takemura, M.; Asahara, H.; Linn, S.; Yoshida, S.; Koiwai, O.; Sugawara, F.; Yoshida, H. & Sakaguchi, K. (2002). Petasiphenol: a DNA polymerase λ inhibitor. *Biochemistry*, Vol.41, No.49, pp. 14463-14471, ISSN 0006-2960
- Mizushina, Y.; Hirota, M.; Murakami, C.; Ishidoh, T.; Kamisuki, S.; Shimazaki, N.; Takemura, M.; Perpelescu, M.; Suzuki, M.; Yoshida, H.; Sugawara, F.; Koiwai, O. & Sakaguchi, K. (2003). Some anti-chronic inflammatory compounds are DNA polymerase λ -specific inhibitors. *Biochemical Pharmacology*, Vol.66, No.10, pp. 1935-1944, ISSN 0006-2952
- Mizushina, Y. (2009). Specific inhibitors of mammalian DNA polymerase species. *Bioscience, Biotechnology, and Biochemistry*, Vol.73, No.6, pp. 1239-1251, ISSN 0916-8451
- Nakamura, Y.; Murakami, A.; Ohto, Y.; Torikai, K.; Tanaka, T. & Ohigashi, H. (1998). Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1'-acetoxychavicol acetate. *Cancer Research*, Vol.58, No.21, pp. 4832-4839, ISSN 0008-5472
- Rahman, I.; Biswas, S.K. & Kirkham, P.A. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. *Biochemical Pharmacology*, Vol.72, No.11, pp. 1439-1452, ISSN 0006-2952
- Ramadan, K.; Shevelev, I.V.; Maga, G. & Hubscher, U. (2002). DNA polymerase λ from calf thymus preferentially replicates damaged DNA. *The Journal of Biological Chemistry*, Vol.277, No.21, pp. 18454-18458, ISSN 0021-9258
- Sakaguchi, K.; Sugawara, F. & Mizushina, Y. (2002). Inhibitors of eukaryotic DNA polymerases. *Seikagaku*, Vol.74, No.3, pp. 244-251
- Singhal, R.K. & Wilson, S.H. (1993). Short gap-filling synthesis by DNA polymerase β is processive. *The Journal of Biological Chemistry*, Vol.268, No.21, pp. 15906-15911, ISSN 0021-9258
- Sobol, R.W.; Horton, J.K.; Kuhn, R.; Gu, H.; Singhal, R.K.; Prasad, R.; Rajewsky, K. & Wilson, S.H. (1996). Requirement of mammalian DNA polymerase- β in base-excision repair. *Nature*, Vol.379, No.6561, pp. 183-186, ISSN 0028-0836
- Sugo, N.; Aratani, Y.; Nagashima, Y.; Kubota, Y. & Koyama, H. (2000). Neonatal lethality with abnormal neurogenesis in mice deficient in DNA polymerase β . *The EMBO Journal*, Vol.19, No.6, pp. 1397-1404, ISSN 0261-4189
- Takata, K.; Shimizu, T.; Iwai, S. & Wood, R.D. (2006). Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. *The Journal of Biological Chemistry*, Vol.281, No.33, pp. 23445-23455, ISSN 0021-9258



Edited by Sonya Vengrova

Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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