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DNA Repair
An Update

Edited by Maddalena Mognato

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Meet the editor

Dr. Maddalena Mognato is a researcher in cell biology at the Department of Biology, University of Padova (Italy). She obtained a master's degree in Biological Sciences from the University of Padova in 1996 and received her PhD in Environmental Medicine in 2001. Her research activity is focused on cytotoxic and genotoxic effects induced by physical and chemical agents in human and mammalian cells, both primary and established. Her research is mainly focused on the DNA-damage response signaling pathway, in particular on the repair of radio-induced DNA double-strand breaks and on gene expression modulation mediated by microRNAs. Her research activity is documented in international peer-reviewed articles and numerous abstracts in international and national congresses. Since 2007, she has held courses for the students of molecular biology at the University of Padova.

Contents

Preface

During the last few years, many advances in the knowledge of DNA repair mechanisms were made in eukaryotic cells, thanks to innovative technologies in cellular and molecular biology. However, due to the complexity of cellular physiology, the whole mechanism is still under investigation, highlighting different factors that affect DNA repair efficiency in human cells. The role of proteins involved in DNA repair has been widely studied, but the modality and power of extrinsic and intrinsic factors in influencing protein functionality and correct protein-protein interactions represent a research area under constant investigation. Among intrinsic factors affecting DNA repair processes, there is epigenetics, which strongly impacts on gene expression regulation of DNA repair genes and the complex network of DNA-damage response-related genes. The structure and function of the epigenome under physiological and pathological conditions in the presence of DNA damage are an open and rapidly growing research field. Moreover, in mammalian aged cells, accumulated DNA damage is a source of genomic instability if proper repair is not carried out.

The book is divided into four sections with chapters describing different topics connected to DNA repair in human cells. The first section contains the introductory chapter dealing with the subjects of the book. The second section is dedicated to the role of protein-protein interactions during DNA repair in nuclear and mitochondrial compartments. The third section is dedicated to the relationship between the epigenome and DNA repair in normal and cancer cells. The fourth section is about the interconnection between aging and DNA repair. This last section also contains a chapter on the relationship between the angiogenesis of cancer cells and DNA damage repair and a chapter on the DNA repair-enhancing property of glucan.

I acknowledge the authors that contributed to this book and hope that the topics here discussed may suggest readers to explore new avenues and aspects of the interconnection between different DNA lesions and responses essential for the maintenance of nuclear and mitochondrial genome stability.

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Section 1

Introduction

Chapter 1

Introductory Chapter: DNA Repair in Human Cells - A Daily Challenge

Maddalena Mognato

1. Introduction

The faithful repair of DNA is a challenge that human cells have to fight every day to maintain genomic stability. The type and frequency of DNA lesions are related to both endogenous and exogenous sources of DNA damage. In addition to normal metabolism, which is responsible for a great number of DNA lesions (approximately 70,000 per cell) [1, 2], environmental agents (i.e., ionizing radiation, UV light, and chemicals) contribute to enhance such number. The capacity of cells to faithfully repair their proper DNA is the primary goal to safeguard the genome integrity. To this purpose, eukaryotic cells have evolved accurate repair systems to overcome the different lesions induced by both external and internal sources of DNA damage. A lot of information is now available for most repair systems, and in the last decades, a lot of efforts have been made in the comprehension of the role of DNA repair proteins, in relation to the type of damage and the effectiveness of repair carried out by different complexes. Besides the molecular role of proteins in such pathways, several other important factors can affect the efficiency of DNA repair, including epigenetics, chromatin structure, mitochondrial function, and aging.

Epigenetics regulate gene function through posttranslational modifications of histones, DNA methylation noncoding RNAs, and when DNA is damaged, epigenetic alterations can occur at sites of lesions. Epigenetic alterations that occur during DNA repair are mostly transient, being the original epigenetic marker restored. However, sometimes, epigenetic alterations can persist after DNA repair as a sort of "scars" [3]. What is the role of such epigenetic markers left after repair? Epigenetic modifications occur either in normal cells or in cancer cells, representing a further element for cancerogenesis in this last case. Numerous studies reported gene expression changes in human cancers and found signature for specific type of tumors. Each cancer has its own genetic and epigenetic profile, which increases the difficulty to comprehend the process of tumorigenicity. In this regard, the response to each tumor to different DNA-damaging agents is related to the characteristics of its genetic and epigenetic landscape.

The structure of chromatin around DNA damage changes significantly to promote DNA repair proteins accessibility. During DNA repair, the structure of chromatin is modified as a consequence of new histone incorporation, replacement, and modification. The coordination of DNA repair protein interactions is a critical process which needs to be fully elucidated, also in relation to the specific DNA-damaging agent.

Mitochondria, with their own DNA, are organelles that are on the rise for several reasons, including the repair of their proper DNA, the mtDNA. Mitochondrial DNA is different from the nuclear one, being circular, without histones, and present in multiple copies. The repair of mtDNA relies on the activity of proteins encoded by

nuclear DNA, and the efficiency of repair is crucial for the maintenance of mtDNA integrity. What happens when the mitochondrial genome is affected by improper DNA repair and mutations arise? To address this question, studies should take into account that the multiplicity of mtDNA genomes inside the same cell originates a coexistence of mutant and wild-type genomes [4].

Notably, the accumulation of DNA damages during the cell lifespan threatens the fidelity of repair. According to the candidate hallmarks of aging in mammalian cells, recently reviewed by Lopez-Otin et al. [4], it appears evident how the process of DNA repair is tightly linked to genomic instability, cellular senescence, epigenetic alterations, and mitochondrial dysfunction. In humans, alterations in nuclear DNA repair are present in several syndromes characterized by premature aging, and epigenetic modifications in histones and histone-modifying enzymes affect chromatin structure in an age-related manner. Several studies attempted to elucidate the linkage between mitochondria dysfunction and aging. Indeed, when the mitochondrial function is impaired, the result is an increase of oxidative stress that triggers a cascade of toxic effects on cellular environment.

Finally, the connection between DNA repair process and angiogenesis is another open research field. Angiogenesis is a physiological process that allows the regeneration of blood vessels following injuries. However, angiogenesis is extremely harmful in pathological conditions, such as in tumoral tissues, characterized by the uncontrolled growth of new blood vessels. Mutations or alterations in genes involved in the cellular response to DNA damage can affect the angiogenic response.

Many questions are still open and further investigations are needed to shed light on the whole mechanism of DNA repair, either nuclear or mitochondrial. To this purpose, the present book offers a collection of chapters dedicated to the interplay between DNA repair and epigenetics under physiological and pathological conditions, aging, mitochondrial function, angiogenesis, and the contribution of base excision repair process to oxidative damage, giving a contribution to cancer biology and clinical management. **Figure 1** shows some of the principal aspects discussed in this book.

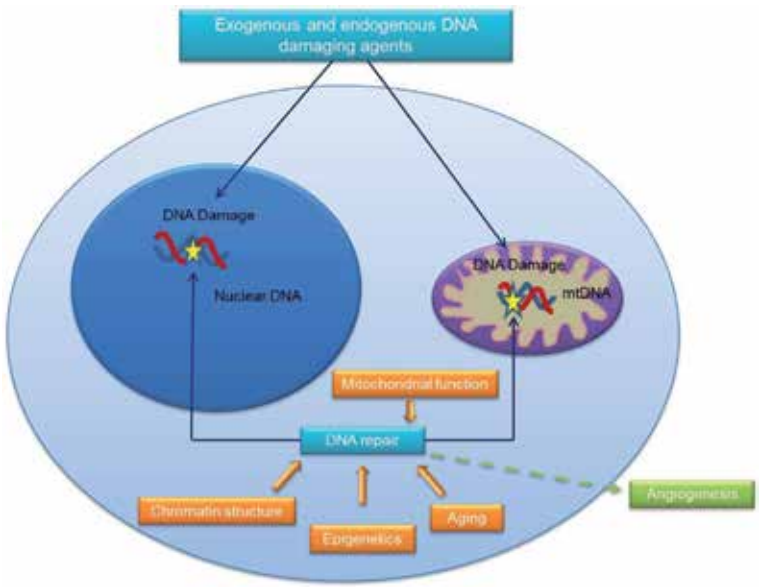

Figure 1.
Example of intrinsic factors affecting the repair of DNA damage induced by exogenous and endogenous sources in nucleus and mitochondria of eukaryotic cells.

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Section 2

DNA Repair Protein Interactions

Chapter 2

Coordination of DNA Base Excision Repair by Protein-Protein Interactions

Nina Moor and Olga Lavrik

Abstract

The system of base excision repair (BER) evolved to correct the most abundant DNA damages in mammalian cells is the most essential for maintaining the genome integrity. The multistep BER process involves several enzymes and protein factors functioning in a coordinated fashion that ensures the repair efficiency. The coordination is facilitated by the formation of protein complexes stabilized via either direct or indirect DNA-mediated interactions. This review focuses on direct interactions of proteins participating in BER with each other and with noncanonical factors found recently to modulate the efficiency of BER. All the known partners of main BER participants, the sites responsible for their interaction, and the characteristics of protein-protein affinity are summarized. Well-documented evidences of how DNA intermediates and posttranslational modifications of proteins modulate protein-protein interactions are presented. The available data allow to suggest that the multiprotein complexes are assembled with the involvement of a scaffold protein XRCC1 and poly(ADP-ribose) polymerase 1, a key regulator of the BER process, irrespective of the DNA damage; the composition and the structure of the complexes are dynamically changed depending on the DNA damage, its chromatin environment, and the step of BER process.

Keywords: base excision repair, protein-protein interactions, noncanonical factors, posttranslational modifications of proteins, coordination of DNA repair

1. Introduction

Many forms of DNA damage are generated due to permanent action of endogenous and exogenous factors. In order to maintain genome integrity, cells have evolved several specific pathways to repair DNA lesions. Base excision repair (BER), which ensures correction of the most abundant damages—modified nitrogenous bases and apurinic/apyrimidinic (AP) sites—is critically important for survival of human cells [1–3]. Enzyme and protein factors of BER also participate in the repair of DNA single-strand breaks (SSBs) considered as a separate pathway of the BER system [4, 5]. The other repair systems (**Figure 1**) deal with bulky nucleobase lesions (NER), DNA double-strand breaks (HR; NHEJ), and mismatched bases (MMR). Impaired DNA repair is associated with embryonic lethality, rapid aging, and a variety of severe human hereditary diseases as well as development of cancer [7, 8]. The balance of DNA damage and DNA repair is highly relevant to both

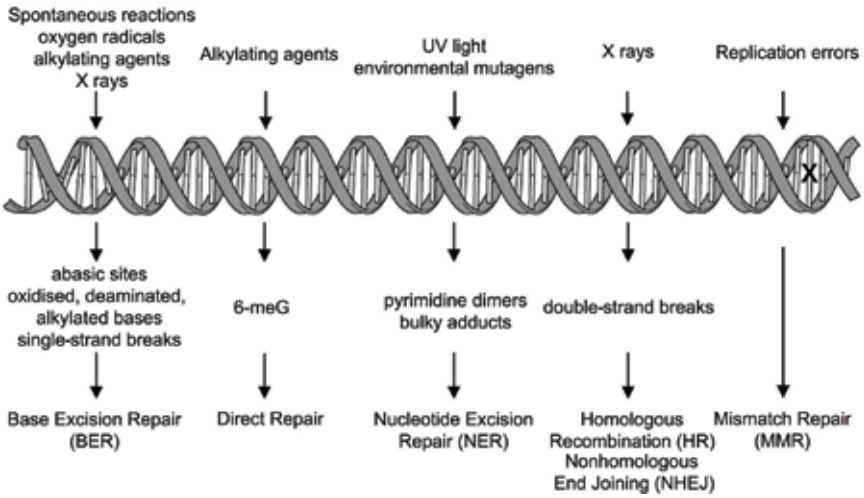

Figure 1.
DNA damages generated by endogenous and exogenous factors and specific systems of their repair. Letter X in DNA duplex marks mismatched base pair. Reproduced with modification from [6] with permission of Pleiades Publishing, Ltd.

cancerogenesis and effective anti-cancer therapy due to the ability of cancer cells to repair therapeutically induced DNA damage and impact therapeutic efficacy [9]. Hence, intensive investigation of DNA damage repair is essential to advance our understanding of molecular mechanisms maintaining genome integrity and to develop cancer therapy.

2. Main steps of BER and proteins involved

The widely accepted model for mammalian BER involves several sub-pathways presented schematically in **Figure 2**. The damaged bases are removed by DNA glycosylases specific to the certain type of damage; mono- and bifunctional DNA glycosylases form an intact or cleaved (via β- or β/δ-elimination mechanism) AP site, respectively [10]. The intact AP site is further processed by the main enzymatic activity of multifunctional AP endonuclease 1 (APE1) producing the one-nucleotide gap with 3′-hydroxyl and 5′-deoxyribose phosphate residue (5′-dRp) at the gap margins. Terminal blocking groups in the DNA intermediates produced by bifunctional DNA glycosylases are removed by the phosphatase activity of polynucleotide kinase/phosphatase (PNKP) or 3′-phosphodiesterase and 3′-phosphatase activities of APE1. At the next step, a bifunctional DNA polymerase β (Polβ) catalyzes the removal of the 5′-dRp residue by its dRp-lyase activity and one-nucleotide gap filling by the nucleotidyl transferase activity. The repair of DNA chain integrity via joining of the single-strand break is completed by DNA ligase IIIα (LigIIIα) acting in the complex with X-ray repair cross-complementing protein 1 (XRCC1). This main BER sub-pathway is known as a short-patch repair (SP BER). When the 5′-dRp residue is modified, it cannot be removed by the Polβ-lyase activity, and a long-patch sub-pathway of BER (LP BER) is realized. Polβ initiates the DNA strand displacement synthesis continued by replicative DNA polymerases δ and ε (Polδ and Polε) acting in the complexes with protein factors PCNA and RFC. The flap structure produced at this step is removed by the flap endonuclease 1 (FEN1). According to another model, FEN1 is capable of sequential removing nucleotides at the 5′-end of the break, and the produced gap is filled by the activities of Polβ or

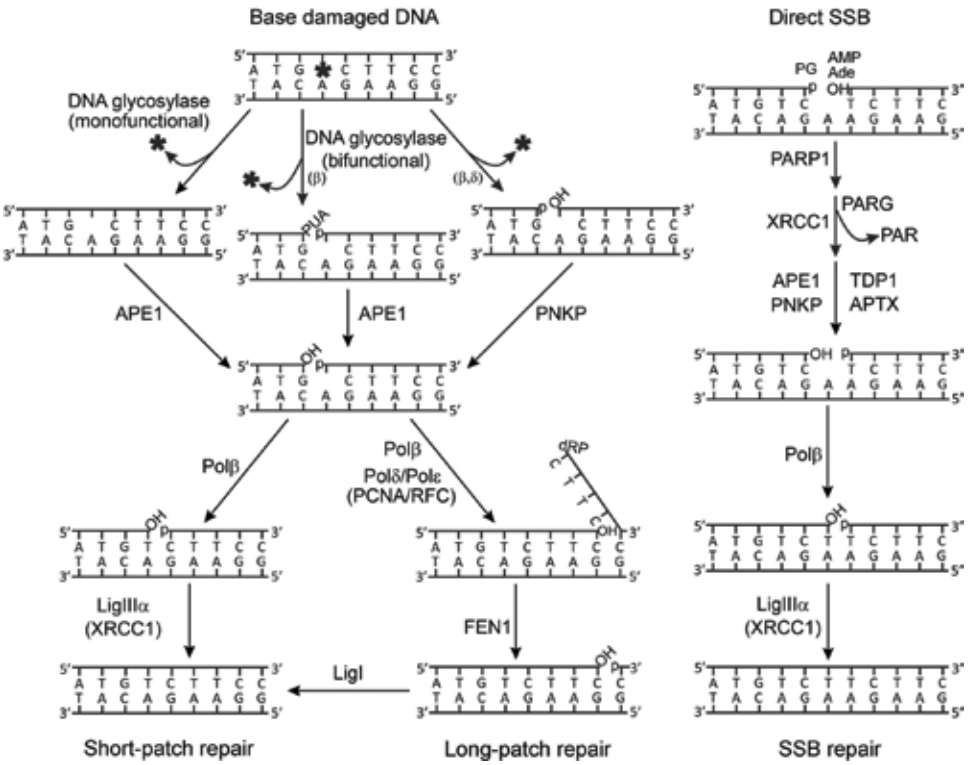

Figure 2.
BER sub-pathways for repair of damaged bases and DNA SSBs. Catalytic steps and proteins involved are schematically presented. The terminal groups in DNA intermediates and SSBs are designated as follows: PUA, 3′-phospho-α,β-unsaturated aldehyde; p, 3′–/5′-phosphate; OH, 3′–/5′-hydroxyl; dRP, 5′-deoxyribose phosphate; PG, 3′-phosphoglycolate; Ade, 5′-aldehyde group; and AMP, 5′-AMP. Reproduced with modification from [6] with permission of Pleiades Publishing, Ltd.

Polλ [11, 12]. Final ligation of the break is catalyzed by DNA ligase I (LigI). A new long-patch sub-pathway of BER that involves formation of a 9-nucleotide gap 5′ to the lesion has been recently discovered; it is mediated by DNA helicase RECQ1 and ERCC1-XPF endonuclease in cooperation with PARP1 and replication protein A (RPA) [13].

Repair of DNA SSBs arising directly via disintegration of the oxidized sugar and as a result of erroneous activity of DNA topoisomerase 1 involves the following steps: (1) detection of the break, (2) removal of blocking groups, (3) filling the gap, and (4) ligation of the break (**Figure 2**). The DNA breaks are detected primarily by poly(ADP-ribose) polymerase 1 (PARP1); the unblocking of 3′- and 5′-ends in breaks is catalyzed by specific activities of APE1, PNKP, aprataxin (APTX), and tyrosyl-DNA phosphodiesterase 1 (TDP1); gap filling and ligation are catalyzed by the same set of enzymes that participate in the respective steps of the short-patch repair of the damaged DNA bases (Polβ and LigIIIα). PARP1 is activated via the interaction with the damaged DNA; it catalyzes the synthesis of poly(ADP-ribose) (PAR) and covalent attachment of the PAR polymer to PARP1 itself and other proteins involved in the DNA repair [4, 5]. The XRCC1 protein is considered to be a main target of PARP1 catalyzed poly(ADP-ribosyl)ation. PARP1 has been suggested to play the main role in recruitment of the XRCC1 protein to the damages of chromosomal DNA [4, 5]. XRCC1 displays no enzymatic activity and is proposed to function as a scaffold protein of the BER process. PARP2 is another enzyme from the PARP family that is activated via binding with DNA SSB and catalyzes PAR

synthesis [14, 15]. The importance of both PARP1 and PARP2 for DNA repair is indicated by knockout studies revealed that knocking out the *parp1* gene activity increased the sensitivity of cells to DNA-damaging agents, while *parp1* and *parp2* double knockouts caused early embryonic lethality [16]. The role of PARP2 in BER processes and its possible synergism with PARP1 action are under intensive investigation [17, 18]. Poly(ADP-ribosyl)ation of proteins is a transient modification that turns over rapidly due to the enzymatic activity of poly(ADP-ribose) glycohydrolase (PARG) [19]. Another important function of PARP1 in DNA repair is remodeling of chromatin structure via poly(ADP-ribosyl)ation of histones and binding of the remodeling proteins with the synthesized PAR polymer [20].

Coordinated action of the enzymes catalyzing the sequential individual reactions of the multistep BER process is required for efficient repair of damaged DNA. One of the coordination mechanisms proposed previously is the "passing the baton," that implies the transfer of the DNA intermediate from the enzyme remaining bound to the product to the next enzyme [1, 21]. This model is supported by numerous data on mutual modulation of activities of the BER enzymes [2, 21]. The stimulating effect of APE1 on the catalytic activity of DNA glycosylase OGG1 explored in detail recently does not require direct interaction between the proteins and is adequately described by the "passing the baton" model [22]. Another mechanism of coordination implies the formation of multiprotein complexes (so-called repairosomes) composed of enzymes and scaffold proteins [2]. XRCC1 is a striking example of the scaffold protein involved in BER. The existence of "repairosomes" is evidenced by multiple interactions between enzymes and protein factors of BER detected even independent of the DNA damage. Most likely both mechanisms are relevant to coordination of the BER process.

3. Proteins involved in BER interact directly with each other

Many protein participants of BER have been shown to interact physically with each other. Data on their direct interactions and structural domains involved are summarized in **Table 1**. Interactions of the XRCC1 protein with multiple partners have been explored in the greatest detail. The structure of XRCC1 is composed of three domains linked with disordered fragments (linkers XL1 and XL2), one of which (XL1) contains a nuclear localization signal (**Figure 3**) [23]. The availability of two BRCT domains (BRCTa and BRCTb) mediating protein-protein interactions (for review, see [24]), in addition to the N-terminal domain (NTD) involved in DNA binding, favors the main function of XRCC1 as scaffold in structural organization of "repairosomes". Interestingly, the binding sites of four enzymes catalyzing sequential steps of BER—APE1, PNKP (N-terminal domain), Polβ, and LigIIIα—are localized in different structural modules of XRCC1 (**Figure 3**). A second PNKP interaction site localized recently in XRCC1 (linker XL1) binds PNKP (catalytic domain) with lower affinity; this interaction has been proposed to stimulate PNKP activity, in contrast to the high-affinity interaction responsible for PNKP recruitment to DNA damage [25]. At the same time, the binding sites of various DNA glycosylases in XRCC1 overlap with those for APE1, Polβ, and PARP1 (**Figure 3**). It is likely that the enzymes initiating the repair of damaged bases form dynamic contacts with XRCC1 and other constituents of "repairosome." Direct interactions of DNA glycosylases NEIL1, NEIL2, and MYH with other enzymes of SP and LP BER (APE1, PNKP, Polβ, LigIIIα, Polδ, FEN1, and LigI) have been shown (**Table 1**). The multiprotein complexes of XRCC1 detected in many studies to be formed by recombinant proteins and cell extracts contain Polβ, PNKP, and LigIIIα as stable partners, and their presence enhances the interaction of XRCC1 with

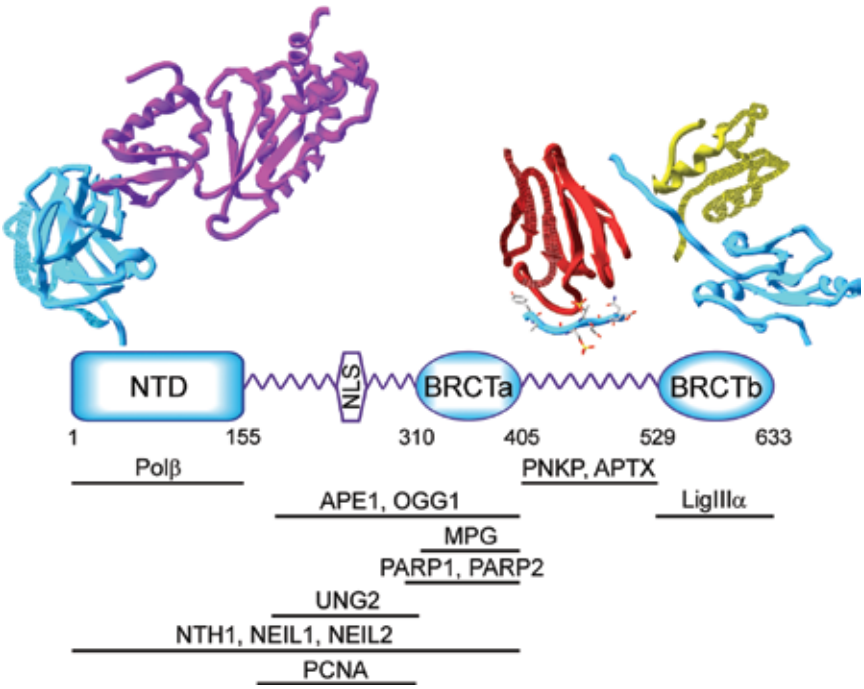

Figure 3.
The multidomain structure of XRCC1 and specific regions responsible for its scaffold function in BER. Protein partners and their binding sites in XRCC1 are shown schematically in the upper part of the figure. At the top, 3D structure models determined for the N-terminal domain, a fragment of XL2 linker, and the BRCTb domain crystallized as complexes with the respective domains of Polβ, PNKP, and LigIIIα (PDB codes: 3K75, 2W3O, and 3QVG) are presented. Reproduced with modification from [6] with permission of Pleiades Publishing, Ltd.

DNA glycosylases [30, 31, 33, 52]. PNKP and LigIIIα are the constituents of another multiprotein complex containing XRCC1 and TDP1 [53].

The PARP1 protein consists of multiple structural modules constituting an N-terminal DNA-binding domain and a C-terminal catalytic domain in addition to the central BRCT domain [55, 57]. The coordinating function of PARP1 in BER can be realized via direct interaction with some enzymes (PNKP, Polβ, LigIIIα, and TDP1) or indirect interaction mediated by the XRCC1 protein. The binding sites for main BER enzymes (Polβ and LigIIIα) and the scaffold XRCC1 protein are localized in the DNA binding and BRCT domains, while that for TDP1 is completed by the catalytic domain of PARP1 (**Table 1**). As a consequence, TDP1 is capable of the formation of a stable ternary complex with PARP1 and XRCC1 [53]. The overlapped binding sites for the majority of PARP1 partners create prerequisites for dynamic contacts in the preformed multiprotein assemblies, which can be stabilized in the complex with auto-modified PARP1 (PAR-PARP1). Many BER participants such as XRCC1, Polβ, PNKP, APTX, TDP1, LigIIIα, and LigI contain PAR-binding motifs, and some of them (XRCC1, LigIIIα, and TDP1) have been shown to interact with PAR-PARP1 more efficiently than with the unmodified PARP1 [46, 47, 58, 59]. The poly(ADP-ribose) acceptors have been identified in all the structural domains of PARP1; this expands significantly the platform for the formation of the "repairosomes" [60]. In contrast to PARP1, PARP2 does not have the BRCT domain and specialized zinc-fingers for DNA binding [15, 61]. The nonconserved WGR domain of PARP2 is responsible for the interaction with proteins (**Table 1**) as well as for DNA break detection [15]. The function of PARP2 (similar to that of PARP1) in coordination of the DNA repair process can be further mediated through its interaction with XRCC1 [17].

Protein (domain)[a]	Protein partner (domain)[a,b]
XRCC1 (NTD)	Polβ (CD) [23, 26–28]
XRCC1 (NTD + XL1)	NTH1 (CTD); NEIL1 (CTD); NEIL2 (NTD) [29–31]
XRCC1 (XL1)	PCNA [32]; UNG2 (CD) [33]; PNKP (CD) [25]
XRCC1 (XL1 + BRCTa)	APE1, OGG1 [34]
XRCC1 (BRCTa)	MPG, NTH1 (CTD), NEIL1 (CTD), NEIL2 (NTD) [29–31]; PARP1 (DBD, BRCT), PARP2 (WGR) [35, 36]
XRCC1 (XL2)	PNKP (NTD), APTX (FHA) [23, 37–39]
XRCC1 (BRCTb)	LigIIIα (BRCT) [40–42]
XRCC1	TDP1 [43]
PARP1 (DBD + BRCT)	Polβ (CD), PARP1, PARP2 (WGR) [36, 44, 45]; LigIIIα (55–122) [46]
PARP1 (CD)	TDP1 (NTD) [47]
APE1 (CTD)	MYH (293–351) [48]
NEIL1 (CTD)	PNKP, Polβ, FEN1, LigI [49]
Polβ (NTD)	NEIL1 (CTD), NEIL2 (NTD) [30, 31]; LigI (NTD) [50]
Polβ (CD)	PARP2 (WGR) [36]
Polβ	APE1 [51]; PNKP [52]
LigIIIα (BRCT)	NEIL1 (CTD), NEIL2 (NTD) [30, 31]; PARP2 (WGR) [36]; PNKP [52]; TDP1 (NTD) [53, 54]

[a]Protein domain(s) responsible for the interaction with protein partner(s) is shown in brackets. Structural composition of multidomain proteins: XRCC1: NTD 1–155, XL1 156–309, BRCTa 310–405, XL2 406–528, BRCTb 529–633; [23] PARP1: ZnF1 1–96, ZnF2 97–206, NLS 207–240, ZnF3 241–366, BRCT 381–484, WGR 518–661, CD 662–1014; [55] PARP2: NTD 1–63, WGR 64–198, CD 199–559; [36] LigIIIα: ZnF 1–100, linker 101–170, DBD 171–390, CD 391–836, BRCT 837–922 [56]. Designations: NTD/CTD, N-/C-terminal domain; CD, catalytic domain; DBD, DNA-binding domain; XL1/XL2, linker 1/2 in XRCC1 protein; NLS, nuclear localization signal; ZnF, zinc finger; FHA, forkhead-associated domain. The data for human and mouse (PARP2) recombinant proteins are presented.

[b]Techniques used in studies: affinity coprecipitation [25, 26, 29–36, 40, 41, 44–50], two-hybrid analysis [27, 30, 31, 35, 37, 46, 51–54], gel filtration [27, 28, 41, 42], ultracentrifugation [27, 50], coimmunoprecipitation [29, 31–33, 36–39, 41, 43, 46, 47, 52–54], fluorescence titration [38], fluorescence polarization [39], surface plasmon resonance [41], small-angle X-ray scattering [42], X-ray crystallography [23, 42, 45], and NMR [48].

Table 1.
Interactions between main proteins involved in BER.

Direct interactions between the enzymes catalyzing different, usually sequential, steps of the BER process have been demonstrated in several studies (**Table 1**). Interestingly, the enzyme of the final step of SP BER—LigIIIα has direct binding partners among the enzymes involved in both the initial and middle steps of the process (NEIL1, NEIL2, PNKP, and TDP1), utilizing the BRCT domain for the interaction. Data reported recently indicate the ability of this enzyme to control the assembly of multiprotein complexes on single-strand DNA damages similar to PARP1, thus suggesting a scaffolding function of LigIIIα in the coordination of BER [62].

Most interactions between proteins involved in BER have been detected using the affinity coprecipitation, two-hybrid analysis, and immunoprecipitation techniques (**Table 1**). These techniques provide no information on physicochemical, structural, and conformational parameters of the complexes, leaving open many questions on the mechanisms of their functioning, such as the relative contribution of the proteins to the formation of macromolecular associates and their stoichiometry, the roles of dynamic interactions, conformational changes, and DNA intermediates in the formation of functional assemblies. Information on the structural

organization of these complexes is very limited. The 3D structures determined by X-ray crystallography are known for the isolated domains/fragments of the XRCC1 protein in complexes with the respective domains of its stable partners Polβ, LigIIIα, and PNKP (**Figure 3**). It is interesting to note that the specific contact region of the XRCC1 protein with LigIIIα (not involved in XRCC1 homodimerization)—a polypeptide consisting of hydrophobic amino acid residues at the N-terminus of the BRCTb domain—was revealed in the X-ray study [42]. The binding sites localized in proteins by the traditional nonequilibrium techniques participate obviously in the most stable interactions. The available structural data are not sufficient to decipher the molecular mechanisms of BER coordination.

Using quantitative equilibrium techniques—fluorescence titration and fluorescence (Förster) resonance energy transfer (FRET)—we have characterized several homo- and hetero-oligomeric complexes of various BER proteins (**Figure 4**). *N*-hydroxysuccinimide esters of 5(6)-carboxyfluorescein (FAM) and 5(6)-carboxytetramethylrhodamine (TMR) were used for N-terminal fluorescent labeling of proteins. Direct (not mediated by DNA or other proteins) interactions of APE1 with Polβ, TDP1, and PARP1 and of Polβ with TDP1 as well as homo-oligomerization of APE1 have been detected for the first time. The apparent equilibrium dissociation constant (K_d) of the complexes is in the range of 23 to 270 nM. The XRCC1-PNKP complex characterized previously by using a similar approach has a K_d value in the same range [64]. The highest stability of the XRCC1 complex with Polβ was confirmed by the nonequilibrium approach, size exclusion chromatography coupled with multi-angle laser light-scattering (SEC-MALLS) [63]. Model DNAs imitating various DNA intermediates of BER have been shown to modulate the structure of protein complexes and their stability to different extents, depending on the type of DNA damage [63]. The DNA-dependent effects on the protein affinity for each other were most pronounced for the complexes of APE1 with different proteins (Polβ, XRCC1, and PARP1). Our findings advance understanding of the mechanisms underlying coordination and regulation of the BER process. The dependence of the efficiency of APE1 interaction with Polβ on the type of DNA intermediate indicates that functions of the two key enzymes are coordinated not only due to the differences in their affinity for DNAs as proposed previously in [65] but also due to the strength of their interaction with each other, which is controlled by DNA at different steps of repair. The higher affinity of APE1 for Polβ in the presence of AP-site containing DNA than in the complex with the incision product suggests that the efficient repair is facilitated by the transfer of the DNA intermediate to Polβ immediately during the incision step. The higher affinity of APE1 and Polβ for PARP1 than for each other in the presence of SSB containing DNA suggests that the regulation of functions of the BER participants via DNA-dependent modulation of their affinity for each other represents a common mechanism for various proteins. On the contrary, the stability of the XRCC1-Polβ complex does not depend on the presence of DNA intermediates, even though the most pronounced effect of different DNAs on the FRET signal, which reflects structural rearrangement of the complex, was detected for this complex. Our data indicate that this complex revealed in [66] to protect each protein from proteasome-mediated degradation may also serve as a stable component of the multiprotein assemblies, similar to the XRCC1-LigIIIα complex. Moreover, the XRCC1 binding sites with Polβ and LigIIIα do not overlap with regions mediating interactions with most other protein partners, thus enabling participation of the preformed ternary Polβ-XRCC1-LigIIIα complex in the entire Polβ- and XRCC1-dependent BER sub-pathway. Formation of the stable ternary complex *in vivo* is evidenced by synchronous accumulation of XRCC1, Polβ, and LigIIIα at the damage sites of DNA [67, 68].

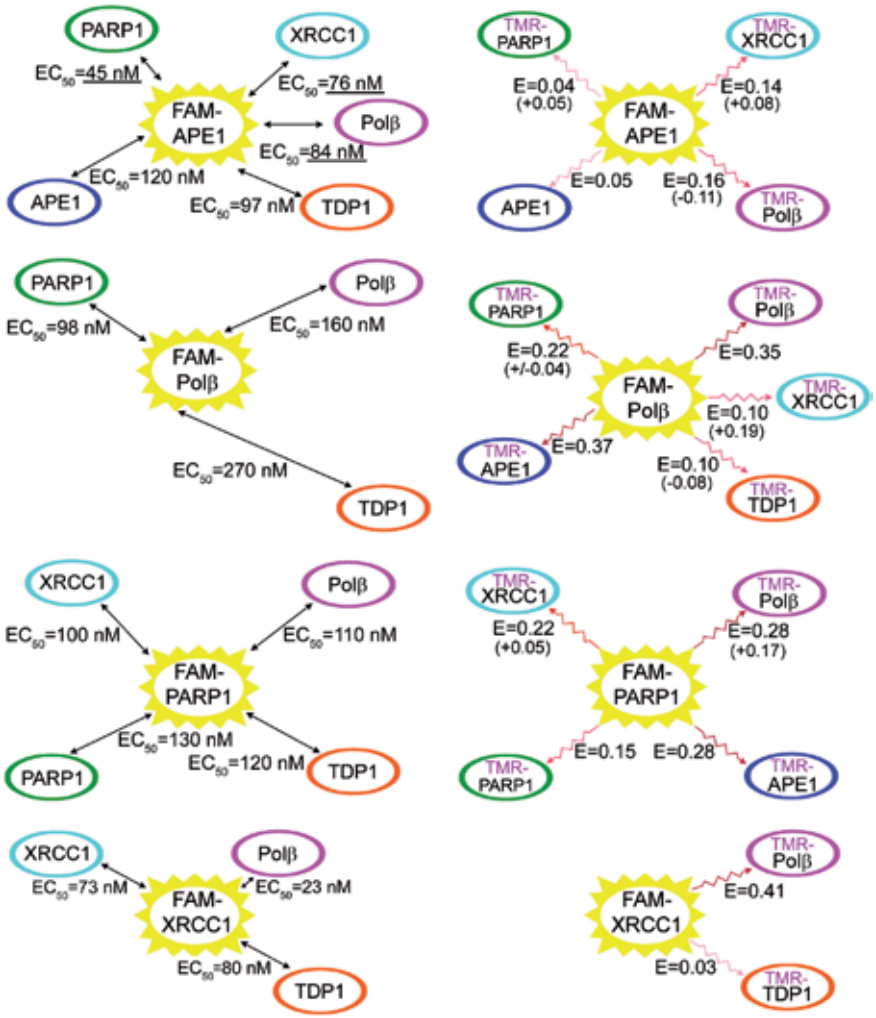

Figure 4.
Direct interactions between BER proteins detected by fluorescence titration and FRET [63]. The EC_{50} values represent apparent equilibrium dissociation constants of the complexes (determined as half-maximal effective concentrations of protein partners); the length of black arrows connecting the protein pairs is proportional to the binding affinity; the underlined EC_{50} values have changed remarkably in the presence of DNA intermediates. The interaction in each pair of FAM- (donor) and TMR-labeled (acceptor) proteins is characterized by FRET efficiency (E); the highest change of the E value induced by DNA intermediates (increase/decrease with +/– sign) is presented in brackets. Reproduced with modification from [6] with permission of Pleiades Publishing, Ltd.

Recently, the oligomeric states of BER proteins and their complexes have been estimated based on hydrodynamic sizes determined by using dynamic light scattering (DLS) technique [69]. All the proteins have been proposed to form homodimers upon their self-association. The most probable oligomerization state of the binary complexes formed by PARP1 with various proteins is a heterotetramer. The oligomerization state of the binary complexes formed by XRCC1 varies from heterodimer to heterotetramer, depending on the partner.

Interaction of PARP1 with Polβ and APE1 detected in our study [63] in both the absence and presence of DNA may contribute to regulation of the BER process. Cooperation between PARP1 and BER enzymes at different steps of DNA repair is evident from our previous studies. Interaction of PARP1, Polβ, and APE1 with the "central" DNA intermediate in BER established by photoaffinity labeling

of BER proteins in the cell extract suggests interplay between these proteins during repair synthesis catalyzed by Polβ [70]. The ability of PARP1 to compete with APE1 for the binding of an AP-site containing DNA indicates possible cooperation between the proteins upon the recognition and further incision of the AP site [71]. Following the incision of AP site, PARP1 can catalyze the synthesis of poly(ADP-ribose). According to the initially proposed mechanism of its action, PARP1 dissociates from the complex with DNA after covalent attachment of the negatively charged PAR polymer. Further studies of an active role of PAR in the formation of the repair complexes have modified this hypothesis. It was established that following poly(ADP-ribosyl)ation, PARP1 was capable of covalent binding to the photoreactive DNA intermediate; the lifetime of such complexes was shown to depend on both the size of covalently bound PAR and the initial affinity of PARP1 for the DNA damage [70]. Complexes of PAR-PARP1 with damaged DNA have been detected by atomic force microscopy [72]. Recently, kinetics of poly(ADP-ribosyl)ation and PAR homeostasis (but not the PARP1 protein) have been proposed to play a primary role in protection of cells from acute DNA damage [73]. Hence, the formation of BER complexes on the damaged DNA can be regulated via either poly(ADP-ribosyl)ation of proteins or their interactions with PAR polymer synthesized by PARP1 and PARP2. Poly(ADP-ribose) is the most important cell regulator of protein-protein and protein-nucleic acid interactions. [20, 74–78].

4. Interactions of BER proteins with noncanonical factors contribute to the regulation of DNA repair

Many proteins with various cellular functions, not considered previously to be involved in BER, have been shown to regulate this process via interactions with main participants. The HMGB1 protein—a chromatin architecture factor—interacts directly with three BER enzymes (APE1, Polβ, and FEN1), modulates their catalytic activity in the process of DNA repair, and, hence, ensures regulation of the process via the SP or LP BER sub-pathway [79–81]. Human DNA-binding proteins hSSB1 and SATB1 form complexes with DNA glycosylase OGG1, thus enhancing its efficiency in recognition of DNA damage and its repair [82, 83]. The human mitochondrial single-stranded DNA binding protein (mtSSB) interacts with NEIL1 in the presence and absence of a DNA substrate revealed to modulate the oligomerization state and stability of the NEIL1-mtSSB complex [84]. Protein factors of unknown nature that are not involved in chromatin structure remodeling form complex with DNA glycosylase NTH1 and stimulate its activity in BER initiation [85]. The SSRP1 protein entailed in chromatin disassembly as a histone H2A/H2B chaperone interacts with both PAR-PARP1 and XRCC1 and facilitates repair of SSBs [86]. In general, the mechanisms of BER functioning within chromatin are largely unexplored (for example, see [87]), remaining possibility to discover new noncanonical factors of BER.

In addition to multiple enzymatic functions in DNA repair, APE1 is known to play a regulatory role in the transcription processes, RNA processing, and ribosome biogenesis [76, 88]. The activities of the multifunctional enzyme, its expression level, and intracellular localization are regulated by its interaction with the multifunctional protein nucleophosmin (NPM1) [89]. Direct interactions of APE1 and several DNA glycosylases (TDG, NEIL2, NTH1, OGG1, and UNG2) with protein factors of nucleotide excision repair (XPC, XPG, CSB, and RPA) and homologous recombination (Rad52) have been shown to play a regulatory role in the overlapping repair pathways [90].

PARP1 forms stable complexes with Ku70/Ku80 proteins, and this interaction has been proposed to be an important regulator of the Ku70/80 heterodimer function in the repair of DNA double-strand breaks (DSBs) [91, 92]. Recent studies have demonstrated the involvement of Ku70 and Ku80 proteins in different steps of BER [93]. Septin4, a member of GTP binding protein family considered to be an essential component of the cytoskeleton, is a novel PARP1 interacting protein, and the interaction is enhanced under oxidative stress [94]. PARP1 interacts with NR1D1 protein, a nuclear receptor subfamily 1 group D member 1; the interaction is enhanced under oxidative stress and inhibits the catalytic activity of PARP1 [95]. Whether the interaction of these noncanonical factors with PARP1 may contribute to regulation of BER remains to be explored. The protein DBC1 (deleted in breast cancer 1), one of the most abundant yet enigmatic proteins in mammals containing a conserved domain similar to Nudix hydrolases (hydrolyzing nucleoside diphosphates) but lacking catalytic activity, interacts directly with the BRCT domain of PARP1; the strength of the interaction shown to inhibit the catalytic activity of PARP1 is modulated by NAD+ concentration [96]. Thus, a novel function of NAD+ to directly regulate protein-protein interactions, the modulation of which may protect against cancer, radiation, and aging, has been discovered.

The multifunctional Y-box-binding protein 1 (YB-1) is another noncanonical factor of BER. The proteolytic fragment of this positively charged intrinsically disordered protein localized in the nucleus is formed in response to DNA damage [97]. We have shown that YB-1 interacts with poly(ADP-ribose) and could be an acceptor for PARP1/PARP2 catalyzed poly(ADP-ribosyl)ation *in vitro* [98]. Several proteins essential for BER—APE1, Polβ, NEIL1, PARP1, and PARP2—directly interact with YB-1, although most complexes being less stable than the complexes of BER proteins with each other (the apparent K_d values are in the range of 340 to 810 nM as compared to those presented in **Figure 4**) [99]. A strong interaction detected between APE1 and YB-1 could be an important factor for the cooperative action of these multifunctional proteins in transcription regulation [100]. Interactions of YB-1 protein with BER enzymes could be responsible for the regulation of their activities: the AP-endonuclease activity of APE1 and 5′-dRp-lyase activity of Polβ are inhibited in the presence of YB-1, while the AP-lyase activity of NEIL1 is stimulated [99]. YB-1 was found to stimulate the catalytic activity of PARP1 via strong binding with poly(ADP-ribose) linked to PARP1, which increased the lifetime of this complex in DNA [99]. Acting as a cofactor of PARP1, YB-1 decreases the efficiency of PARP1 inhibitors [101].

5. Intersection of posttranslational modifications and protein-protein interactions in BER coordination

Posttranslational modifications (PTMs) of proteins involved in BER modulate catalytic and DNA-binding activities of individual proteins, their expression, intracellular localization, structure, and stability as well as protein-protein interactions and may therefore contribute to regulation of DNA repair either directly or indirectly. Numerous studies of PTMs and their functions in BER have been reviewed previously [90, 102–106]. As mentioned above, PARP1 modifies itself and binding partners with poly(ADP-ribose). Among the targets of PARP1 catalyzed ADP-ribosylation are two key BER proteins—XRCC1 and Polβ, and XRCC1 negatively regulates PARP1 activity [35, 107]. The automodification of PARP1 has been shown to enhance its interaction with XRCC1, LigIIIα, and TDP1; the length of

PAR polymer determines the efficiency of PAR-mediated accumulation of XRCC1 on DNA damage [46, 47, 108]. Recent studies have identified other PTMs, such as phosphorylation, acetylation, and methylation, to regulate the activity of PARP1 [104, 106]. Phosphorylation of PARP1 mediated by protein kinase CDK2 represents a novel DNA-independent mechanism of PARP1 activation [106]. Modifications of PARP1 mediated by AMP-activated protein kinase (AMPK) and lysine acetyltransferase 2B (PCAF) modulate both the activity of PARP1 and ADP-ribosylation of other proteins [106]. Polyubiquitination of PARP1 by E3 ubiquitin protein ligase is promoted by the automodification of PARP1 and targets PAR-PARP1 for proteasomal degradation [106]. It has to be noted that nonproteolytic roles of protein ubiquitination in regulation of DSBs repair and NER have been demonstrated [105]. PARP1 modification (at Lys486 residue) with small ubiquitin-like protein catalyzed by SUMO E3 ligase (SUMOylation) enhances p300-dependent acetylation of PARP1, while it has no effect on its activity [106].

The most abundant evidence on PTM-mediated regulation of protein-protein interactions is available for the XRCC1 protein. XRCC1 is an extensively phosphorylated protein with more than 45 phosphorylation sites localized in the linker regions and BRCTa domain [109]. Catalyzed by p38 MAPK kinase phosphorylation of the BRCTa domain (at T358 and T367 residues) has been shown to regulate PAR-mediated recruitment of XRCC1 to DNA damage [109]. The phosphorylation of XRCC1 by checkpoint kinase 2 (CHK2) at Thr284 residue *in vivo* and *in vitro* increases the affinity of XRCC1 for DNA glycosylase MPG, facilitating thereby initiation of BER [110]. As shown recently, the same kinase interacts with PARP1 and modifies the BRCT domain; the CHK2-dependent phosphorylation of PARP1 stimulates its catalytic activity and interaction with XRCC1 [111]. Seven sites of XRCC1 phosphorylation mediated by kinase CK2 (localized in the XL2 linker) are necessary to modulate the interaction of XRCC1 with end-processing enzymes—PNKP, APTX, and PNK-like factor APLF—and the efficiency of repair of chromosomal DNA SSBs [37–39, 112, 113]. Notably, the phosphorylated and unmodified forms of XRCC1 bind different structural domains of PNKP and modulate the kinase activity of PNKP or its accumulation on DNA damage, respectively [37, 38]. The oxidized form of XRCC1 stabilized by a disulfide bridge between Cys12 and Cys20 residues forms a more stable (in comparison with the reduced form) complex with Polβ; an increase in the number of intermolecular contacts in this complex has been confirmed by X-ray analysis of the complex [23]. The existence of oxidized form of XRCC1 *in vivo* is essential to protect cells against extreme oxidative stress [114]. XRCC1 is a substrate for SUMOylation promoted by DNA damage-induced PARylation; SUMOylation of XRCC1 contributes to regulation of BER via increasing its binding affinity for Polβ [115].

The most frequent PTMs discovered for the multifunctional protein APE1 include phosphorylation, acetylation, S-nitrosylation, S-glutathionylation, formation of disulfide bonds, and ubiquitination [90, 102]. Most modifications modulate redox activity of APE1 and its regulatory function in transcription. As Cys residues are targets of different modifications, it is essential to understand the competition between these PTMs and their roles in APE1 function. Numerous studies on APE1 phosphorylation by a variety of protein kinases provide contradictory data on modulation of the repair activity of APE1 [102]. Recently, it has been shown that acetylation of APE1 (at Lys residues in the mammalian-conserved N-terminal extension) enhances both the AP-endonuclease activity and the interaction with XRCC1 and XRCC1-LigIIIα complex, ensuring cell survival in response to genotoxic stress [116].

Acetylation of DNA glycosylase TDG weakens its interaction with APE1 and produces opposite effects on the excision activity of the enzyme toward various

types of base damages; repair of damage induced by the chemotherapeutic action of 5-fluorouracil is enhanced by the TDG acetylation [90]. Based on these data, the acetylation status of TDG within tumor cells was proposed to impact the chemotherapy efficacy. Phosphorylation of the flexible N-terminus of DNA glycosylase UNG2 (at Thr6 or Tyr8 residues) shown to disrupt interaction with the PCNA factor, without affecting the UNG2 catalytic activity or its RPA interaction, has been proposed to regulate the formation of the ternary PCNA-UNG2-RPA protein complex [117].

Various PTMs of Polβ (acetylation, phosphorylation, and methylation) modulate its 5′-dRp-lyase and nucleotidyl transferase activities; the only example of PTMs impacts on protein-protein interaction is inhibition of Polβ-PCNA interaction due to PRMT1-dependent methylation of Arg137 [102]. The enzymes completing BER—LigIIIα and LigI—undergo posttranslational modification *in vitro* and in *vivo*; however, the intersection of their PTMs with protein-protein interactions is yet unknown [102].

6. Conclusions

Intensive studies of DNA repair system ensuring repair of damaged bases and single-strand DNA breaks (BER) in recent decades have made impressive progress in establishing the participants of the repair process, main sub-pathways, and auxiliary mechanisms activated when the main BER sub-pathways are inefficient. In addition to the enzymes responsible for catalytic steps of BER, several proteins, such as XRCC1, PARP1, PARP2, and others, have been identified as BER participants essential for assembling and functioning of the dynamic multiprotein system. Multiprotein complexes of various compositions can be formed without the involvement of DNA, but their structure and stability are modulated by the damaged DNA and intermediates formed in different steps of BER. Interactions of individual BER enzymes with DNA substrates and products have been deciphered in detail by X-ray studies. This method is of little use to explore dynamic supramolecular structures operating in DNA repair. The next step is required to clarify how the BER system functions upon association of the multiprotein complexes with chromatin; novel methods in structural analysis, such as electron microscopy, and more complex models imitating DNA repair in chromatin structure might be helpful to apply. How protein-protein interactions and posttranslational modifications coordinate BER with other DNA repair systems requires future studies. Elucidation of molecular mechanisms underlying efficient BER and its dysregulation in pathological states will help broaden our understanding the origins of diseases and provide novel strategy of their treatment.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Chapter 3

Mitochondrial Genome Maintenance: Damage and Repair Pathways

Ulises Omar García-Lepe and Rosa Ma Bermúdez-Cruz

Abstract

The mitochondrial genomic material (mtDNA), similarly to nuclear genome, is exposed to a plethora of exogenous and endogenous agents, as well as natural processes like replication that compromise the integrity and fidelity of the mtDNA, despite the abovementioned, the mtDNA does not contain genes involved in DNA repair, therefore mitochondria completely depend on the importation of nuclear-encoded elements to achieve genome maintenance, which implies a coordinated crosstalk between these two organelles. It has been determined that to counteract damage, mitochondria possess well-defined repair pathways quite similar to those of the nucleus, among which are: base excision repair (BER), mismatch repair (MMR), single-strand break repair (SSBR), microhomology-mediated end joining (MMEJ), and probably homology recombination dependent repair (HRR). If these repair pathways are nonfunctional and the lesions remain unrepaired, the emergence of mutations, deletions, and other insults may result in compromised cellular viability and disease.

Keywords: mitochondria, mtDNA, damage, repair, BER, MMR, SSBR, HRR, MMEJ

1. Introduction

The mitochondrion is an essential organelle involved principally in the production of ATP and other metabolites which are important to several cellular functions, besides this organelle participates in other processes as iron-sulfur cluster biogenesis, heme production, and calcium regulation [1]. The mitochondrion possesses its own circular genomic material (mtDNA), which is exposed to the same DNA lesions as nuclear genome is, however, unlike the latter, mtDNA does not encode for genes involved in DNA maintenance or repair which implies that these processes completely depend on nuclear-encoded elements translocated to mitochondria. It was first thought that mitochondria lacked the ability to repair its DNA material, and this assumption was originated due to the observation of the absence of pyrimidine dimer resolution after ultra violet light exposition in mammalian cells [2]; however, nowadays, the study of mtDNA repair pathways has evolved into a complete research area that is constantly growing, since it has been observed that mitochondria not only possess some of the nuclear-conserved mechanisms like: base excision repair (BER), mismatch repair (MMR), single-strand break repair (SSBR), microhomology-mediated end joining (MMEJ), and homologous recombination

dependent repair (HRR), additionally mitochondria have evolved specific unique methods to deal with mtDNA insults based on the redundancy nature of mtDNA and mitochondrion itself, if the damage surpasses its repair capabilities, the mtDNA molecules can be destroyed and replicated again or even the whole organelle can be degraded [3]. Of importance, lesions that remain unrepaired in mtDNA such as deletions, mutations, inversions, and other rearrangements have been linked to several heritable disease syndromes [4]; further, mtDNA rearrangements and deletions have been associated with aging and cancer (www.mitomap.org/org/MITOMAP) [5]. In this chapter, we will summarize the different mechanisms by which the mammalian mtDNA can be damaged and the described pathways that are involved in maintenance of fidelity and integrity of mitochondrial genome.

2. The mitochondrial genome

One of the features of mammalian cells is that they have two DNA-containing compartments: nuclei and mitochondria. Nuclear genome is large, diploid, and linear; in contrast, mitochondrial genome is polyploid and quite small, since is formed by a 16,569 pb circular molecule that accounts for 0.0005% of the human genome and 0.1% of the total number of genes in the human; mtDNA is redundant, since a few hundred to few thousand copies can be found per cell [3], when all the copies are identical, the genotype is termed homoplasmy, instead when multiple forms exist within the same tissue or cell; the genotype is called heteroplasmy [6]. The mitochondrial genome presents 37 genes, 13 of which encode for proteins oxidative phosphorylation chain specific and the remaining are implicated in translation: 2 ribosomal RNAs (small of 12S and large of 16S) and 22 tRNAs. The grade of compaction of mtDNA is interesting since it has no introns, and the intergenic regions are almost absent, additionally there are two noncoding regions: one of approximately 1 kb known as noncoding region (NCR) and another small of 30 bp, both implicated in regulation of replication and transcription [7]. The NCR presents a triple stranded region, named D-loop, which occupies most of its extension and is related to the start of transcription [8], besides it has been observed that some genes overlap and others lack termination codons; therefore, it has been established that the promoters produce polycistronic transcripts which are further processed to generate mature RNA molecules [9]. As mentioned above, some of the proteins involved in respiratory system and ATP synthesis, which are extremely important to cellular functions, are encoded by mtDNA and not the nuclear genome, thus it is important to maintain mitochondrial genome integrity to preserve homeostasis [10].

Despite the advances made in the study of mtDNA replication mechanism, the exact machinery and steps involved in this procedure are not fully known; however, it has been determined a general head core to this process which consists of the polymerase gamma (Polγ), a DNA helicase named Twinkle, and the mitochondrial single-stranded binding protein (mtSSB) [11]. Nowadays, there are three proposed models to explain mtDNA replication: (1) the first is quite similar to nucleus DNA replication, with standard leading and lagging strand replication, (2) a strand displacement model, where the lagging strand is synthetized once the leading has advanced and synthetized a long fragment, and (3) in this model, the lagging strand is hybridized with complementary RNA, a mechanism termed RNA incorporation throughout the lagging strand (RITOL) [8]. Another interesting feature about mtDNA replication is that contrary to what occurs in nuclear genome, mitochondrial genome replication is not limited to S phase of the cell cycle [12].

Unlike the nucleus, where the DNA forms part of nucleoprotein complexes, consisting of DNA molecules wrapped around histone structures, the mitochondrial

genome does not present histones. It has been thought that this lack is responsible of the high rate of mtDNA mutagenesis, which is 10-fold greater than that in nucleus; however, this hypothesis is controversial since experimental evidence has suggested that histones might provoke DNA damage instead of preventing it [3]. Despite the above, mitochondrial genome is not naked; it is packaged into protein-DNA complexes, which are termed mitochondrial nucleoids due to its similarity to bacterial chromosomes [13]. The most abundant nucleoid-associated proteins are mtSSB, transcription factor A of mitochondria (TFAM), Polγ, mitochondrial RNA polymerase (POLRMT), and Twinkle DNA helicase [14].

3. Sources of mtDNA damage

Mitochondrial genome is exposed to almost the same insults that nuclear genome is, which can be originated by internal and external sources. Six types of DNA damage have been proposed to be the more relevant in mitochondria [3].

3.1 Alkylation damage

This kind of lesion may be due to exposition to exogenous agents as chemotherapeutic drugs, diet, and tobacco smoke; however, DNA alkylation damage can also be generated from the interaction of DNA with endogenous molecules [15], such as betaine, choline, and S-adenosylmethionine (SAM); the latter is the most relevant alkylating agent in the cell; SAM is a co-substrate involved in the transfer of methyl groups, when incubated with DNA in aqueous solutions leads to base modification, forming small amounts of 7-methylguanine and 3-methyladenine nonenzymatically, therefore SAM acts as a weak DNA-alkylating agent [16]. Of interest, these DNA modifications, in specific 7-methylguanine can trigger the formation of mutagenic apurinic sites (AP) and imidazole ring opening which results in the stoppage of replication machinery [17]; moreover, 3-methylguanine itself is a cytotoxic DNA lesion that also blocks replication [15]. Interestingly, mitochondria store about 30% of total hepatic SAM [18], thus mtDNA is constantly exposed to this alkylating agent, which threats its stability and integrity.

3.2 Hydrolytic damage

There are two types of hydrolytic damage, the first is the formation of AP sites as a product of hydrolysis of the glycosidic bonds between bases and deoxyribose, and these lesions could appear due to heating, alkylation damage (previously mentioned) or by the action of N-glycosylases [19]. It has been estimated that AP is one of the most frequent lesions in the DNA, with approximately 10,000 lesions per cell, per day [20]. Interestingly, typical AP sites generate base pair modifications, since there is a preference to incorporate adenine opposite to AP by polymerases during DNA replication [21]. The other form of hydrolytic damage is the hydrolytic deamination of bases, where cytosine and its homolog 5-methylcitosine are mainly affected. It is noteworthy that the conversion of cytosine to uracil may introduce punctual mutations to the genome during replication if left unrepaired [20].

3.3 Formation of adducts

This type of lesions can be generated for exposition to ultraviolet type B and C light which produce bulky DNA adducts termed photodimers, in addition, activated metabolites of several organic contaminants, for example, polycyclic aromatic

hydrocarbons and mycotoxins may bring about adducts [1]. On the other hand, adduct formation can also be stimulated by endogenous factors, for example, it has been demonstrated that reactive intermediate products of diethylstilbestrol metabolization form DNA adducts preferentially with mitochondrial genome, where these insults are suggested to avoid replication and/or transcription, thus producing mtDNA instability in vivo [22].

3.4 Mismatches

During replication, polymerases can introduce base to base mismatches as well as generate nucleotide insertions or deletions in mitochondria, which are normally known as insertion-deletion loops (IDLs). One important source of mismatches are damaged deoxyribonucleotide triphosphates (dNTPs), predominantly oxidized, which can be incorporated to DNA during synthesis [3, 10].

3.5 DNA strand breaks

These injuries are divided based on the breaking of one or both strands. Single strand breaks (SSBs) can be generated by normal cellular procedures that went wrong, such as erroneous or abortive activity of DNA topoisomerase I (Top1), which presents mitochondrial localization, and when it fails may produce protein-linked DNA breaks [23], also SSBs are produced by ineffective base excision repair (BER), or by oxidative stress [24]. One lesion related to SSBs is the formation of a covalently linked AMP to a 5′ phosphate, product of an unsuccessful DNA ligase activity [25]. On the other hand, double strand breaks (DSBs) are the most harmful, since they can provoke global cellular responses that involve many aspects of cell metabolism [26]. These lesions may occur by endogenous agents like reactive oxygen species (ROS), errors in DNA metabolism by topoisomerases, and nucleases or detention of replisome. On the other hand, lesion can be caused by exogenous insults such as ionizing radiation and chemotherapeutic drugs [1].

3.6 Oxidative damage

In living organisms, ROS are normally produced as a consequence of endogenous metabolic reactions and also by external factors. ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and single oxygen (O_2), all of them can oxidize DNA molecules and generate several types of damage including oxidized bases and single- and double-strand breaks [15]. Oxidative damage is by far the most prevalent and studied in mtDNA, since mitochondria are an important contributor in the creation of ROS [27], generated by the leakage of electrons from the electron transportation chain (ETC) [28], and there are at least nine sites responsible for generating the superoxide anion [29]. The importance of ROS affecting mtDNA lies in the observation that oxidative damage accumulates in several human diseases [30]. Of interest, it has also been reported that reactive nitrogen species (RNS) are able to oxidize or deaminate DNA and produce strand breaks, lesions that could be possible in mtDNA since these RNS can be found in mitochondria [31, 32].

4. Mitochondrial DNA repair machinery

Most of the repair pathways used by mitochondria to deal with its damaged DNA are quite similar to those operating in the nucleus; this observation makes

sense when we realize that mitochondrion relies completely in import of DNA repair elements encoded by nuclear genome, despite that mitochondrial genome does not contain any gene implicated in repair; it appears that the mitochondrial version of the repair machinery operates with fewer proteins than the nuclear counterparts [33] (**Figure 1**).

4.1 Base excision repair (BER)

Base excision repair (BER) is the commissioned pathway to removed nonbulky lesions like alkylated, deaminated, and oxidized bases from the DNA. This pathway has been well studied in the nucleus and was the first repair mechanism reported in mitochondria and to date the best characterized in this last organelle [33, 34]. BER mechanism is highly conserved from bacteria to humans and basically comprises five stages: (1) recognition and excision of the damaged base, (2) removal of the abasic site formed, (3) DNA end processing, (4) repair synthesis, and (5) ligation [35], and at the same time, BER can be divided into two branches (both founded in mitochondria): short patch and long patch, where the difference lies in the procedure used to repair, while short patch forms a single nucleotide gap, long patch forms a bigger one of 2–10 nucleotides [36]. One of the main elements involved in BER processes are the DNA glycosylases, enzymes that catalyze the excision of the N-glycosidic bond between the altered base and its corresponding carbohydrate, thus creating an abasic site, and activity that was first observed by Lindahl et al. [37]. These glycosylases can be subdivided into monofunctional or bifunctional, depending on whether they have lyase activity or not, ability that determines the type of damage they can repair, while monofunctional glycosylases focus on nonoxidative damage, bifunctional exert their action against oxidized DNA bases [35]. In mammalian mitochondria, seven glycosylases have been reported, three monofunctional: uracil-DNA glycosylase (UNG1), *E. coli* MutY homolog (MYH), and N-methylpurine DNA glycosylase (AAG/MPG) and four bifunctional: 8-oxoguanina-DNA glycosylase (OGG1), *E. coli* endonuclease III homolog (NTH1) endonuclease VIII-like glycosylase 1 and 2 (NEIL1 and 2) [38–41]. After the action of glycosylases, the next step, DNA end processing, is catalyzed by nuclear BER enzymes that are also found in mitochondria, as apurinic-apyrimidinic endonuclease 1 (APE1) and polynucleotide kinase-3′-phosphatase (PNKP) [35], further the

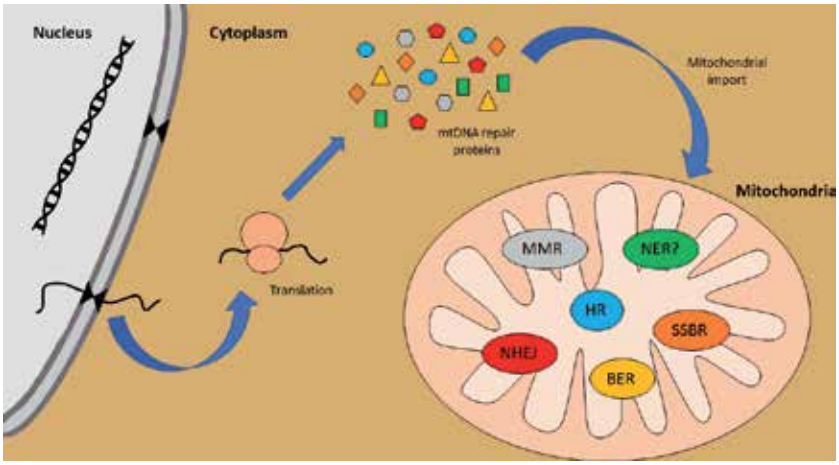

Figure 1.
Mitochondrial import and mtDNA repair pathways. The elements that participate in mitochondrial genome maintenance are nuclear encoded; therefore, mitochondria need to import these effector proteins, which are involved in different repair pathways.

synthesis is carried out by the main mitochondrial polymerase (Polγ). It was recently reported that polymerase beta (Polβ) also localizes within mitochondria, whereas similar to the nucleus, it has a relevant role in mtDNA maintenance and mitochondrial homeostasis through its participation in BER [42]. It has also been observed that FEN1, DNA2, and EXOG are involved in the removal of DNA flap generated by polymerase strand displacement synthesis [43]. Finally, the ligation activity is made by mitochondrial ligase III [44]. In conclusion, despite that BER is the most characterized pathway in mitochondria, it is still unknown whether all the nuclear elements are conserved in mitochondrial BER, or on the contrary, if this pathway have specific proteins that do not participate in nuclear version of BER, it is not well understood either which are the regulatory signals that controls the import of these elements to mitochondria.

4.2 Nucleotide excision repair (NER)

Nucleotide excision repair (NER) pathway is a complex machinery involved in recognition of lesions, adducts or structures that modifies the DNA double helix structure, having the possibility of blocking transcription, replication, and thus affecting DNA stability. One of the most important features of NER is its versatility on a wide kind of lesions since they are detected and repaired. Roughly, the process starts with damage recognition, next, the damaged strand is cleaved at both sides of the lesion to be removed later, then the missing sequence is synthesized using the complementary strand as a template, and finally the ends are ligated, thus restoring DNA sequence and integrity [45].

There is a large body of evidence reporting the absence of nucleotide excision repair activity in mitochondria. The first observation about this lack was made by Clayton et al. in 1974 [2], who reported that mitochondria were unable to remove UV-induced pyrimidine dimers. As a result, lesions that are normally repaired in nucleus by NER could persist in mitochondrial genome; this damage is not only restricted to photodimers, since NER also participates in repair of other bulky lesions and a subset of oxidative DNA damage [1]. In the nucleus, bulky lesions can also be overcome through the use of specialized translesion synthesis (TLS) polymerases; however, in mitochondria, the major polymerase (Polγ) presents a weakly thymine dimer bypass activity in vitro and at present, it is unknown whether this activity is conserved in vivo [46]. On the other hand, it was recently reported that polymerase theta (Polθ), an enzyme that acts as a translesion bypass polymerase, thus promoting the pass of replicative stalling lesions in the nucleus [47], is localized to mitochondria. Additionally, it was observed that there is an increase of Polθ localization to mitochondria after treating the cells with an oxidative agent, suggesting that the enzyme is recruited to the organelle when this kind of damage is inflicted, where it could facilitate translesion bypass synthesis. Interestingly, in POLQ KO cells, the rate of point mutation in mtDNA was significantly reduced after oxidative treatment; this observation indicates that Polθ is involved in a pathway of error-prone DNA synthesis that may facilitate replication in mitochondria [48].

Despite that the nucleotide excision repair pathway has not been clearly recognized in mitochondria, there are several proteins involved in the nuclear version of this pathway that not only localize to the organelle but also they accumulate upon oxidative damage, and when they are depleted from cells, the number of point mutation is increased, observations that strongly suggest their participation in mtDNA maintenance. Some of these proteins are: Cockayne syndrome A (CSA), Cockayne syndrome B (CSB), and Rad23A [48–50]. Therefore, further investigation is needed to completely rule out NER pathway from mitochondria, or to elucidate whether these mentioned proteins are involved in other pathways different to nucleus.

4.3 Mismatch repair (MMR)

MMR is a highly conserved pathway involved in the correction of misincorporation and slippage mistakes committed by polymerases during DNA replication, and base mismatches generated by base deamination, alkylation, and oxidation [3]. In general terms, MMR process presents the next steps: localization of the mismatch and identification of the newly synthesized strand, excision at both extremes of the mismatch lesion, DNA resynthesis, and finally ligation to complete the process [10].

The first demonstration of mammalian mitochondria capable to repair mismatch lesions was done by Mason et al. [51]. They observed that rat liver mitochondrial lysates repaired G-T and G-G mismatches; however, by using immunodetection no MSH2, a key nuclear element in MMR, was detected in these lysates, suggesting that mitochondrial MMR activity uses different elements to those of the nucleus. Posteriorly, in the pursuit of proteins responsible for the activity observed, one study reported that mismatches and small IDLs in mitochondrial genome are recognized by the Y-box binding protein 1 (YB1), which also localizes to nucleus where it exerts other functions, and its depletion in cultured cells triggers an increased mutagenesis in mtDNA. In addition, it was demonstrated that MMR activity is independent of MSH2 and that MSH3, MSH6 or MLH1 are not present in human mitochondria, at least under the experimental conditions employed [52]. In contrast to the previous observation, it was later demonstrated that MLH1 do localizes to mitochondria [53]. Through overexpression of *Mlh1* or *Msh2* in retinal endothelial cells, it was determined that MLH1 has a protective role in mtDNA after glucose-induced DNA damage, and on the other hand, this protective effect was not detected when *Msh2* was overexpressed, observation that suggests no participation in mtDNA maintenance [54], in accordance with previous studies. Additionally, it was reported that the incidence of base-mismatches in mtDNA in diabetic retina is a consequence of expression silencing of MLH1 by methylation of its promoter, activity performed by Dnmt1, enzyme overexpressed in diabetes. Thus, these observations propose that MLH1 has an important role in mtDNA maintenance, since its silencing by methylation triggers mtDNA damage [55]. In summary, MMR pathway is involved in mitochondrial genome maintenance; however, not all the elements implicated have been found. It could be possible that the proteins of mitochondrial MMR may have a different splicing or post translational versions than nuclear ones, which impairs their identification through antibody-based techniques, or in the other hand, mitochondrial MMR could not depend of all the elements involved in the canonical nuclear form, as it was seen with the participation of YB1 [52]. In any case, more research is needed to find more MMR nuclear factors within mitochondria or to discover new ones and be able to catalog the mitochondrial MMR as an original pathway.

4.4 Single strand break repair (SSBR)

The repair of SSBs in mitochondria is achieved through a BER subpathway known as base excision/SSB repair (which is also present in nucleus), since both mechanisms share common component, especially in the last steps: gap filling and DNA ligation [10, 24]. Indeed, most of the SSBs can be repaired by elements of BER pathway: APE1, PKPK, and Polγ [24]. Other members of mitochondrial SSB repair include: PARP1, a protein implicated in the detection of SSBs in the nucleus and also more recently observed in mitochondria, where not only it binds to mtDNA, but also when is depleted, this provokes accumulations of DNA damage, thus confirming its participation in mtDNA maintenance [56]. Besides, the participation of ExoG in SSB repair has been elucidated, since its depletion induces mitochondrial persisting SSBs that eventually lead to apoptosis [57].

As was previously mentioned, there are some lesions associated to SSB, like trapped topoisomerase 1 (Top1), damage that can be repaired through the action of tyrosil-DNA phosphodiesterase 1 (TDP1), an important enzyme involved in the release of covalently trapped Top1 with DNA that was first described in yeast [58]. In addition to its well characterized function, it has been observed that TDP1 also removes other types of 3′-blocking lesions, resulting oxidative damage [59–61]. A fraction of TDP1 (nuclear encoded) localizes to the mitochondria, where it has been implicated in mtDNA repair, since the treatment with chain terminator nucleotide analogs (CTNAs), which are also substrates of this enzyme, in *tdp1*$^{-/-}$ cells generate a reduction in mtDNA copy number, whereas wild type cells remain unaffected [61, 62]. These findings confirm the involvement of TDP1 in mtDNA damage repair, in this case induced by CTNAs. Another SSB-related lesion is the generation of a covalent binding of adenine monophosphate (AMP) to the 5′end of mtDNA, and this error is promoted by abortive ligase activity. The resolution of such damage relies in aprataxin (APTX) protein that is able to remove 5′-adenylate groups. APTX localizes to mitochondria, whereas its depletion generates a decline of mtDNA copy number as well as higher levels of DNA damage, observations that suggest a direct role of this enzyme in mtDNA maintenance [63]. If any of the lesions mentioned remains unrepaired, further complications may appear, since SSBs may progress to DSBs, which are more deleterious to cells.

4.5 Double strand break repair (DSBR)

In general, cells of higher eukaryotes use two main approaches to repair DSBs. The first approach is through the union of the ends in a nonhomologous dependent way, this pathway is termed nonhomologous end joining (NHEJ); it has been determined that NHEJ possesses some alternatives versions that use noncanonical elements, these sub pathways are known as alternative NHEJ (A-NHEJ); the repair with these mechanisms guarantee the restoration of DNA integrity but not sequence.

4.5.1 Nonhomologous end joining (NHEJ)

Nonhomologous end joining (NHEJ) is one of the two main pathways used by the cells to repair DNA double strand breaks. Similar to most DNA repair processes, NHEJ is based on three general steps: the action of a nuclease to resect the damaged DNA, next, the fill-in to make new DNA by a polymerase, and finally the participation of a ligase to restore the integrity of the strands. One of the most interesting features of NHEJ is the diversity of substrates that can use and convert to joined products [64]. By virtue of its template-independent operation, NHEJ is associated with insertions and deletions and hence with a lack of reliable restoration [26].

It has been observed that mammalian mitochondria do possess the capacity to bind DNA ends, activity that is retained even in Ku-deficient cells [65], additionally, the efficiency and precision of this activity apparently depend on the structure of the ends generated, since blunt-ended DNA fragment repaired are less conserved than sticky ends in comparison to the original [66]. Tadi et al. [67] demonstrated that mitochondria have a noncanonical version of NHEJ, also named alternative NHEJ (A-NHEJ). The repair by this pathway is based on microhomology and is sometimes associated with long deletions, and hence it is described as microhomology-mediated end joining (MMEJ). In this same study, using rodents and human mitochondrial extracts, a lack of end-to-end joining of nonligatable broken DNA was observed, the fact that suggests the absence of a functionally operative canonical NHEJ in mitochondria, or at least is undetected with the techniques used. In contrast, mitochondria have the ability to join oligomeric dsDNAs

harboring direct repeats (microhomology) that vary in length, from 5 to 22 nt, with an efficiency that is enhanced with the increase in the length of homology. These results are supported by a previous observation, where DSBs are induced in mice through mitochondrially targeted restriction endonuclease (*Pst* I), and the repaired mtDNA presented small directed repeats (a few nucleotides) at the breakpoint [68], resolution that fits with the repair manner of MMEJ, and in addition, these repair products have also been observed in most of the mtDNA deletions associated with human diseases, which are mostly (~85%) flanked by small direct repeats [69]. Besides, it has been determined that this mitochondrial MMEJ activity involved the proteins CtIP, FEN1, Mre11, PARP1, and ligase III [67].

Therefore, MMEJ has been proposed as a central pathway in the repair of double strand breaks and maintenance of mammalian mitochondrial genome, and the use of this pathway and possibly not C-NHEJ may explain the Ku independence proteins to exert the joining activity as was previously mentioned. The possible absence of C-NHEJ activity in mitochondria is contrasting with the observation that antibodies to KU70 and KU80 cross-react with proteins from mitochondrial extracts with DNA end-binding activity [10, 70]. Furthermore, it has been determined that XRCC4, a mediator protein of nuclear DSB repair pathway, is present in mitochondrial, where it is indeed involved in mtDNA repair and possibly associated with DNA ligase III [48]. In summary, at present no NHEJ activity has been described in mitochondria; however, this organelle presents the ability to join broken DNA ends, and it appears that this action depends on the structure of the ends generated and in the presence of homology at both ends of the DSB, further, the repair by MMEJ may explain the deletions observed in the majority of mitochondrial diseases.

4.5.2 Homologous recombination (HR)

Homologous recombination (HR) is a ubiquitous process conserved from bacteriophages to humans and is one of the most important pathways used by the cells to deal with DNA double strand breaks. To achieve the restoration of molecular integrity and sequence in a free-error manner, HR needs a homologous sequence to use it as a template [26, 71].

It has been determined that HR is essential for preservation of mtDNA in plants, yeast, and fungi, and on the other hand, although there is evidence about HR in mammalian, its significance in vivo is not clear [1]. One of the first reports about mitochondrial homologous repair capabilities was made by Thyagarajan et al. [72] where they observed that mitochondrial protein extracts from mammalian cells catalyzed homologous recombination repair of plasmid DNA substrates, therefore concluding that mitochondria do possess the machinery to perform this process. Additionally, after preincubating protein extracts with anti-RecA antibodies, an inhibition of the reaction was observed, fact that suggests the participation of a mammalian mitochondrial RecA homolog. Supporting this evidence, in 2010, Sage et al. [73] demonstrated that Rad51 and the related proteins, Rad51C and XRCC3, localize to human mitochondria, and they also reported that the protein levels were enriched after stress induction and that depletion of any of these elements generates a dramatic decrease in mtDNA copy number, these results strongly suggest some type of HR participation in mitochondrial genome maintenance. Other proteins involved in HR have been observed in mitochondria, and their participation in mtDNA repair has been validated, such as Dna2 [74] and Mre11 [67, 75, 76]; moreover, it has been suggested that ExoG could supply Exo1, and therefore, many of the factors needed to perform HR process are present in mitochondria.

Recently, using biochemical assays, it was determined that HR is the major DSBR mechanism, where it has a role in maintenance of mitochondrial genome

integrity, since the induction of DSBs significantly enhanced this process. Besides the participation of Rad51, Mre11 and Nibrin relevance in HR was confirmed by suppression of HR-mediated repair after immunodepletion of these proteins in the mitochondrial extracts [76]. The process of mitochondrial HR may proceed in two ways, one through intramolecular recombinant events, where a sole mtDNA molecule recombines with itself, and a second form, where a molecule can recombine with another one homologous or heterologous [77, 78]. Despite the knowledge of the elements involved in mitochondrial HR have increased over the last years, the exact mechanism about how HR is achieved in mitochondria is lacking in comparison to the nuclear models [33].

The second approach is through the use of nondamaged homologous sequences; this kind of repair restores molecular integrity as well as sequence; another pathway that uses homology sequences is single strand annealing (SSA), which needs directed repeats in both ends of the DSB, in such a way that when repairing, it restores integrity and sequence but at the expense of a variable length deletion [26].

4.6 Other pathways

As it has been previously described in this chapter, mitochondria have a repertoire of elements to deal with DNA damage, even it has been observed that mitochondria possess a mechanism to "prevent" further lesions (described below). However, if the mtDNA lesions surpass the mitochondrial repair capabilities, the cell maintains other options to avoid a higher damage, and in these circumstances, it is possible to degrade the unrepairable mtDNA, the organelle or even the whole cell [10].

4.6.1 Sanitation of the dNTP pool

The DNA is not the only molecule susceptible to chemical damage, the deoxyribonucleotide triphosphates (dNTPs) pool is also affected, being oxidative damage one of the most recurrent alterations [79]. If unrepaired, these lesions could become a source of mismatch errors during DNA synthesis [3]. To cope with this threat, mitochondria have MTH1, an specialized enzyme also found in the nucleus, which can hydrolyze oxidized dNTPs such as 8-oxo-20-deoxyguanosine triphosphate (8-oxo-dGTP), 8-oxo-20-deoxyadenosine triphosphate (8-oxo-dATP), and 2-hydroxy-20-deoxtadenosine triphosphate (2-hydroxy-ATP) to corresponding monophosphates, which cannot be assembled in the DNA by polymerases [41, 80]. In 2008, Pursell et al. [81] reported that 8-oxo-dGTP exists in some rat tissues at levels that are potentially mutagenic; therefore, these data suggest that oxidized dNTP precursors could generate mutagenesis in vivo and consequently promote mitochondrial dysfunction. In addition, it was reported that a pathogenic variant of Polγ, which is present in patients with progressive external ophthalmoplegia (PEO), increases 8-oxo-dGTP misincorporation, observation that establishes a relationship between the oxidative lesions and increased mtDNA damage observed in other models with this pathogenic version, and misincorporation of oxidized nucleotides [82]. In summary, although sanitation of premutagenic free nucleotides is not properly a DNA repair mechanism, its participation prevents the formation of mismatches in mitochondrial genome and therefore reduces the probability of mutagenesis.

4.6.2 mtDNA degradation

Compared to nuclear genome, mitochondrial genomic material has a remarkable advantage about DNA damage and repair, its redundancy, consisting of hundreds to thousands of copies per cell. Due to this characteristic, mitochondria

can dispose of a considerable fraction of mtDNA, where its repair capabilities were exceeded; however, it does not compromise organelle functions, and this is not an option for nucleus, where the diploid genome cannot be submitted to degradation without affecting the cellular homeostasis [3]. It is thought that after mtDNA degradation, the lost molecules are restored by mitochondrial genome turnover, a process that was first described several decades ago [83] (**Figure 2**). There is a wide body of evidence that supports this hypothesis of mtDNA degradation after unrepairable insults; for instance, it was observed that when one of the initial steps of the BER repair pathway is inhibited by methoxyamine drug, the increase of incidence of oxidative and alkylating damage enhanced the mtDNA degradation [84]; additionally, through qPCR analysis, it has been shown a mtDNA amount decrease after persisting exposure with the oxidizing agent hydrogen peroxide [85, 86]. Furthermore, the absence of mutation fixation after persisting cell treatment with alkylating agents which have a high mutagenic potential suggests that due to the lack of mechanisms for repairing bulky lesion, the mtDNA could be selectively degraded and to prevent further modifications [87]. Nowadays, it is not completely clear how the mitochondrion degrades its damaged DNA; however, it has been recently determined that endonuclease G (EndoG) has an important role in mtDNA depletion, since it promotes cleavage of mtDNA as a response to oxidative and nitrosative stress, action that subsequently generates an upregulation of mtDNA replication as an indirect outcome [88]. This evidence is supported by the fact that endo G is the most abundant and active nuclease within mitochondria, and it has a preference on oxidized DNA harboring single-strand breaks or distorted DNA product of crosslinking agents to exert its endonuclease activity in vitro [89], also it has been reported that this nuclease preferentially cleaves 5-hydroxymethylcytosine an oxidized product of 5-methylcytosine [90]. In conclusion, despite that additional research is needed to elucidate the whole mechanisms and elements that participate in mtDNA degradation, this pathway emerges as a unique and mitochondrial specific method to maintain DNA integrity.

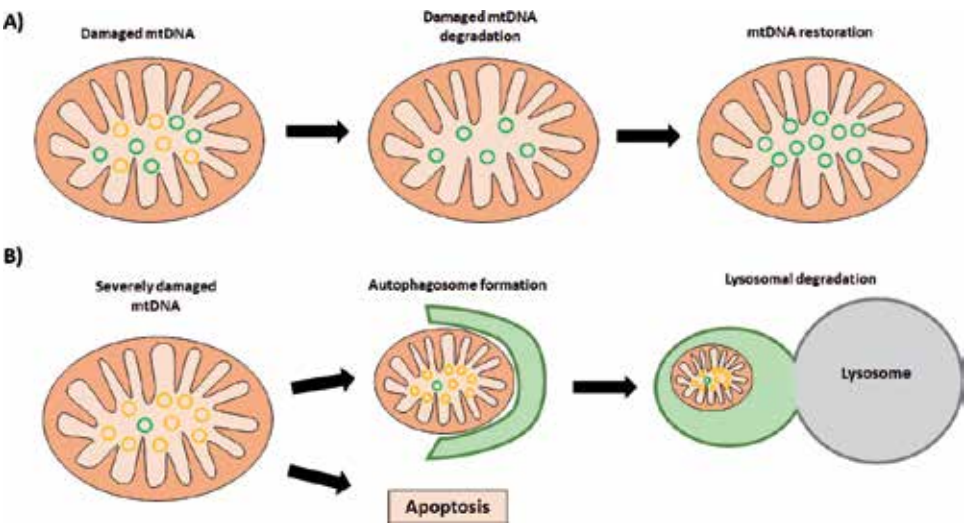

Figure 2.
mtDNA degradation and mitophagy. (A) Damaged mtDNA (yellow circles) can be selectively degraded inside mitochondria, thus keeping "healthy" mtDNA (green circles), then this can replicate to re-establish mitochondrial genome homeostasis. (B) If the mtDNA is severely damaged and the repair mechanism is surpassed, injured mitochondria can be selectively degraded by the formation of an autophagosome and subsequent fusion with lysosomes [91]. On the other hand, the mtDNA lesions can also trigger cell apoptosis.

4.6.3 Mitochondrial clearance, dynamics, and apoptosis

In general terms, autophagy is a highly conserved degradative mechanism used by cells to maintain homeostasis [92]. This is a finely regulated process that takes part in cell growth, development, and in the maintenance of an equilibrium between synthesis, degradation and recycling of cellular elements including whole organelles [11]. There is a specialized sub pathway of autophagy, which is specifically involved in degradation of damaged and dysfunctional mitochondria, and this procedure is known as mitophagy or mitochondrial clearance. Although mitophagy can emerge as a programmed cellular event, like the one that is observed during erythroblast maturation in order to generate mature red blood cells lacking mitochondria [11], it has been proposed that mitophagy could participate in the elimination of organelles harboring low levels of DNA damage stress. On the other hand, when the DNA lesions are too many to handle with mtDNA repair mechanism or by mitochondrial clearance, the cellular response could trigger apoptosis [93], therefore the choice of which pathway must be used depends on the degree of DNA damage (**Figure 2**). In accordance with the previous mechanism, Suen et al. [94] observed selectively degradation by mitophagy of organelles harboring deleterious COXI mutations after overexpressing the protein Parkin, which translocated to affected mitochondria and induced autophagic elimination, thus this selection enriched cells for nonmutated mtDNA and restoring cytochrome c oxidase activity [95].

It appears that mitophagy is closely associated with mitochondrial dynamics processes: fission and fusion [96]. Fusion is the joining of two organelles to form one, this mechanism allows mitochondria to distribute mtDNA and to replenish it when is damaged, therefore safeguarding mtDNA integrity and protecting it from mutations [97]. On the other hand, fission is the division of a single organelle to create two, this process is very important to cellular viability, it contributes to symmetrical distribution of mitochondria during mitosis, and promotes the removal of lesioned organelles by partitioning the damaged elements (like mtDNA) to a derived mitochondria that can fuse to a healthy one with the intention of recovering functionality or to be degraded by mitophagy. Therefore, mitochondrial removal by mitophagy is preceded by mitochondrial fission, which is capable of dividing the organelle into smaller pieces to be degraded easily [98]. When mitochondrial clearance, fusion, or fission are dysfunctional, the cells could be severely affected, since it has been observed that in these situations, an increase in mtDNA instability and generation of neurodegenerative, cardiovascular, and age-related diseases were obtained [99].

5. mtDNA repair, diseases, and aging

Mitochondrial diseases are a heterogeneous group of illnesses affecting multiple organs and leading to eventual degeneration and in some cases premature death. These affectations have origin in mutations on mtDNA, which are generated by errors during DNA replication, exogenous sources, and ROS; however, mitochondrial dysfunction may also arise from mutations in nuclear genes which encode proteins with mitochondrial function, involved in several processes like biogenesis, transcription, replication, mitochondrial dynamics, and mtDNA repair, among others. Of interest, neurodegeneration is a prevalent trait in mitochondrial diseases, maybe because the brain needs a higher demand of energy in comparison with other tissues [11]. On the other hand, there is a large body of evidence that underscores the relationship between mitochondrial disorders and aging; however, there is still

controversy about whether these mutations in the mtDNA are the product of age-related disorders or they are themselves the cause [100].

About the genes involved in mtDNA maintenance, it has been well established that failure of the mtDNA repair pathways may promote diseases and age-related disorders in humans [11, 28]; in addition to mutations, the reduction of mtDNA copy number has also been associated with neurodegeneration, aging, diabetes, and cancer [101]. For example, it has been observed that the lack of proofreading activity of Polγ in mice generates multi-systemic disease and phenotypes resembling to premature aging [102], furthermore, over 200 mutations in POLG have been associated with mitochondrial diseases, these POLG-related disorders can be classified into five main phenotypes of neurodegeneration: Alpers-Huttenlocher syndrome (AHS), childhood myocerebrohepatopathy spectrum (MCHS), myoclonic epilepsy myopathy sensory ataxia (MEMSA), ataxia neuropathy spectrum (ANS), and PEO [28], besides, mutations in Twinkle helicase often causes infantile onset spinocerebellar ataxia (IOSCA), which usually appears in early childhood [103]. Other mtDNA repair elements, such as APTX and TDP1, implicated in SSBR, are related with the generation of ataxia with ocular motor apraxia (AOA1) when are mutated [10, 104]. Also, defects in the proteins CSA and CSB, implicated in the possibly mitochondrial DNA repair transcription coupled-NER pathway, are related with the development of progressive cerebellar pathology [105]. Furthermore, alterations in fusion, fission, or mitophagy processes due to mutations in the proteins involved generate mtDNA instability, which in turn may induce neurodegenerative, cardiovascular, and age-related diseases [99], such is the case of MFN2, which is implicated in mitochondria fusion, and its alteration lead to organelle fragmentation and causes axonal Charcot-Marie-Tooth disease (CMT2A) [106], also mutations in OPA1, lead to optic atrophy, affectation that can be accompanied with hearing loss and ophthalmoplegia [107]. Additionally, mutations in DNA2 and mitochondrial genome maintenance exonuclease 1 (MGME1) nucleases are implicated in ophthalmoplegia, myopathy, and mtDNA depletion [108, 109]. In conclusion, the importance of mtDNA maintenance lies in the observation that when the repair elements are affected, or the mechanisms exceeded, the risk of disease development increases, thus the understanding of these alterations may shed light for clinical targets to prevent diseases or treat them.

6. Concluding remarks

After decades of study, it has been concluded that like nucleus, mitochondria do possess specific mechanisms to maintain integrity of its small and polyploid genome, and although nowadays, the complete repertoire of elements participating in mtDNA repair has not been identified, it appears that these pathways resemble those of nucleus but operating with fewer elements. In addition, mitochondria have evolved organelle specific mechanisms which work as a backup when the repair pathways are surpassed by the amount of damage and that would be impossible to carry out in nuclear genome. In conclusion, the repair of mitochondrial genome is a field in continuous growth that promises new discoveries in the years to come.

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Chapter 4

Cellular Responses to Aflatoxin-Associated DNA Adducts

Michael Fasullo

Abstract

Aflatoxin B1 (AFB1) is the most potent known hepatocarcinogen. The signature p53 mutation (p53 249^{ser}) that is found in AFB1-associated liver cancer suggests that AFB1 is a potent genotoxin. AFB1 is not genotoxic *per se* but is metabolically activated by cytochrome P450 enzymes that convert the promutagen into a highly reactive epoxide, which primarily reacts with the N^7 group of guanine, forming 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N^7-dG). While this primary adduct is unstable, the subsequent trans-8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy aflatoxin B1 (AFB1-Fapy)-derived adducts are stable and are mutagenic. Studies have revealed that nucleotide excision repair (NER), base excision repair (BER), recombinational repair, and DNA replication bypass are all involved in conferring AFB1 resistance. To minimize the genotoxicity of AFB1, pathways function to detoxify the metabolically active intermediate, excise resulting DNA adducts, bypass unrepaired adducts, and repair secondary DNA breaks. How these repair pathways functionally cooperate to minimize AFB1-associated genetic instability phenotypes is not well understood. Insights can be gained from epidemiological research and model organisms. Gene profiling and next-generation sequencing are facilitating how pathways and tissue-specific differences are induced. This review will encompass studies concerning human genetic susceptibility to AFB1 and pathways that repair and tolerate AFB1-associated DNA damage.

Keywords: aflatoxin B1, liver carcinogenesis, DNA damage tolerance, oncogenes, p53

1. Introduction

The mycotoxin aflatoxin B1 (AFB1) is the most potent known liver carcinogen [1] and is also a lung [2] and esophageal carcinogen [3]. The International Agency for Research and Cancer (IARC) has classified AFB1 as a Group 1 human carcinogen [1]. AFB1 was discovered as the causative chemical agent in Turkey X disease, so named after a 1960 occurrence where 100,000 turkeys in Great Britain died after feeding on contaminated peanut meal imported from Brazil [4]. Its notoriety is underscored by its persistence in grain supplies, ground nuts and animal feed, which must be continually monitored [5]. Produced by aspergillus parasiticus and aspergillus flavis, the mycotoxin is a particular problem in subtropical areas of China, and in tropical areas of Southeast Asia and Africa [6, 7]. In temperate climates, such as in North America, high levels of AFB1 contamination have been found in corn and nuts, such as almonds and pistachios [8]. To minimize health risks in humans, the Food and Drug Administration (FDA) mandates that the

human food supply contain no more than 20 ppb AFB1 [5]. While human food supply is relatively protected in developed countries, outbreaks of acute mycotoxin contamination have been noted in third world countries and among animals, as recently as 2006 [5]. Although the incidence of acute aflatoxicosis is rare, it is estimated that a large fraction of the population in the developing world are chronically exposed to AFB1 and thus at a higher risk for aflatoxin-associated cancer, especially liver cancer [6].

Liver cancer ranks third in all worldwide cancer mortalities [9–11] and ninth in cancer mortalities in the United States [12, 13]. 4–28% of cancer cases are related to AFB1 exposure [2]. Most liver cancer is characterized as hepatocellular carcinoma (HCC). HCC is highest where there is both a high rate of hepatitis B (HBV) [14, 15] and C virus (HCV) infection [15–17] and high levels of AFB1 contamination in the human food supply, especially in areas of Southeast Asia, China and Africa [6]. Interestingly, the incidence for liver cancer is higher in men than women, regardless of whether the cancer is associated with AFB1 exposure [18]. Because diagnosis is often late and there is no effective treatment for late-stage cancer, the five year survival rate is low in both men and women [12, 13]. The carcinogenic potency of AFB1 is correlated with AFB1 being a strong genotoxin, the signature p53 mutation, p53 249^{ser} [19, 20], is found in 40–60% of all liver cancer derived from patients in heavily contaminated areas [2]. Animal studies have further strengthened the idea that AFB1 carcinogenicity is associated with its genotoxicity; AFB1-associated DNA adduct levels are directly proportional to the number of the animals stricken with liver cancer [21, 22].

Observations that HCC incidence is correlated to AFB1 exposure continues to motivate biomedical researchers to study the repair and toleration of AFB1-associated DNA adducts, the cellular response to these DNA adducts, and associated factors that may enhance or mitigate the high mutagenicity of the DNA adducts in humans. This review will address (1) associated risk factors that enhance or synergize with AFB-associated DNA adducts that increase liver cancer incidence, (2) genetic instability phenotypes associated with AFB1-associated DNA adducts, and (3) repair mechanisms that have been elucidated in model organisms and conserved in humans, (4) cellular responses that enhance repair mechanisms, and (5) future directions in understanding the contributions of genes in AFB1-associated DNA repair. In particular, novel research that addresses epigenetic factors that can alter the repair of AFB1-associated genotoxic damage will be addressed.

2. Progression of HCC

Liver cancer progression is slow and the median age of onset is 60–65 years [11]; populations in areas that are at high risk for environmental and life-style factors are exceptions. For example, the incidence of liver cancer in the Qidong province of China peaks at 45 years [9, 11]. HCC generally develops as a consequence of liver injury, whether caused by chronic hepatitis or cirrhosis, which leads to chronic inflammation and deposition of connective tissue. Chronic hepatitis leads to upregulation of mitogenic pathways, partially through epigenetic mechanisms [23]. Monoclonal populations of dysplastic hepatocytes may exhibit telomere erosion and re-expression of telomerase to maintain viability. Eventual malignant cells accumulate irreversible genetic alterations [23]. As the transformed phenotype advances, the rate in the accumulation of genetic alterations increases [24]. The exact threshold for the number of mutations or alterations present in liver cancer has not been established. Thus, the progression of liver cancer is associated and is accelerated with the accumulation of genetic mutations and altered gene expression patterns.

2.1 Mutations that contribute to liver cancer

Understanding which HCC-associated genetic changes are associated with AFB1 exposure requires a comparison of the genomic alterations that occur in sporadic HCC or HCC associated with other causes. For sporadic HCC, similar to solid tumors, there is both a multiplicity and heterogeneity in genetic alterations in HCC [23–25]. In general, these genetic alterations can be grouped into those that result in loss of function and those that result in gain of function. Genetic alterations that result in loss of function include dominant negative mutations and recessive mutations, which are expressed after loss of heterozygosity (LOH).

Among sporadic tumors, both loss of heterozygosity (LOH) and mutations have been found in HCC tumors. Among 363 patients, The Cancer Genome Atlas Research Network [25], report that the most heavily mutated gene was TP53 (31%), encoding p53, followed by WNT pathway member CTNNB1 (27%), encoding β-catenin, and AXIN (8%), encoding a WNT signaling scaffolding protein, and chromatin remodeling genes (12%) [25]. In greater than 10% of HCC, mutations are found in CDH1, TP53, IGF2H, RB1, CDKN2A, PTEN, KLC, TP73, EXT, MLH1, THRB, THRA, E2F5, and CTNNB1 [23]. Whether these mutations occur early or late in the etiology of liver cancer is still not understood. While the p53 gene functions in controlling the DNA damage response and apoptosis, the WNT pathway is important in controlling cell proliferation [23]. Many of the mutagenic events result from G to T transversions, unlike events found other tumors. The strong bias for G to T transversions suggests that these genetic alterations likely result from chemical DNA damaging agents, rather than spontaneous events, such as cytosine deamination [23]. While the heterogeneity in genetic mutations may reflect multiple mechanisms for liver cell transformation, identifying alterations in HCC are informative in understanding the etiology and possible treatment of individual cancer cases. For example, β-catenin defective liver cancer may be easier to treat than liver cancer resulting from multiple mutations [23–25].

In addition to mutation and LOH events, gain-of-function genetic alterations may confer higher levels of oncogene expression and thereby accelerate carcinogenesis [26–29]. Such alterations could include gene amplification events, such as c-N-methyl-N′-nitro-N-nitroso-guanidine HOS transforming gene (c-MET) and cyclin D (CCND1) [27]. Other gains of function mutations include mutations in the promoter for telomerases reverse transcriptase (TERT) promoter. TERT mutations frequently were shown to be among the earliest and most prevalent neoplastic events in HCC [28, 30].

Both epidemiological and molecular pathology studies have facilitated the identification of which genetic alterations are likely to be associated AFB1 exposure. Mutated genes found in HCC from areas with high AFB1 exposure include p53 and β-catenin [30]. The p53 249^{ser} mutation shows a strong correlation with HCC associated with AFB1 exposure, while is less frequent or absent in HCC from localities where there is little AFB1 exposure [19, 20]. For example, among have HCCs from southern Guangxi province of China, an area of high AFB1 exposure, the p53 249^{ser} mutation was found in 36% of tumors [30]. *CTNNB1* mutations and β-catenin protein accumulation in human hepatocellular carcinomas is also associated with high exposure to AFB1, although it is less clear whether these mutations directly result from AFB1-associated DNA adducts [30]. Whether these mutations must occur early or late in cancer progression is still unclear. One hypothesis is that initial mutations confer a higher level genetic instability that is aggravated by further exposure to genotoxic agents.

To determine whether mutations found in HCC confer higher levels of genetic instability and a higher probability of liver cancer when present in a non-cancerous

liver, scientists have constructed transgenic mice that exhibit similar genotypes found in human cancer. Ghebranious and Sell [31] constructed transgenic mice that were both homozygous and heterozygous for the p53ser246 gene, equivalent to the human p53 249^{ser} mutations. Male mice expressing p53ser246 increased the incidence of AFB1-associated high-grade tumors to 14%, compared to 0% exhibited by p53+ (wild type) mice [31]. These studies indicate that the mutant p53 249^{ser} may also be a driver of AFB1-associated liver cancer.

The role of inflammation in liver cancer has led to insights into the gender bias of its incidence. Men are afflicted more than women in nearly all age groups; however the prognosis of liver cancer in either sex is about the same [9]. Naugler et al. [18], have shown that inflammatory cytokines, such as IL-6, are more prevalent in men than women, estrogen having a negative effect on IL-6 production. This gender difference is not only true for humans [32, 33] but also for rodents, including mice and rats [31]. The gender bias underscores the notion that inflammatory responses play a role in liver cancer etiology.

2.2 Associated risk factors that accelerate AFB1-associated liver cancer: role of HBV and HCC virus

The incidence of HCC synergistically increases when individuals are both exposed to AFB1 and infected with either HBV or HCV virus. Interestingly, the incidence of high grade tumors in p53ser246 transgenic mice that are HBsAg-positive is 100% [31]. The common molecular mechanisms by which HBV and HCV infection stimulates AFB1-associated genetic instability phenotypes are still not completely understood; HBV is a DNA virus that replicates by reverse transcription while HCC is a RNA virus that replicates by RNA replication and encodes a single polycistronic message [34, 35]. While 257 million individuals are estimated to be infected with HBV, 140 million individuals are estimated to be infected with HCV; and chronic HBV and HCV infection is the leading cause for 60–70% of HCC [35, 36]. Although HCC contains no oncogenes *per se*, HCV-associated carcinogenesis is associated with increase in reactive oxygen species (ROS), ROS-associated genetic instability, inflammation, and hepatocyte proliferations [36]. Similarly, HBV-associated HCC is associated with inflammation and necro-inflammatory liver damage [16, 36]. Both viruses are not cytopathic *per se*; liver damage caused by HCV and HBV is likely induced by viral-specific $CD8^+$ T and natural killer cells (NK) [35, 36]. Thus, both HCV and HBV create an inflammatory cellular environment that stimulates repopulation of hepatocytes, enhancing AFB1-associated genetic instability.

However, different pathologies of HBV and HCV infection may accelerate HCC progression at different rates. While the median onset age for HBV-associated HCC is 55 years that of HCV is 65 years [11]. HBV can chronically infect children after transmission from the mother [11, 36]. Once HBV is stably integrated into the host genome, HBV can promote chromosomal rearrangements and mutations in cancer-associated genes and interfere with checkpoint controls [37, 38]. For example, HBV integration can occur in TERT promoters, stimulating expression of telomerase, and near LINE sequences [39]. The HBV-encoded oncogene HBx can activate both Src and Ras signaling and is essential for viral DNA (cccDNA) replication. To facilitate replication, HBx mediates chromatin changes by recruiting histone acetyltransferases to acetylate histone H3. HBx is also thought to interact with p53 249^{ser}, and attenuate DNA repair and apoptosis [20].

Besides stimulating host cell replication, HBx may also interfere with the host cell's DNA repair pathways and promote genetic instability and replication [38, 40–44]. HBx binds to DNA damage binding protein 1 (DDB1) and cullin-4 (Cul4), which form a ubiquitinase complex, and can perturb the stability of structural

maintenance of chromosome proteins 5 and 6 (Smc5/6) and thus affect DNA replication and DNA damage tolerance [35]. HBx may also interfere with nucleotide excision repair (NER) of AFB1-associated DNA adducts [40–42, 44]. Although less substantiated, HBx is also thought to interfere with PARP1 and decrease excision repair of DNA adducts. Thus HBx drives carcinogenesis by multiple mechanisms that accelerate carcinogenesis.

2.3 Gene polymorphisms associated with AFB1-associated liver cancer

While HBV and HCV infections are the primary factors that aggravate the risk for AFB1-associated HCC, genetic risk factors have also been postulated [45]. With the advent of technologies that accelerate genome sequencing, such as next generation sequencing (NGS), epidemiologists have identified candidate polymorphic genes that increase the risk for aflatoxin-associated liver cancer. Single nucleotide polymorphisms (SNPs) may be located in the amino acid coding region, the introns, or the promoter regions of the candidate genes. Risk factors generally can be grouped into those that (1) are associated with AFB1 metabolic activation and detoxification and (2) that function in DNA repair or DNA damage tolerance genes.

To understand genetic risk factors that affect metabolic activation and detoxification of AFB1 it is necessary to identify genes involved in these pathways. AFB1 is activated by cytochrome P450 enzymes that hydroxylate AFB1 so that the metabolized carcinogen can be rendered hydrophilic and effectively excreted; for review, see [46–48]. Referred to as phase I enzymes and monooxygenases, the cytochrome P450 enzymes contain a heme group at their active sites and catalyze the transfer of single oxygen to specific sites on the target molecule [46]. Cytochrome P450 enzymes require NADPH oxidoreductase (POR) to maintain activity [46]. The P450 enzymes are located in the endoplasmic reticulum in the vicinity of the POR [46]. Of the characterized enzymes expressed by 57 CYP450 genes, CYP1A2 is liver specific and has a high affinity for AFB1, while CYP3A4 constitutes approximately 50% of the hepatic P450 activity. While there have been disagreements over which cytochrome P450 enzymes is chiefly responsible for AFB1 activation in the liver [46, 49, 50], several reports favored CYP3A4 [50, 51], while another report suggested that CYP3A5 has the highest catalytic activity [52]. Among extrahepatic CYPs, CYP2A13 activates AFB1 in the lung, while CYP1A1 catalyzes the formation of AFM1, a hydroxylated AFB1 derivative that can be excreted in milk, which is still carcinogenic [2]. A transient intermediate in the hydroxylation pathway is a highly reactive epoxide, referred to as AFB_1-8,9-exo-epoxide (AFBO) (**Figure 1**). This epoxide can be effectively detoxified by either epoxide hydrolases (EHs) or glutathione S-transferases (GSTs), referred to as phase II enzymes [47, 48]. While multiple cytochrome P450s can activate AFB1, the highly reactive epoxide is thought to be the predominant reactive intermediate in all P450 reactions. Thus, gene polymorphisms that increase the risk of HCC could: (1) increase P450 enzyme levels or activation, (2) downregulate phase II enzymes, (3) decrease the repair of DNA existing lesions, and (4) channel the repair of the DNA lesions into mutagenic pathways.

One source of polymorphic enzymes that can influence the fate of AFB1 is glutathione S-transferases that are present in the liver [53]. In the mouse, knock-out of GSTa3 confers extreme AFB1-associated toxicity [54] and GSTa3 expression levels correlate with AFB1-associated liver cancer in young mice [55]. In humans, HCC risk is dramatically increased by SNPs in glutathione S transferase mu1 (GSTMI) and (glutathione-S-transferase theta1) (GSTT1) [56]. Expression of epoxide hydrolase in yeast also leads to detoxification of AFBO [57]; however, polymorphisms associated with epoxide hydrolase only have a weak association with liver

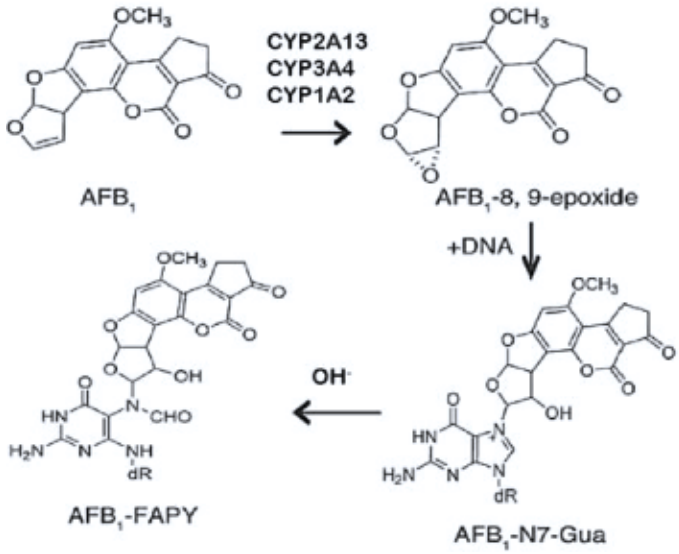

Figure 1.
CYP-mediated metabolic activation of AFB1 to the activated AFB1-8,9 epoxide and adduct formation and conversion to AFB1-Fapy. Adapted from Ref. [130].

cancer [45]. These studies support the idea that detoxification of the highly reactive epoxide is critical in reducing AFB1 toxicity.

While diminished ability to detoxify AFBO is a risk factor for AFB1-associated liver cancer, higher or altered P450 activity could also increase HCC risk. HBx activates the pregnane receptor (PXR) and stimulates expression of CYP3A4 [58]. Particular CYP3A5 alleles, such as CYP3A5*3, are correlated with higher levels of expression and aflatoxin-protein adducts in individuals from Gambia, Africa [59]. CYP3A5*3 is present in a high percentage of individuals in Gambia but not in the Caucasian population [59]; the allele found in the Caucasian population confers an altered spliced mRNA, which is poorly expressed [60]. However, establishing correlations between HCC and increased expression of other P450 genes is complicated by the multiple interactions between P450 enzymes.

Genetic risk factors have also been identified among polymorphic alleles of DNA repair and cell cycle checkpoint genes, which may increase chromosomal instability in cells chronically exposed to AFB1. These risk factors have been found in p53, XRCC1, XRCC3, and ERCC1. The combination of p53 codon 72 Arg72Pro and MDM2 (mouse double minute 2 homolog) SNP309 (T>G) increases the risk of HCC in individuals infected with HBV [61]; p53 codon 72 Arg72Pro affects the frequency of double strand breaks and is associated with hyper-methylation of promoters in tumor suppressor genes [61]. XRCC3 (X-ray complementing defective repair in Chinese hamster cells) encodes a Rad51 paralog which is involved in double-strand break repair and could be involved in error-free by pass of AFB1-associated DNA lesions. The XRCC3 rs861539 allele (codon Thr241Met polymorphism) is a risk factor for HCC, and the risk is aggravated if individuals are exposed to AFB1 [62–64]. Other alleles that have been associated with higher risk for HCC include those participating in the base excision repair (BER) and NER pathways, such as XRCC1 rs25487 polymorphism (codon Arg399Gln polymorphism) [65, 66] and XPD rs25487 polymorphism, respectively [67]. These studies reinforce the idea that AFB1-associated genotoxicity can accelerate HCC progression. To understand the genotoxicity in more detail it is important to understand the nature of the AFB1-associated DNA adducts.

2.4 AFB1-associated DNA adducts and cellular targets

AFB1-associated DNA adducts have been characterized *in vitro* and isolated from organisms that were exposed *in vivo*. DNA exposed to synthesized AFBO reacts predominately with the N^7 group of guanine bases forming 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N^7-Gua), as identified by mass spectrometry analysis. In the presence of hydroxyl ions (base), N^7-guanine DNA adduct is unstable and decays into an apurinic site and a AFB_1 formamidopyrimidine (Fapy, **Figure 2**) DNA adduct; for review, see [68]. It is unclear whether both apurinic sites and AFB1-Fapy DNA adducts are equally generated; based on mutations generated by DNA lesions constructed *in vitro*, it has been suggested that AFB1-Fapy DNA adducts are the primary source of genetic mutations [69, 70], especially G to T transversion mutations that are found in AFB1-associated liver cancer [71, 72]. The AFB1-Fapy adduct is stable and can be present in two anomer forms; the alpha and the beta forms. While the beta form is highly mutagenic in *Escherichia coli* [69], the alpha form can stabilize the duplex helix and interfere with DNA replication [70]. In the rat liver, the half-life for AFB1-N^7-Gua is 7.5 h, while that for AFB1-Fapy is at least 24 h [68]. While the AFB1-N^7-Gua is unstable, the accumulation of AFB1-Fapy in the rat liver may also result from differential repair of the two types of DNA adducts.

AFB1 exposure also generates oxidative stress (ROS) in exposed cultured cells *in vitro* and in the liver and lung *in vivo* [73]. Multiple factors may contribute to

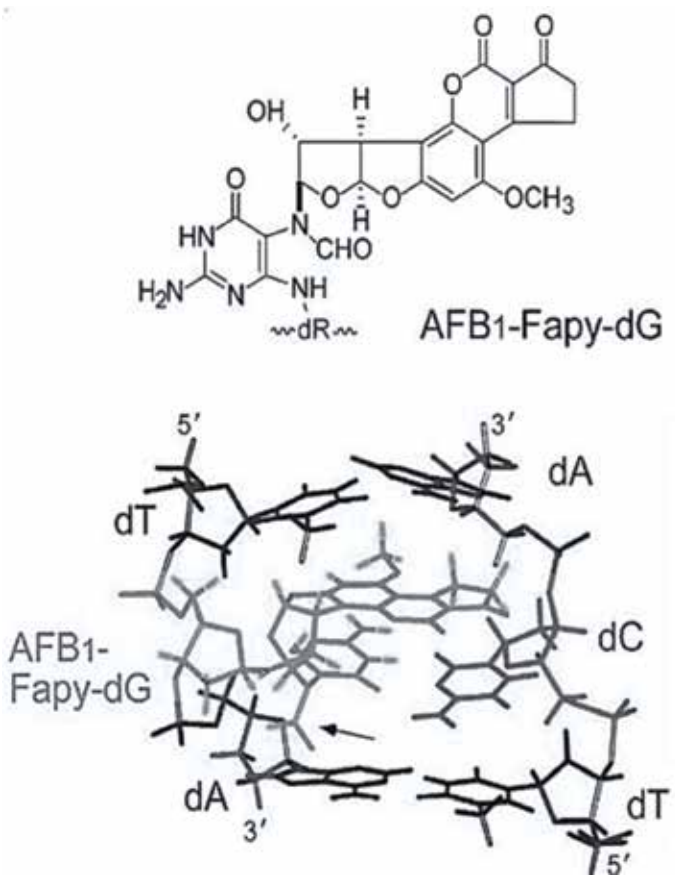

Figure 2.
Intercalation of the AFB1-Fapy-dG in duplex DNA. The adduct is in lighter tone; adapted from Ref. [121].

AFB1-associated oxidative stress including cytochrome P450 activity that involves iron-catalyzed reactions and Kupffer cells [68]. Oxidative stress generates hydroxyl radicals that form 8′-hydroxy-2′-deoxyguanosine (8oxodG) DNA damage. AFB1 exposure increases the 8OH-dG in the livers of ducks [74] and rats [75] and cultured woodchuck hepatocytes [68].

Interestingly, Niranjan et al. [76] observed that in rats, AFB1 bound to mitochondria l DNA exceeded the amount that was bound to the nuclear DNA and persisted for a longer period of time [76]. Furthermore, the persistence of mitochondrial DNA adducts correlated with a longer delay in expression of mitochondrial proteins, compared to that of nuclear-encoded proteins. The authors speculated that the persistence of AFB1 in the mitochondria may result from the lack of NER in the mitochondria. These studies support the notion that mitochondria are a prime target for acute effects of AFB1 exposure, and oxidative stress associated with AFB1-exposure could be indirect due to damage to mitochondria and the generation of superoxide.

To further elucidate the pathological consequences of AFB1-associated mitochondrial DNA lesions, Liu and Wang [77] measured AFB1-associated mitochondrial damage in primary broiler hepatocytes by monitoring mitochondrial membrane potential (MMP), ROS generation, apoptosis, and nuclear factor erythroid 2-like factor 2 (Nrf2)-related signal pathway. They observed mitochondrial ROS generation, decreased MMP and induced apoptosis. The increase in apoptotic cells correlated with an increase expression of caspase-9 and caspase-3. They concluded that AFB1 exposure results in a disruption of mitochondrial functions, generating more ROS, and consequently inducing apoptosis while triggering the Nrf2 signaling pathway [77].

2.5 Epigenetic changes associated with AFB1-associated damage

While genetic instability associated with AFB1 have been described, less well known are epigenetic changes. Epigenetic changes are inheritable changes that result in phenotypic changes without affecting the DNA sequence. Epigenetic changes can result from DNA methylation (hypermethylation) or demethylation (hypomethylation), histone modifications, and changes in microRNA (miRNA) expression [78]. AFB1-associated epigenetic changes have been observed in cell cultures, animal studies, and human tumors (**Table 1**). Hypomethylation has been observed to increase the expression of oncogenes and repetitive sequences, while hypermethylation may decrease expression of DNA repair and tumor suppressor genes (**Table 1**). Zhang et al. [79] observed global hypomethylation in AFB1-associated cancers, where particular genomic repetitive elements, such as LINE-1 elements, were hypomethylated; correlating with increased retro transposition and genetic instability [80]. Hypomethylation also correlated with increased expression of the oncogene c-MET, which is associated with accelerated liver cancer progression and poor prognosis [81]. Hyper-methylated genes include the DNA repair gene methylguanine methyl transferase (MGMT) and p16, which have a negative effect on DNA repair and apoptosis [82]. Thus, methylation patterns may possibly serve as biomarker that can indicate increased risk for HCC [83–85].

Additional biomarkers that indicate AFB1 exposure include alterations in miRNA expression. miRNAs are small noncoding RNAs that are generally 19–25 nucleotides in length and regulate gene express at the post-transcriptional level. They are important factors in regulating HCC development in mammalian organisms [87, 88], and a list of miRNAs that correlate with AFB1 exposure is shown in **Table 1**. This comprises a partial group of total miRNAs that have been associated with HCC. Several miRNAs upregulated after rats or liver cell lines are exposed to

Epigenetic change	Gene functions affected	Consequence	Context/ref.
DNA methylation			
DNA hyper methylation	MGMT expression decreased	DNA Repair downregulated	Human tumor tissue [82]
DNA hypo methylation	c-MET, RAB27A, TXNRD1 expression increased LINE 1	Growth and metastasis increased, decreased expression of GSTs, LINE1 transposition increased	Human tumor tissue [79, 81, 82]
miRNA expression			
miR-429 ↓	Downregulates Rab23	Metastasis increased when miR-429 decreased	HCC tumor tissue [83, 85]
miR-4-34a ↑	Downregulation of WNT/β-catenin pathway	Tumor suppressor effect; p53 enhances its expression	HepG2 cell lines [83]
miR-33a ↑	Downregulation of WNT/β-catenin pathway	Tumor suppressor effect; p53 enhances its expression	HepG2 and normal cell lines [83]
miR-24 ↑	Inhibition of apoptosis	Larger tumor size	HCC tumor tissue [83]
miR-34a-5p ↑	c-MET, CCND1, CCNE2 suppressed	Cell cycle arrest	*In vivo* rat livers [83]
miR-122 ↓	CUTL1 suppressed in mice	Required for tumor differentiation	Human studies [83, 88]
miR-138-1* ↓	PDK1 and indirectly PI3K/PDK/Akt	Inhibits colony formation, migration, invasiveness	P450-B-2A13 human cell culture [83]
Histone modification			
H3K9me3 ↑	Repression of gene expression (multiple)	Reprogramming of pluripotency	Porcine oocytes [83]
H3K27me3 ↓	Repression of gene expression (multiple)	Developmental gene programing in stem cell differentiation	Porcine oocytes [83]
H3K4me2 ↓	Activating gene expression (multiple)	Developmental gene programing in stem cell differentiation	Porcine oocytes [83]

↑, *up arrow designates upregulation;* ↓, *down arrow designates down regulation.*

Table 1.
Epigenetic changes associated with AFB1 exposure.

AFB1 may be protective by downregulating cell proliferation, while upregulated miRNAs found in AFB1-associated HCC may promote tumor size or carcinogenesis. While an individual microRNA may target multiple genes, the expression of individual microRNA can be influenced by multiple transcriptional and epigenetic factors, as well as by genomic changes. These factors include CpG methylation, c-Met signaling, and gene copy number.

Among HCC tumor cells associated with AFB1 exposure, upregulation of several miRNAs, such as miR-429 and miR-24 [86], are associated with larger tumor size [83]. In human bronchial epithelial cells that express CYP2A13 (P50-B-2a13 CELLS), AFB1 exposure induces malignant transformation of immortalized cells [89]. Among transformed cells, one downregulated miRNA was miR-138-1, observed to inhibit proliferation, colony formation, and transformation of P50-B-2a13 CELLS [89]. This miRNA preferentially inhibits 3-phosphoinositide dependent protein kinase-1 (PDK1), which lowers the expression of the P13K/PDK/Akt pathway [89]. These studies indicate that changes in miRNA expression in AFB1-associated HCC may promote carcinogenesis.

HBV infection also upregulates the expression of miRNAs in hepatocytes and may promote HBV-associated HCC. The expression of miR106b-25 is upregulated in HCC patients in general, and in HCC patients infected with HBV [90]. Hep 3B cells transformed with an HBx expression plasmid also express higher levels of miR106b-25, compared to cells that do not express HBx. The miR106b-25 is a member of a cluster of miRNAs in MCM7 that downregulate the expression of several tumor suppressors, including p21, E2F, BIM, and pTEN [91]. Thus, HBV infection may not only interfere with DNA repair mechanisms but also epigenetically silence tumor suppressor genes and accelerate HCC progression.

2.6 Mutagenic signatures associated with AFB1-associated DNA adducts

Mutation signatures are useful biomarkers to determine AFB1 exposure and HCC progression. AFB1 is known to induce mutations in *E. coli*, *Saccharomyces cerevisiae* (budding yeast), and in mammalian cells. AFB1 was one of the original carcinogens published in the Ames assay [92, 93]. While G to T transversions are considered associated with chronic AFB1 exposure in humans [19, 20, 68, 71], in *E. coli*, carcinogen-induced transversion mutations require over-expression of expression of MucAB, which encodes the polV error-prone polymerase [94]. In budding yeast expressing either human CYP1A2 or CYP1A1, AFB1 has been shown to increase mutation frequencies at a *CAN1*, *LYS2*, and *URA3*; however the mutagenic signature of AFB1 in yeast has yet to be identified [95, 96]. The mutagenicity of AFB1 in yeast, however, is low compared to many alkylating agents, such as ethyl methane sulfonate (EMS) [95].

While AFB1 is well-known to cause G to T transversion mutation in mammalian cells, other nucleotide substitutions occur, some of which are in the vicinity of the AFB1-DNA adduct. Investigators have used two approaches to determine the DNA sequence context of AFB1-associated mutations; one technique utilizes PCR (QPCR) and ligation-mediated PCR (LMPCR), and the second technique utilizes whole genome sequencing. Using the first technique, Denissenko et al. [97] mapped total AFB1 adducts in genomic DNA treated with AFB_1-8,9-epoxide. In a second experiment, Denissenko et al. [97] mapped total AFB adducts in hepatocytes exposed to either AFB1 activated by rat liver microsomes or AFB1 activated by human liver microsomal preparations. The p53 gene-specific adduct frequencies in DNA, modified in cells with 40–400 μM AFB1, were 0.07–0.74 adducts per kilobase (kb). *In vitro* modification with 1–4 ng AFB_1-8,9-epoxide per microgram DNA produced 0.03–0.58 lesions per kb. The adduct patterns obtained with the epoxide

and the different microsomal systems were virtually identical indicating that AFB1 adducts share similar sequence-specificity whether occurring *in vitro* and *in vivo*.

With the advent of next generation sequencing (NGS) [98], investigators have studied the entire genome and determine whether particular mutation signatures. Huang et al. [99] determine whole genome sequencing data to determine the position of >40,000 mutations in two human cell lines, and in liver tumors from wild type mice and a transgenic mouse carrying the hepatitis B surface antigen. The mutational signature from all four experimental systems was remarkably similar and compared well with experimental mutational signatures derived from sequenced HCCs form Qidong County in China, an area of high AFB1 exposure [100]. The Catalog of Somatic Mutations in Cancer (COSMIC) mutational signature 24 [101], previously associated with AFB1-associated liver cancer, was confirmed and also shown to be present in a high proportion (16%) in HCC from Hong Kong, but in 1% or less from HCC from Japan or North America. The COSMIC mutation signature 24 indicates guanine damage with a very strong transcriptional strand bias for C>A mutations. Additional studies being performed by multiple research groups [25, 102] confirm the presence of signature 24 in human HCC tumors and in tumors induced by AFB1 in mice. In addition to signature 24, investigators have also noted the presence of transition mutations that might also occur in the context of oxidative stress. It has not been determined which of these minor mutation classes drive HCC.

2.7 AFB1 is a potent recombinagen

In budding yeast expressing CYP1A2, AFB1 is potent recombinagen but a poor mutagen [95]. Exposure to AFB1 stimulates homologous recombination between sister chromatids (sister chromatid exchange or SCE), chromosome homologs, and repeated sequences located on non-homologous chromosomes. Using a recombination assay involving truncated fragments of *his3* [103] positioned on non-homologous chromosomes, Sengstag et al. [95] showed that homologous recombination could be stimulated 50-fold in contrast to a less than 10-fold stimulation of mutations. AFB1 concentrations as low as 5 μM were shown to be effective at stimulating the formation of reciprocal translocations, and the karyotypes were confirmed by pulse field gel electrophoresis [103]. AFB1 is also a recombinagen in human and Chinese hamster ovary (CHO) cells and can increase the frequencies of SCE [104–107]. It is unclear whether the same AFB1-associated DNA lesions can stimulate both mutations and recombination. For example, it could be possible that particular lesions that stall DNA replication and generate breaks generate more recombination events while other lesions that can be bypassed by DNA polymerases generate more mutagenic events. These studies thus demonstrate that the genotoxicity of AFB1 extends beyond making mutations and involves stimulating chromosomal rearrangements in model eukaryotic organisms and in humans.

2.8 Repair of AFB1-associated DNA damage

Considering the genotoxicity of AFB1-associated DNA adducts and possible hindrance of DNA replication, it is important to identify which DNA repair pathways and which replication bypass mechanisms are used to tolerate the most persistent AFB1-associated DNA adducts. There are several pathways that are involved in repairing AFB1-associated DNA damage. Among these repair pathways are nucleotide excision repair NER, BER, and recombinational repair; for a general review see [108]. Post-replication repair pathways to bypass DNA adducts involve (1) either error-prone or error-free DNA polymerases, or (2) template switch mechanisms. The later

mechanism involves DNA recombination mechanisms, which are utilized in tolerating UV-induced DNA damage and alkylated DNA bases. While in some organisms there are preferred pathways, a general theme in DNA repair is that organisms have evolved redundant DNA repair mechanisms. A prediction of redundant DNA repair pathways is that eliminating genes in two or more repair pathways should effectively lead to a synergistic decrease in AFB1 resistance, while eliminating genes in the same pathway should confer no greater sensitivity than the most sensitive mutant.

Nucleotide excision repair (NER) involves the recognition of the DNA adduct, the opening of the helix at the DNA damage site, the excision of the DNA adduct and the re-synthesis of DNA using the non-damaged DNA strand as a template. In general, 12–13 nucleotides are excised in prokaryotes (for review see [109]) while 24–32 nucleotides are excised in eukaryotes. Global genome repair (GGR) can occur on either the transcribed or non-transcribed strand. Transcription-coupled repair (TCR) does discriminate and preferentially repairs the transcribed strand. The mechanistic difference between the two pathways is how the DNA adduct is recognized; in GGR specific proteins recognize the DNA helical distortion while in TCR, the RNA polymerase stalled complex is recognized; for general review see [110]. In eukaryotes and prokaryotes, both mechanisms are used. While the mechanism is widely conserved among eukaryotes, the mechanism differs between prokaryotes and eukaryotes in the amount of DNA that is excised.

NER is likely to be the predominant mechanism for the repair of AFB1-associated DNA damage in many eukaryotic and prokaryotic organisms [68]. The AFB1-N^7-guanine adduct is fairly unstable while the AFB1-Fapy DNA adduct can insert between the base pairs of the DNA double helix [111]. UvrABC from *E. coli* can effectively excise both DNA adducts, although the AFB1-Fapy adduct appears to be more chemically stable [68, 112]. The excision of the DNA adducts does not depend on the SOS response; thus, basal levels of the DNA repair enzymes appear to be adequate in repairing the DNA lesions. In *E. coli*, both AFB1-N^7-Gua and AFB1-Fapy adduct appear to be excised at a similar rate. One explanation is that the UvrABC complex does not rely on helix distortion to repair the DNA adduct, but rather size and structure of the aromatic rings [68, 112].

Other insights from model organism yeast revealed that the NER genes are required to excise AFB1-associated DNA adducts [96, 113, 114]. *RAD14* (XPA) and *RAD1-RAD10* (XPF-ERCC1) are required for AFB1 resistance. Failure to repair the DNA adducts in a *rad4* (XPC) haploid mutant results in S phase arrest, supporting the notion that particular AFB1-associated DNA adducts interfere with DNA replication [113, 114]. In addition, in *rad4* mutants the level of AFB1-N^7-Gua DNA adducts was reported to increase three fold [114]. These studies support the notion that the yeast NER pathway recognizes and repairs AFB1-N^7-Gua DNA adducts.

In mammalian cells, the NER pathway preferential repairs AFB1-N^7-Gua DNA adducts but still participates in the repair of AFB1-Fapy DNA adducts [115, 116]. In XPA human fibroblast cells, the loss of AFB1-N^7-Gua DNA is much slower and the accumulation of the AFB1-Fapy DNA adducts is greater compared to wild type cells [116]. $XPA^{-/-}$ deficient mice are also more susceptible to AFB1-associated tumorigenesis compared to wild-type mice [117]. Since the accumulation of DNA adducts correlate with the increased carcinogenicity of the DNA adducts [71, 72, 118], it is likely that the burden of AFB1-associated DNA adducts increases the frequencies of carcinogen-associated mutations in the XPA deficient mice.

The second major pathway to repair DNA involves the BER pathway (for review, see Fortini and Dogliotti [119]). As in NER, the DNA damage base is excised and new DNA is synthesized using the undamaged DNA as template for repair (**Figure 3**). In BER, the modified DNA base is recognized and excised by a specific enzyme that generally referred to as a glycosylase. Subsequently, a apurinic endonuclease (APE1)

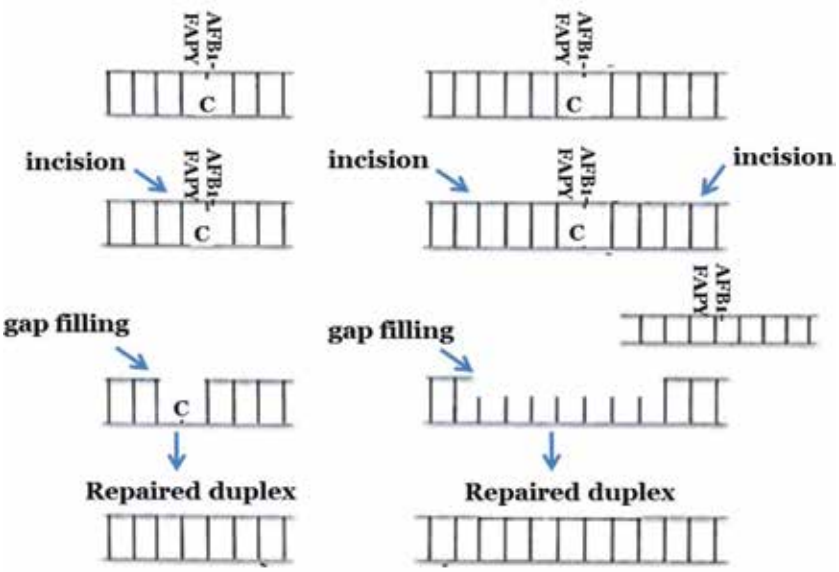

Figure 3.
BER (left) and NER (right) mechanisms to repair the AFB1-Fapy adduct. Both mechanisms involve incision and excision of the damage base, followed by unscheduled DNA synthesis.

generates a 3′OH for primer recognition and new DNA synthesis. In mammalian cells, polymerase β synthesizes new DNA across the gap and removes the deoxyribose residue, and XRCC1/Ligase III cooperate to seal the nick An alternative pathway that does not involve APE1, employs endonuclease VIII like-1 (NEIL1). Following excision of the damaged base by a βδ excision mechanism, the 3′ phosphate is excised by polynucleotide kinase (PNK) to yield a 3′OH for primer recognition and new DNA synthesis. For long patch repair, DNA polymerase δ/PCNA/RFC synthesizes across the gap, the displaced oligonucleotide is excised by FEN1, and the nick is sealed by Ligase I [119]. Poly(ADP-ribose) polymerase PARP1 generally protects the single-strand gap from being subjected to further cleavage or from serving as a substrate for recombinational repair proteins although additional pathways have been proposed [120].

Interestingly, while BER mechanisms have been thought to play a minor role for DNA repair of some AFB1-associated DNA adducts in yeast, BER mechanisms for AFB1-associated DNA adducts can occur in mammalian cells. In budding yeast, the *apn1/apn2* haploid double mutant is no more AFB1 sensitive than the haploid wild type [96]. However, AFB1-associated mutagenesis is lower in the *apn1/apn2* haploid double mutant compared to wild type [96], suggesting that either Apn1 or Apn2 still function in processing the AFB1-associated adducts for post-replication repair. One interpretation of these results is that there is redundancy in both NER and BER mechanisms for conferring AFB1 resistance, while another interpretation is that budding yeast lack the BER enzymes, such as NEIL1, which may actively participate in the repair of AFB1-associated DNA adducts.

In mice, the NEIL1 gene has been isolated and knock-out of the gene leads to higher levels of AFB1-associated DNA adducts and AFB1-associated HCC [121]. The NEIL1 enzyme recognizes and excises AFB1-Fapy-dG adducts in "bubble" DNA structures, such as the one described by Brown et al. [70, 111]. One idea is that AFB1-Fapy-dG adducts may stably intercalate in the helix and be recognized by NEIL1-dependent BER pathway but not by the NER pathway; the repair pathway may thus depend on the DNA sequence context of the AFB1-Fapy-dG adduct. Knock-out of NEIL1 in mice leads to an increase of AFB1-associated tumors and

an accumulation of Fapy-adducts [121]. Vartanian et al. [121] assert that the AFB1-associated carcinogenicity in *Neil1*$^{-/-}$ mice is as high if not higher than that observed in *Xpa*$^{-/-}$ mice, noting that both the size and number of tumors are greater in the *Neil1*$^{-/-}$ mice compared to the *Xpa*$^{-/-}$ mice. However, the investigators indicate that spontaneous tumors arise at a much higher frequency in *Xpa*$^{-/-}$ mice, so that the increase in AFB1-associated tumors were measured until the mice were 11 months in age and not when the mice were 15 months in age.

AFB1 exposure is also associated with oxidative stress, as evident by the accumulation of 8-oxodG lesions. It is particularly interesting whether 8-oxodG accumulates in particular DNA repair mutants and contributes to genotoxicity and the etiology of liver cancer. The contribution of 8-oxodG to overall AFB1-associated genotoxicity is unclear; *Ogg1*$^{-}$*/Ogg1*$^{-}$ transgenic mice do not exhibit more AFB1-associated lung tumors than those that are wild type, but do exhibit increased weight loss and mortality [122]. However, *Ogg1*$^{-/-}$ null mice succumb to other cancers after being exposed to oxidizing agents and carcinogens [123]. These studies suggest that AFB1-associated 8-oxodG lesions are not the causative lesions in liver or lung cancer.

The third major pathway in cells that function in AFB1-associated DNA damage is recombination repair. Knocking out *RAD51* in either *rad14* or *rad4* cells leads to a synergistic increase in AFB1 sensitivity in yeast [96, 113]. There are two different explanations. One explanation is that some AFB1-associated DNA lesions that accumulate in *rad4* cells are converted into single or double-strand breaks and require recombinational repair. A single double-strand break has previously been shown to be lethal in strains defective in homologous recombination [124]. An alternative explanation is that cells require *RAD51* to bypass the DNA lesions and accumulate stalled replication forks. Studies have shown that *RAD51* is required for DNA damage-associated SCE [125], which likely occur by replication bypass mechanisms. This second reason is also supported by the notion that *rad4* cells tend to arrest in a small budded stage upon entry into the cell cycle.

2.9 DNA damage tolerance and AFB1-associated DNA damage

DNA damage tolerance pathways allow cellular replication mechanisms to bypass blocking DNA adducts, such as the AFB1-Fapy DNA adduct, resulting in persistence of the DNA adduct in the divided cells. These mechanisms are divided into error-free mechanisms where the original "correct" base is opposite the modified base and error-prone mechanisms where an "incorrect" base is inserted opposite the damaged base, thereby generating mutations. The insertion of the "incorrect" base is generally accomplished by substituting a "high fidelity" polymerase with a lower fidelity polymerase that also has lower processivity. The polymerase switch mechanism is accomplished by a series of ubiquitination reactions on PCNA, which is the processivity factor for DNA polymerase on the DNA template; for review, see [126, 127]. The first ubiquitination reaction of PCNA is a monoubiquitination reaction catalyzed by Rad18/Rad6. Subsequent polyubiquitination of PCNA by Rad5/Ubc13/Mms2 is required for error-free by-pass mechanisms, which includes template-switch mechanisms. Both *RAD18/RAD6* and *RAD5* genes are well conserved in eukaryotes.

The function of replication bypass in conferring AFB1 resistance has been validated in model organisms. In budding yeast, *RAD18*, *RAD5*, *REV1*, and *REV7/REV3* are required for AFB1 resistance [96]. These genes are also required for AFB1-associated mutagenesis [96]. These results indicate that all three translesion polymerases are required for AFB1 resistance, while it is unclear which gene is required for replication bypass of individual AFB1-associated DNA adduct.

While there are only three translesion DNA polymerases in budding yeast, in humans, there are at least 11 translesion polymerases, forming the majority of the 15 DNA template-dependent DNA polymerases [128]. Both the AFB1-N7-Guanine and

the AFB1-Fapy DNA adducts can be bypassed by translesion polymerases [129, 130]. Of the translesion polymerases, DNA polymerase ζ, also referred to as Rev3L the Rev3 homolog in humans, limits chromosomal damage and promotes cell survival following AFB1 exposure [131]. The authors suggest that Rev3 is required for progression through S phase since mouse embryonic fibroblasts, derived from *Rev3L*$^{-/-}$ knock down mice, arrest in S/G2 after AFB1 exposure [131]. These cells also exhibit an increase in gamma-H2AX foci, micronuclei, and chromosomal aberrations; the kinetics of micronuclei formation support a replication-dependent mechanism that results in the accumulation of unrepaired DSBs in. The Rev3 requirement for DNA replication of an AFB1-associated DNA adduct was also demonstrated for a single lesion present on a replicating plasmid in HEK239 cells [131]. Considering the number of mammalian translesion polymerases [128], the Rev3 requirement for replication bypass may reflect the efficiency by which AFB1-associated DNA adducts block other polymerases or Rev3's ability to minimize detrimental chromosomal damage [132].

2.10 Template-switch mechanisms as an alternative mechanism for tolerating DNA damage

Exposure to AFB1 stimulates SCE in multiple organisms. One possible mechanism is that in post-replication repair, processing of AFB1-associated DNA damage generates apurinic sites and/or subsequent DNA single-strand gaps, which initiate SCE by serving as substrates for DNA recombination proteins. Template switch mechanisms are another mechanism (**Figure 4**) that avoid the necessity of using error-prone polymerase for replication bypass. In support of the role of template switching in AFB1-associated SCE, studies have been performed in budding yeast indicating that *rad51* null mutants, deficient in DNA damage-associated SCE [125], exhibit higher frequencies of AFB1-associated mutations [96, 133]. In addition, Rad51 foci appear as cells enter S phase [114] and not in G2, suggesting that the appearance of Rad51 foci are replication-dependent and not associated with double- or single-strand breaks after replication. However, it is possible that multiple mechanisms are involved.

AFB1-associated SCE are also observed in human and mammalian cells. SCEs have been detected in human lymphocytes, Chinese hamster V79 cells, rat and mouse hepatocyte cell lines [104–107]. It has not yet been determined whether mammalian cells defective in homologous recombination exhibit more AFB1-associated mutations. Nonetheless, it is interesting that polymorphisms of XRCC3 [62–64], which functions in homologous recombination, are a risk factor for HCC.

2.11 Tissue specificity of DNA damage repair of AFB1-associated DNA adducts

Since AFB1-associated DNA adducts are found in different tissues, the question can be asked whether there are tissue-specific differences in repair mechanisms. Mudler et al. [134] addressed the question whether oxidative damage caused by AFB1, 8-oxodG, was repaired more efficiently in the mouse lung compared to the mouse liver. They exposed mice to a low chronic amount of AFB1 (0.2 or 1.0 ppm AFB1) and then assayed for the amount of dGTP incorporation. Interestingly, they found that although Ogg1 was present in both the lung and the liver, there was a lower repair efficiency in the liver after exposure to 1.0 ppm AFB1. The lower efficiency of the repair in the liver did not correspond to AFB1-associated cytotoxic effects, and they speculated that the differences could result from AFB1 directly inhibiting Ogg1 [134].

Bedard et al. [135] asked the question whether AFB1-N^7-Gua and AFB1-Fapy DNA adducts were repaired more efficiently in the mouse liver or lung. They also compared the efficiency of repair in the rat liver and the mouse liver. After exposing mice to 50 mg/kg AFB1, extracts were obtained from the various tissues and used to determine the repair of plasmid DNA AFB1-N^7-guanine or AFB1-Fapy adducts as

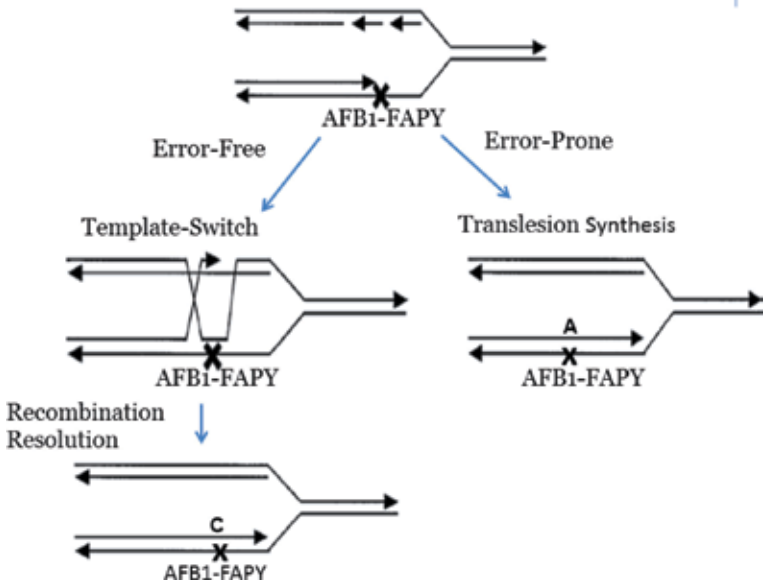

Figure 4.
DNA damage tolerance mechanisms used to bypass a AFB1-Fapy DNA adduct blocking the leading strand polymerase on a growing replication fork. Error-free (left) bypass uses a template switch mechanism while error-prone (right) bypass uses a low fidelity DNA polymerase, resulting in the insertion of an A opposite the DNA adduct.

substrates. Mouse liver extracts repaired AFB1-N^7-guanine and AFB1-Fapy adducts 5- and 30-fold more effectively, respectively, than did extracts from the mouse lung. Mouse liver extracts also repaired the adducts 6-fold and 4-fold more effectively, respectively, than did liver extracts from rats. They conclude that there is a tissue-specific induction in repair in the mouse liver that renders the mouse liver more resistant to AFB1-associated carcinogenesis. However, further studies are needed to determine which NER and BER enzymes are preferentially induced in the liver.

2.12 AFB1-induction of DNA repair and protective mechanisms

The redundancy in repair mechanisms for DNA adducts in yeast and in mammalian organisms provokes the question of which genes are transcriptionally induced after the exposure of AFB1. Two complementary studies have been performed using budding yeast and several studies have been performed in mammalian cells. While studies in yeast utilized microarrays, more recent studies in mammalian cells have used RNAseq and NGS technology. The common genes that are induced have provided clues into which pathways are shared among eukaryotic organisms.

Keller-Seitz et al. [113] determined which budding yeast genes were induced after exposure to AFB1. Essentially, an exponentially grown culture was concentrated to 4 × 10e8 cells/ml and then exposed to 25 μM AFB1 in phosphate buffer (pH 7.5). After RNA was extracted, cDNA was synthesized and labeled for analysis on microarrays. Fourteen DNA repair genes were upregulated more than two-fold, with *RAD51* being upregulated more than seven-fold. Among NER genes, *RAD16*, *RAD3*, and *RAD1* were AFB1-inducible. The upregulation of selected genes was verified by RT-PCR. Additional genes that were induced included those involved in mismatch repair and DNA synthesis, while genes participating in NHEJ were downregulated.

A similar study was done by Guo et al. [136], except AFB1-inducible genes were identified in actively growing cultures. Similar to the Keller-Seitz study [113], *RAD51* was upregulated over seven-fold. However, additional genes involved in

regulating dNTP levels were also upregulated including *DUN1*, which encodes a DNA damage-signaling kinase, and *RNR2* and *RNR4*, which are subunits of ribonucleotide reductase. Although the functional significance of the AFB1-associated inducibility is unknown, there is good overlap with a cluster of genes identified as DNA damage-inducible but not generally stress-inducible [137]; DNA damage-inducible genes from multiple studies include *DUN1*, *RAD51*, *RNR2*, and *RNR4*. In contrast to the previous study by Keller-Seitz, NER genes were not upregulated. In both studies, the DNA damage-inducibility of *RAD51* is *MEC1*-dependent; *MEC1* is the ATM/ATR orthologue of yeast. The functional significance of the upregulation was illustrated by showing that the recombination deficiency exhibited by *mec1* mutants could be partially suppressed by over-expression of *RAD51* [113]. Thus, upregulation of particular DNA repair genes could enhance AFB1 genotoxic effects.

Additional genes that were upregulated in both studies included genes involved in cell cycle control, protein transport, DNA metabolism, and ion homeostasis [113, 136]. Although the functional significance of the upregulation of each of these genes is unknown, many of these genes are involved cell cycle regulation. Interestingly, genes involved in histone biosynthesis were downregulated, reflecting a delay in S phase [136]. The delay in S phase may result from the stability of the AFB1-Fapy DNA adduct during the exposure time.

Identification of AFB1-inducible genes in mammalian cells revealed broader classes of upregulated genes, compared to the yeast studies, reflecting the hepatic cell's ability to metabolize and neutralize xenobiotic agents. Merrick et al. [138] performed RNA seq analysis on liver cells after the rat was injected with AFB1. In brief, RNA was obtained from male rats exposed 1 ppm AFB1 in feed for 90 days, and RNA seq analysis was performed using the appropriate number of unexposed rats as controls. 1026 differentially induced transcripts were identified. Genes upregulated more than five-fold relevant to hepatocellular proliferation include follistatin (442-fold), Aldh3a1 (302-fold), Mybl2 (21-fold), Mybl1 (6-fold), and Sox9 (6-fold). Genes upregulated and involving the E2f1 transcription factor included Cdk1, Mdm2, Ect2, Mad2L1, and Nuf2. Of those genes that were upregulated, of particular interest are those involved in DNA damage tolerance and repair. A two to four-fold increase was observed for Mgmt, Top2a, Rad51, Rad18, Xrcc6, Mnd1, and Tynns [138]. These studies indicate that chronic AFB1 exposure in animals can also induce DNA repair genes that are involved in cell cycle regulation and DNA replication bypass.

2.13 Signal transduction and checkpoint activation

Both studies in yeast and in mammalian cells indicate that AFB1 triggers a checkpoint response that delays cell cycle progression so that DNA damage can be repaired. The mechanism by which the AFB1 DNA adducts are sensed is unknown. However, it is likely that DNA replication stress triggers S phase delay that is associated with Rad53 (Chk2 orthologue) phosphorylation [133]. In budding yeast, exposure to 50 μM AFB1 is sufficient to delay S phase [133, 135]. The Rad53 phosphorylation is dependent on MEC1, the ATM/ATR orthologue. Fasullo et al. [133] observed that the downstream effector of Rad53, DUN1, was required for both AFB1-associated mutation and AFB1-associated recombination. However, the substrates for the signaling cascade that affect AFB1-associated recombination and mutation are unknown. One possibility is that Rad55 phosphorylation is important in triggering AFB1-associated recombination.

In mammalian cells, the DNA damage response to AFB1-associated DNA adducts has been addressed by only a few studies. After exposure to AFB1, HepG2 cells exhibit 53BP1 foci and H2AX foci but not Chk1 or Chk2 activation [139]. However, other studies [140] in other cell lines suggest a robust stimulation of the checkpoint response. In human bronchial epithelial cells (BEAS-2B) expressing CYP2A13 and exposed to

low concentrations of AFB1, AFB1-DNA adducts and 8oxodG significantly increased, along with phosphorylation of ATR and BRCA1. In addition, Mre11, Rad50 and Rad51 were significantly increased. These studies suggest that similar to yeast, checkpoint activation leads to higher expression of DNA recombination genes in3BEAS-2B cells.

3. Conclusions

Liver cancer is the third leading cause of cancer deaths, and unfortunately the incidence of liver cancer is increasing in the USA. Environmental and lifestyle factors include AFB1 exposure and infection with HCV and HBV viruses. AFB1 is a potent liver carcinogen because it is a potent genotoxin and AFB1 exposure is correlated to signature mutations found in HCC. Liver injury and inflammation set the stage for regenerative cell proliferation that enhances AFB1-associated genetic instability. As liver cancer progresses, multiple genetic mutations and epigenetic changes accumulate that eventually accelerate an irreversible path toward malignancy and poor prognosis.

Nonetheless, cellular defense mechanisms have evolved to diminish the AFB1 genotoxicity and repair or tolerate AFB1 DNA adducts so that mutations and chromosomal instability are avoided. First, there are multiple pathways to repair AFB1-associated DNA adducts. These include BER repair involving NEIL1 and NER pathways that excise AFB1-associated DNA adducts. However, it is still unclear which pathway is favored in humans and whether they are redundant. Second, there are common repair and checkpoint pathways that are upregulated in both model organisms and in mammalian organisms; these include ATR signaling pathways and recombinational repair pathways. These pathways may suppress chromosomal instability by error-free mechanisms by which DNA adducts can be bypassed by the DNA replication machinery. One error free mechanism involves recombination-mediated template switch mechanisms. Supporting this idea, RAD51 expression is enhanced in yeast and particular polymorphisms XRCC3, a RAD51 paralogue, may be risk factors for HCC. Nonetheless the DNA repair process can be thwarted by HBV virus, where Hepatitis B virus may directly interfere with NER and perpetuate the replication of cells containing damaged DNA.

The studies presented in this chapter point to future directions in elucidating repair mechanisms of AFB1-associated DNA damage and genetic susceptibility to AFB1-associated cancer. The advent of NGS technology has made it possible to profile the yeast and mammalian genomes for AFB1 resistance which will facilitate identifying the most prominent AFB1 resistant genes. This will facilitate epidemiological studies in determining potential gene polymorphisms that may pose the greatest risk for HCC. NGS technology can facilitate characterizing the DNA sequence contexts where AFB1-associated mutations occur. With the advent of NGS it may be possible to determine the temporal and sequence contexts by with AFB1-associated mutations occur. With the accumulation of genetic information, new biomarkers may be available to aid clinicians and epidemiologists to detect individuals most of risk for HCC and to take appropriate prophylactic actions at earl signs of HCC progression.

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Conflict of interest

The author declares no conflict of interest.

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Section 3

Epigenetics Insight

Chapter 5

Epigenetics: Dissecting Gene Expression Alteration in PDAC

Alia Abukiwan and Martin R. Berger

Abstract

Pancreatic cancer is the fourth leading cause of cancer deaths, with a low 5-year survival rate of about 7% due to its highly invasive nature. Pancreatic ductal adenocarcinoma (PDAC) comprises more than 90% of all pancreatic cancer cases. At the time of detection, around 80% of cases harbor metastases due to the lack of early diagnosis. For decades, scientists have primarily focused on dissecting the origin of pancreatic cancer through genetic alterations and their contribution to diagnosis. Recently, PDAC research has turned into epigenetics to revolutionize our understanding about the silencing of critical regulatory genes. Epigenetic events can be divided mechanistically into various components, including DNA methylation, histone posttranslational modification, nucleosome remodeling, and regulation of transcription or translation by microRNA. The identified epigenetic processes in PDAC contribute to its specific epigenotype and are correlated phenotypic features. Strikingly, some of them have been suggested to have potential as cancer biomarkers, for disease monitoring, prognosis, and risk validation. As epigenetic aberrations are reversible, their correction will become as a promising therapeutic target.

Keywords: PDAC, epigenetics, DNA methylation, histone modification, microRNA, 3' UTR

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) comprises more than 90% of all pancreatic cancer cases. It is highly aggressive, extremely lethal and shows resistance to chemotherapy [1–3]. At diagnosis, around 80% of PDAC cases have already metastasized, thus rendering the current therapeutic options practically ineffective. In line with this, potentially curative surgical resection is limited to a very small portion of patients [4].

On the other hand, cancer metastasis is associated not only with simple gene/protein expression models but also with the existence of epigenetic mechanisms [5], which complicates this process through DNA methylation, histone modifications, and microRNA regulation (see **Figure 1**). Recent studies uncovered the regulatory mechanisms of each process and their key role in EMT and cancer metastasis [6].

2. PDAC from genetics to epigenetics

Historically, the development of PDAC was attributed to DNA mutations, which are classified into three main types: oncogenes (KRAS, BRAF, AKT2, MYB, and AIBI),

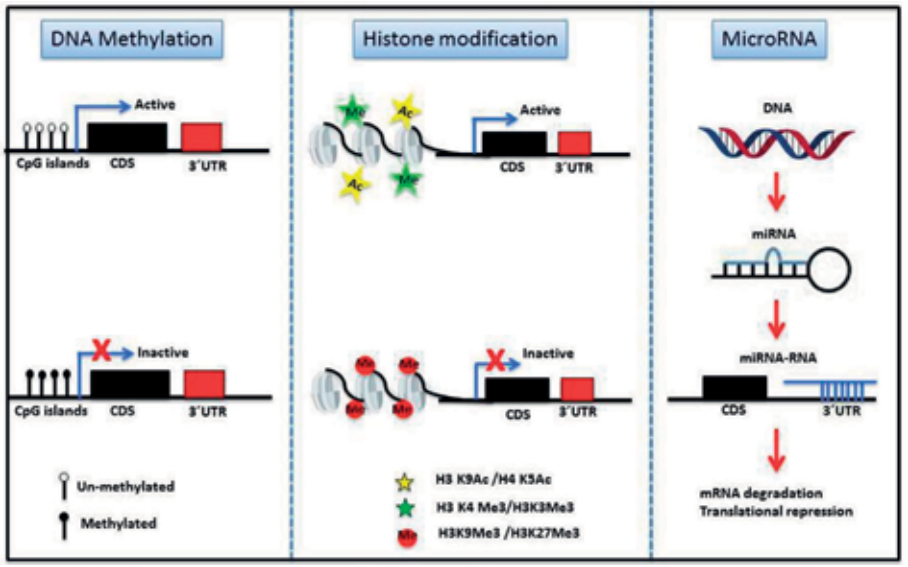

Figure 1.
Schematic diagram of epigenetic mechanisms influencing gene expression. DNA methylation is an epigenetic mechanism through which cytosine residues within CpG regions are covalently modified (left). In addition, the modification of histones has two consequences on genes, which pending on the type of modification and the target residues can either activate or repress the target gene (middle). The epigenetic mechanism is also influenced by microRNAs (miRNAs). These are small noncoding RNAs, which have a proximal length of 22 nucleotides. Functionally, the miRNAs influence gene expression through base pairing with 3′ UTRs of messenger RNAs (right).

tumor suppressor genes (p16, CDKN2A, p53, p21, BRCA2, and SMAD4), and genome maintenance and repair genes (MLH, MSH2, and BRCA2) [1, 2]. Several studies explained the complexity of genetic aberrations and their regulatory signaling pathways [3]. Although a large variety of signal transduction pathways have already been studied in PDAC, much less is known about the cross talk between epigenetic mechanism and signaling pathways typical for PDAC [1]. Strikingly, there are also particular cases where signaling pathways are altered, which directly affect important components of the epigenetic machinery. Therefore, a clear understanding of the epigenetic mechanisms and their implication in PDAC development will open new avenues of therapy. This approach will exploit the intricate process through which cells induce changes at transcription level [4–6].

Epigenetic mechanisms are defined in a way that they can both silence or activate genes without alteration to the DNA sequence itself. Mechanistically, epigenetic changes represent DNA hypermethylation or hypomethylation, histone-based mechanisms that include posttranslational modifications and nucleosome remodeling, as well as aberrant expression of microRNAs [5, 7]. These modifications affect chromatin structure and promoter accessibility, which contribute to genetic alterations [8].

In PDAC, the famous mutant gene KRAS and its downstream signaling cascade are an example for the low therapeutic effect, which is accomplished by current therapies against this gene and its downstream effectors. Interestingly, recent studies demonstrate that dysregulation of epigenetic regulators is essential for PDAC progression as well as for that of many other tumors [9]. Genomic deletions, mutations, and rearrangements frequently target genes encoding components of the chromatin remodeling complex (SWI/SNF), which have been identified in 10–15% of PDAC patients [10].

In general, the discovery of the involvement of the epigenetic effect in cancer added a new concept of gene therapy and informative markers for the diagnosis and prognosis for many malignancies [11]. Whole genomic sequencing studies have revealed driver mutations in epigenetic regulators in various cancer types such as IDH1/2, DNMT3A, KDM6A, DNMT3B, SMARCB1, and CREBBP/EP300 [12]. In PDAC, the sequencing experiments showed more pathogenic mutations in genes encoding multiple components of the SWI/SNF complexes, including ARID1A, ARID1B, PBRM1, SMARCA2, and SMARCA4 [13]. Additionally, recent studies found mutations in important epigenetic regulators like histone methyltransferase enzymes MLL1, MLL2, and MLL3 and histone demethylase KDM6A [13, 14]. Specifically, KDM6A has been found mutated in 5–10% of PDAC patients [15].

Several studies on familial PDAC have shown an association between DNA repair genes' dysfunction and those genes that are responsible for this inheritance (BRCA2, BRCA1, CDKN2A/p16, STK11/LKB1) [13, 16]. Strikingly, the altered DNA repair system is a hallmark of cancer, which causes genome instability and DNA damage [17]. Each cell contains a specific enzymatic system termed the DNA mismatch repair (MMR), which is responsible for detecting and correcting DNA replication errors [18, 19]. Loss or silencing of any protein in this system leads to the accumulation of gene mutations. In this regard, studies reported that MMR inactivation was caused by the epigenetic silencing of the hMLH1 and hMLH2 genes [20, 21]. The hMLH1 protein is one component of a family of seven members of MMR proteins that work coordinately to regulate DNA replication error in humans [20]. In this context, hypermethylation of the hMLH1 promoter has been shown to be an early detection marker of esophageal cancer and also a prognostic marker in colorectal and pancreatic cancers. On the other hand, this mechanism cannot be generalized, as methylation of the hMLH2 gene results less clear into gene inactivation, because the respective promoter is a weak target for CpG island methylation [16, 17, 20–22].

Another example of an affected DNA repair gene is the O^6-methylguanine-DNA transferase (MGMT), which is most intensively regulated by CpG promoter methylation [23]. MGMT is responsible for removing alkyl groups from O^6 in guanine and thus prevents mismatch errors during DNA replication. The silencing of the MGMT activity in human colorectal adenomas has been linked to K-ras GC → AT transition mutations [24, 25]. Interestingly, the epigenetic silencing of MGMT has two main effects in human cancer. First, it reveals a new mutator pathway that causes the accumulation of G-to-A transition mutations. Second, there is a strong and significant positive association between MGMT promoter hypermethylation and enhanced tumor sensitivity to alkylating drugs. These findings highlight the significance of MGMT promoter hypermethylation in translational cancer research [17, 26, 27].

3. DNA methylation

The first epigenetic modification to be identified was DNA methylation [28], which is based on stable and heritable changes in gene structure without a change in DNA sequence [12]. Methylation refers to the addition of a methyl group to the fifth carbon in cytosine forming 5-methylcytosine (5-mC), which is mediated by DNMTs [29]. Generally, methylation occurs in intergenic regions and repetitive sequences such as satellite repeats, and long and short interspersed nuclear elements, while CpG islands of gene promoters often are unmethylated [5, 7, 30]. Interestingly, the global effects of epigenetic alterations in gene regulatory sequences from over 100 cancer cell lines have been identified by the ENCODE project [31]. Normally, DNA

methylation is critical for maintaining pluripotency, X chromosome inactivation, and genome imprinting [12]. Aberrant DNA methylation is one of the hallmarks of cancer [32].

Methylation of DNA is catalyzed by the enzymes DNMT3A and DNMT3B and is then maintained by the major DNA methyltransferase DNMT1, which is also assisted by DNMT3A and DNMT3B [9, 12]. A recent study found that DNMT1, DNMT3A, and DNMT3B are themselves differentially methylated in PDAC [33]. Besides, a very recent finding suggests that the interactions between TP53 and H3K4, MLL3 and MOZ genes play a major role in chromatin regulation [34]. The methylation of tumor suppressor genes is the best-characterized epigenetic event in several malignancies, including PDAC [11]. In fact, several genes such as APC, BRCA1, P16INK4a, P15INK4b, RARβ, and p73 are frequently methylated [10]. Recent studies have revealed that apparent DNA methylation occurred in critical signaling pathways in PDAC such as TGFβ, WNT, integrin, cell adhesion, and axon guidance signaling pathways [35]. Likewise, TGFβ induces epithelial-mesenchymal transition (EMT) by enhancing hypermethylation of CpG islands in the VAV1 gene promoter [36] (see **Figure 2**). Furthermore, the WNT signaling pathway is a target of hypermethylation in PDAC. This has been found for WNT ligands WNT5A, WNT7A, and WNT9A, or the cell surface receptor FZD9, or the cytoplasmic transducer APC2, the nuclear factors SOX1, SOX7, SOX14, and SOX17, and the pathway inhibitors FRZB, SFRP1, SFRP2, KREMEN2, NKD2, and WIF1. Strikingly, the tumor suppressor candidate HIC1 is hypermethylated, which is acting as a transcriptional repressor for abnormal survival circuits of the transcription factors involved in the WNT signaling pathway [33, 37].

Furthermore, several studies demonstrated that promoter DNA hypermethylation is associated with the transcriptional repression of multiple microRNAs (miRNAs). This results into upregulation of oncogenic target genes of the microRNAs, such as observed for the downregulation of miR-181b, which promotes the

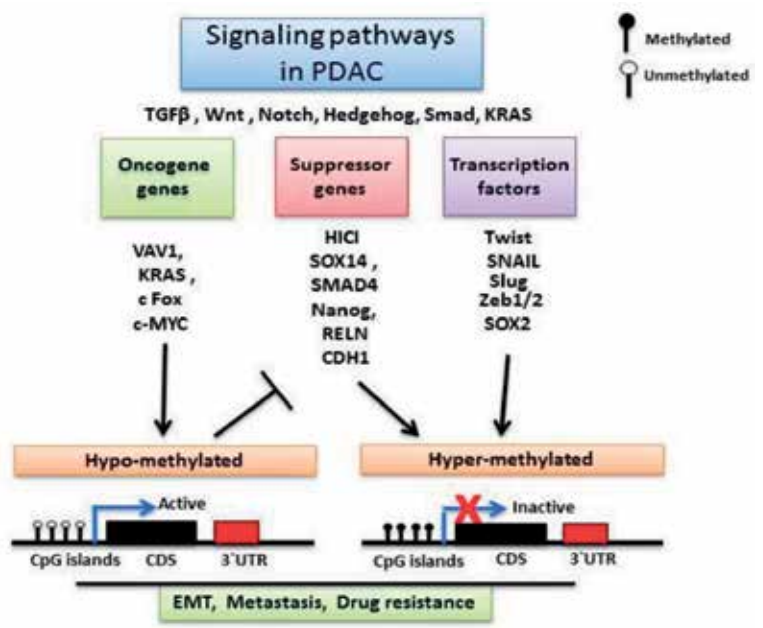

Figure 2.
Schematic diagram of signaling pathways in PDAC, which are deregulated by DNA methylation. Critical tumor suppressors and transcription factors are silenced, whereas oncogenes are activated. This deregulation promotes EMT and metastasis.

Gene	Gene name	Epigenetic alteration	Function	References
CADM1	Cell adhesion molecule 1	Hypermethylation	Cell-cell interaction	[41]
CDH1	Epithelial cadherin	Hypermethylation	Cell adhesion and invasion	[42]
DKK3	Dickkopf-related protein 3	Hypermethylation	Tumor suppressor	[43]
S100A4	S100 calcium-binding protein A4	Hypermethylation	Invasion, motility, and tubulin polymerization	[44]
P16	Cyclin-dependent kinase inhibitor 2A	Hypermethylation	Multiple tumor suppressor	[45]
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	Hypermethylation	Enzyme	[33]
BMP3	Bone morphogenetic protein 3	Hypermethylation	Growth factor	[33]
ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	Hypermethylation	Generation of the cell surface carbohydrate determinants and differentiation antigens	[42]
ST8SIA5	ST8 alpha-N-acetyl-neuraminide α-2,8-sialyltransferase 5	Hypermethylation	A member of glycosyltransferase family	[42]
ST8SIA2	ST8 α-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	Hypermethylation	A member of glycosyltransferase family	[42]
ST8SIA3	ST8 α-N-acetyl-neuraminideα-2,8-sialyltransferase 3	Hypermethylation	A member of glycosyltransferase family	[42]
AKT1	v-Akt murine thymoma viral oncogene homolog 1	Hypermethylation	Kinases	[30]
LCN2	Lipocalin 2	Hypomethylation	Epithelial differentiation	[33]
CCND2	Cyclin D2	Hypermethylation	Cell cycle control	[33]
CLDN4	Claudin-4	Hypomethylation	Cell adhesion	[44]
miR-9-1	MicroRNA-9	Hypomethylation	miRNA translation control	[40]
P59	Cyclin-dependent kinase inhibitor 1C	Hypermethylation	Cyclin-dependent kinase inhibitor	[34]
P16	Cyclin-dependent kinase inhibitor 2A	Hypermethylation	Cyclin-dependent kinase inhibitor	[33]
RARB	Retinoic acid receptor	Hypermethylation	Cell growth control	[33]
SFN	Stratifin (14-3-3sigma)	Hypomethylation	P53-induced G2/M cell cycle arrest	[9]
LCN2	Tissue factor pathway inhibitor	Hypomethylation	Epithelial differentiation	[9]

Gene	Gene name	Epigenetic alteration	Function	References
CDKN1C/ P57	Cyclin-dependent kinase inhibitor 1C	Hypomethylation	Cyclin-dependent kinase inhibitor	[42]
FOXE1	Forkhead box E1	Hypomethylation	Thyroid transcription factor	[42]

Table 1.
A series of methylated genes in PDAC.

expression of BCL 2 [38]. Moreover, downregulation of the miR-29 family was associated with the overexpression of the DNA methyltransferases DNMT3A and DNM3B [39]. The noncoding RNAs and antisense RNA sequences are strongly involved in the respective DNA hypermethylation process, which silences important genes such as polycomb group (PcGs), which in turn may expose these regions to DNA methylation changes [40]. More examples are listed in **Table 1**, all of which are related to PDAC.

4. Histone modification

Nucleosomes are considered to be the basic constituents of chromatin. Each nucleosome is an octamer of histones, which consist of two copies each of histone proteins H2A, H2B, H3, and H4 [46]. The most interesting epigenetic events in PDAC are histone modifications, since several studies revealed that the most frequently mutated epigenetic genes occurred in the histone family [13]. The posttranslational modifications include methylation, acetylation, citrullination, phosphorylation, SUMOylation, and ADP ribosylation. However, the most studied histone modifications in cancer are lysine alterations, including lysine methylation, acetylation, and phosphorylation [47–49]. In normal cell development, histone modifications regulate critical cell processes such as DNA replication and transcription or repair [46], while in cancer, histone modifications contribute to the maintenance of malignant phenotypes. In PDAC, the most common modification includes methylation and acetylation of lysine residues within the N terminal tails of histone proteins [11].

In the context of epithelial-mesenchymal transition (EMT) in PDAC, SNAIL is a critical transcription repressor of E-cadherin in EMT process. It plays a significant role in embryonic development and tumorigenesis [50]. Moreover, SNAIL has an essential function in histone modifications. This includes the activation of a set of chromatin modifiers such as lysine-specific demethylase, euchromatic histone lysine methyltransferase 2 (G 9a), suppressor of variegation 3–9 homolog 1 histone methyltransferases (Suv39H1), SIN3 transcription regulator family member A (SIN3A), and histone deacetylases (HDAC1 and HDAC2) [51, 52].

4.1 Histone methylation

Methylation of histones is coordinated by histone methyltransferases (HMTs) and histone demethylases (HDMs). There are at least 17 different HMTs, all of which share the conserved (Su (var) 3–9, enhancer-of-zeste, trithorax) motif. The lysine methylation residue is most common and is mediated by histone lysine methyltransferases (HKMTs) [53]. Particularly, methylation at H3K9, H3K27, and H3K20 is associated with transcriptional repression, while methylation of H3K4, H3K36, and H3K79 causes transcriptional activation [47]. The silencing of tumor suppressor genes in cancer is caused by the corresponding activities of the HMT and HDMT

enzymes. On the other hand, the H3K27me3-specific HMT EZH2 (enhancer of zeste homolog 2), the catalytic subunit of PRC2, is overexpressed in a broad range of solid tumors, including prostate, lung, breast, colon, skin, and pancreatic cancers [54, 55].

The most frequently altered histone methylated genes in PDAC are KDM6A and MLL2 [33]. KDM6A is an H3K27me3 demethylase, which has a role in endoderm differentiation by regulating the expression of WNT signaling and HOX genes [56]. Other studies found that the loss of trimethylation at K27 of histone H3, which causes nuclear accumulation of EZH2, is strongly correlated with a poor PDAC outcome [57]. Various interactions have been shown to occur between DNA methylation and histone methylation. For example, the interaction between EZH2 and DNMTs renders the EZH2 gene a potential therapeutic target. Mucins (MUCs) are also known to play essential roles in tumor growth and invasion in pancreatic neoplasms. MUC1 and MUC4 are high-molecular-weight transmembrane mucins. Overexpression of mucins in cancer is associated with poor prognosis. It has been shown that mucin expression changes in PDAC are due to DNA methylation of H3 at the lysine9 residue [58, 59].

4.2 Histone acetylation

Histone acetylation is the first discovered histone modification. The acetylation of lysine residues neutralizes their positive charge, which induces chromatin relaxation and activates a set of genes associated with transcription. On the other hand, removal of the acetyl groups is associated with gene silencing. Histone acetylases (HATs) and deacetylases (HDAC) are the required enzymes for this process [60, 61] (see **Figure 3a**).

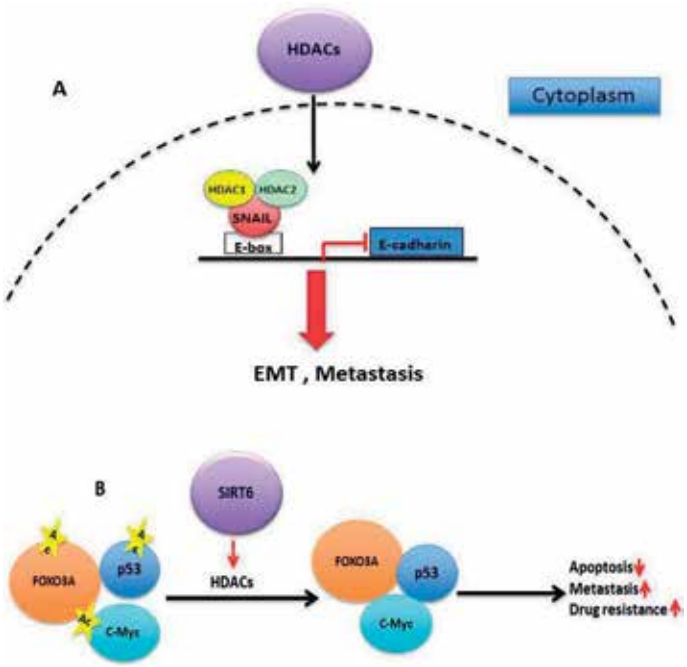

Figure 3.
Schematic diagram on the role of HDACs in PDAC. (A) HDACs mediate E-cadherin translational repression by activating the binding of EMT transcription factors to the E-boxes present in the E-cadherin promoter. (B) SIRT6 mediates the deacetylation of p53, FOXO3A, and C-Myc, which leads to increased metastasis and drug resistance.

Recent studies show a series of significant alterations of the acetylation process in PDAC, as well as mutations in the histone acetylase EP300 [33]. Furthermore, the SIRT6 gene is associated with the deacetylation of histone H3 at lysine residues 9 and 56, thus increasing the expression of the SIRT6 gene associated with PDAC metastasis by deacetylation of p53 and FOXOA3 [62] (see **Figure 3b**). For instance, the activation of KRAS and increased expression of the c-Myc transcription factor promote PDAC metastasis [13]. Also, expression of HDAC7 and HDAC2 has been found increased in PDAC [63]. In addition, HDACs/HATs play important roles in the activation of several tumor suppressor genes in PDAC, such as p53 and EP300 [11, 14, 53, 64].

A recent study identified the acetylation of glutamate oxaloacetate transaminases 2 (GOT2) at three lysine residues (K159, K185, and K404) in PDAC. This promotes the transfer of NADH from the cytoplasm into mitochondria, enhancing PDAC cell proliferation and tumor growth in vivo. On the other hand, the acetylation of GOT2 at only K159 is correlated with downregulation of SIRT3 expression [65].

4.3 Histone phosphorylation

Histone phosphorylation has been associated with different cell processes, including apoptosis, cell cycle, DNA transcription, DNA repair, chromosome condensation, gene regulation, cell signaling pathways, energy, and metabolic pathways [66]. Phosphorylation of histones occurs on serine, threonine, and tyrosine residues, a process mediated by different kinases and phosphatases [46]. In cell development, the most important site for histone phosphorylation is the serine 10 of histone H3 (H3S10P), which is mediated by the Aurora-B kinase. This modification is a critical event in cell mitosis and meiosis [67].

Several studies identified histone phosphorylation changes during DNA damage, such as the phosphorylation of serine 139 on the histone H2A(X). On the other hand, phosphorylation of serines, e.g., 10 and 28 on H3, and serine 32 on H2B have been contributed by the activation of the epidermal growth factor (EGF). Moreover, H3ser28p mediated the expression of c-fos and α-globin [68–70].

It has been shown that H2A T120 is phosphorylated in PDAC by VRK1 on the promoter region of CCND1, which consequently activates the transcription of cyclin D1 [71]. Besides, KRAS is most well-studied and known activated oncogene in PDAC [72]. Other studies have implicated the activation of the Ras-MAPK pathway with the upregulation of phospho-ERK1/2 and their downstream levels of H3 S10ph [73].

5. MicroRNA

MicroRNAs (miRNAs) are small (20–23 nucleotides), endogenous, noncoding, single-stranded RNA molecules, which control the expression of around 60% of the protein-coding genes [74]. Moreover, they can control both physiological and pathological processes, such as development and cancer [75]. In addition, the miRNA machinery is of great importance for drug development, since a functional miRNA machinery is a compulsory prerequisite for any RNA interference (RNAi)-based therapy approach. A total of 700 miRNAs have been discovered in human diseases, and more than 1000 predicted miRNA genes are yet to be experimentally validated [76].

Mature microRNAs require several steps of preprocessing before they can become functional. After they are transcribed by RNA polymerase II/III from intragenic regions or from regions that code for introns, the primary transcript (pri-miRNA) is processed by the ribonuclease Drosha and DGCR8 in the nucleus. The process

produces pre-microRNAs, hairpin-shaped intermediates of 70–100 nucleotides. Exportin-5, a Ran-GTP-dependent dsRNA-binding protein, transports pre-microRNAs into the cytoplasm where they are further processed by the ribonuclease Dicer and TRBP (Tar RNA-binding protein) into a double-stranded miRNA. The strands separate and a mature single-stranded molecule join an RNA-induced silencing complex (RISC). The double miRNA strands are required to interact with RISC complex or to be degraded. Ordinarily, one miRNA strand can give rise to two individual mature miRNA sequences with different targets due to complementary seed sequence [74, 77, 78]. The single-stranded mature microRNA remains stable on the miRISC and induces posttranscriptional silencing of one or more target genes, usually through imperfect pairing with a target sequence in the 3' UTR [74]. However, this is not the only binding region for miRNAs, as there are also binding sites located in 5' UTR or even within the coding DNA sequence of mRNAs [77]. The seed sequence or seed region is a conserved heptametrical sequence, which is mostly situated at positions 2–7 from the miRNA 5' end [79, 80]. Furthermore, degradation of mature miRNAs appears to depend on their activity; in the absence of complementary targets, the miRNA could be released from miRNA-RISC complex, and then its 5′ end becomes accessible to the 5′ → 3′ exonuclease XRN2, which degrades the miRNA [81].

Cancer represents a heterogeneous group of diseases characterized by uncontrolled growth of cells, high proliferation rates, and apoptosis resistance. All of these features result from a complex of structural and expression abnormalities of genes, including those encoding microRNAs [75, 82]. The classification of cancer is more accurately defined with microRNA profiling than with mRNA profiling because of the strong correlation between microRNA expression signatures and tumor origin [75]. In general, microRNAs have two main functions in cancer; they can act as tumor suppressors (TSmiRs) or oncogenes (OncomiRs) [75, 76, 82, 83].

One of the first indications that miRNAs serve as tumor suppressors (TSmiRs) came from Calin and colleagues when they discovered that miR-15a and miR-16-1 were deleted or downregulated in about 68% of chronic lymphocytic leukemia (CLL) samples. MiR-15a and miR-16-1 have been shown to control the expression of VEGF, a key proangiogenic factor involved in tumor angiogenesis. Furthermore, both of them induce the apoptosis of leukemic cells by affecting the antiapoptotic protein BCL2 [75, 84]. Another prominent TSmiR is the let-7 family, located at a chromosomal region, which is usually deleted in human cancers. It has been reported as a TSmiR in lung, breast [84], urothelial, and cervical cancers [85]. Recent studies found that let-7 was able to regulate the RAS oncogene in lung cancer. In addition, let-7 regulates late embryonic development by suppressing a number of genes such as c-Myc, RAS, and HAMGA2 [76, 82]. Taken together, reduced expression of TSmiRs in cancer releases oncogenic genes and promotes tumor initiation and progression.

In contrast, oncogenic miRNAs (OncomiRs) promote tumorigenesis by inhibiting tumor suppressor genes that play roles within other functions, such as cell differentiation and apoptosis. The first OncomiR that was discovered is the miR-17-92 cluster, which encodes miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR19b-1, and miR-92-1. This cluster is located on chromosome 13 and is commonly found to be amplified in human B-cell lymphomas, lung cancer, and anaplastic thyroid cancer cells [86]. Another oncogenic miRNA, miR-21, has been validated in nine solid tumor types (lung, breast, head and neck, prostate, colon, pancreas, esophagus, stomach, and brain). Experimental data confirmed that miR-21 plays a significant role in cancer cell proliferation, apoptosis, and invasion. Accordingly, inhibition of miR-21 induces cell cycle arrest, increased apoptosis, and increased chemosensitivity to anticancer agents [87].

The important emerging role of miRNAs in many cancer types, together with the fact that they can function as TSmiRs or OncomiRs, supports the potential of

miRNAs as a new class of targets in the development of cancer therapies. Several studies have focused on targeting miRNAs as an experimental therapy in vitro or in vivo [85]. Notably, to modulate cancer-associated miRNAs in vivo, two main approaches were established: first, miRNA replacement therapy, which is based on adding the miRNAs missing in cancer cells for restoring their normal functions; second, inhibition of oncogenic miRNAs by using single-stranded chemically modified anti-miR oligonucleotides [85, 88]. The first successful in vivo experiment using anti-miRs in conjunction with locked nucleic acids was successfully applied in African green monkeys with hypercholesterolemia. The experiments resulted in the successful control of triglyceride and cholesterol levels, together with the management of disease manifestations with minimal side effects to herald a new research approach that is equally applicable in cancer [89].

PDAC shares many features with other solid tumors. Numerous studies have reported the significant roles, which miRNAs play in PDAC progression. Furthermore, these studies have also provided important information about cellular features, such as growth, invasive, and metastatic behavior that have been modified or altered in PDAC as a result of miRNAs, thus highlighting, to a large extent, the significance of miRNAs in PDAC progression [90]. High-throughput microarray technologies have been used to extensively profile miRNA signatures in cell lines, normal frozen tissues, formalin-fixed paraffin-embedded tissues (FFPE), blood, and fine needle aspiration biopsy (FNAB) samples, in order to establish a common expression pattern in PDAC [91]. Recently, a meta-analysis reviewed 11 miRNA profiling studies in PDAC and reported 439 miRNAs as deregulated in the 538 PDAC samples that were evaluated [92]. This analysis defines a common pool of

miRNAs	Expression status	Target genes	References
Let-7 family	Downregulated	KRAS, MAPK, c-Myc, STAT3	[94, 104]
miR-181s	Upregulated	TIMP3, TCL1, TGFBI, TRIM2, SIRT1, Bcl2	[91, 105]
miR-26s	Downregulated	MMP2, MMP14, cyclin D1, Mcl-1, Bcl2	[91, 106, 107]
miR-125a	Upregulated	Bcl-w, Bcl2	[108]
miR-192	Downregulated	SERPINE1	[109]
miR-148a,b	Downregulated	DNMT3B, Mtif, CCKBR, BCL2	[90, 91, 110]
miR-200 family	Downregulated	VEGF-A, KRAS, KDR, VEGFR2, ZEB1/2	[100, 111–113]
miR-34a	Downregulated	Notch1/2, Bcl2, SIRIT, CDK4, VEGF	[99, 114–117]
miR-375	Downregulated	PDK1	[90]
miR-124	Downregulated	ITGB1, Rac1, RocK2, EZH1, Bcl2, CDK6	[91, 101, 118]
miR-217	Downregulated	KRAS, SIRIT, c-MYC	[119, 120]
miR-21	Upregulated	PTEN	[121]
miR-132	Upregulated	Rb1, SMAD2	[122, 123]
miR-208	Upregulated	E-cadherin	[106]
miR-196-a	Upregulated	NFKBIA	[124]
miR-100	Upregulated	IGFR1	[90]
miR-155	Upregulated	TP53INP1	[125]
miR-10b	Upregulated	TIP30	[91]

Table 2.
Top frequently deregulated miRNAs in PDAC.

miRNAs that are atypically expressed in PDAC, and the potential renormalization of these miRNAs and/or expression patterns could help create a therapeutic approach in managing this aggressive disease [93].

The commonly deregulated miRNAs are associated with major regulatory genes in several signaling pathways (**Table 2**), which are involved in most aspects of cellular physiology including regulation of cell cycle, differentiation, proliferation, and apoptosis. Notably, altered miRNA expression in PDAC contributes to metastasis and drug resistance [92, 94, 95]. The more frequently deregulated miRNAs in PDAC include miR-21, the expression of which is regulated by KRAS, and correlates with the degree of tumor progression [90]. KRAS is an important molecule in PDAC and is a direct target of miR-96, miR-217, miR-126, and miR-200c. The overexpression of these miRNAs reduces the level of KRAS expression, resulting in decreased cell invasion, migration, and tumor growth [96, 97]. Strikingly, two of these miRNAs, miR-145 and miR-200c, function as a regulatory network in the AKT-PI3K signaling pathway [98]. Conversely, it has been reported that KRAS activation suppresses the expression of the miR-134/145 cluster via the Ras responsive element-binding protein (RREB1) [99]. The miR-200 family is also frequently deregulated in PDAC and plays a significant role in EMT inhibition. One study demonstrated that miR-200 negatively regulates ZEB1 and ZEB2, which are both direct repressors of E-cadherin [100]. In the context of epigenetic modifications, several studies have found TSmiRs in PDAC, including miR-9-1, miR-124s, miR-192, miR-615-5p, and miR-1247, which were hypermethylated [44, 101–103].

6. Conclusions

For the high mortality, poor prognosis, and undefined therapeutic targets in PDAC, the unraveling of the complex molecular layers driving this lethal cancer is a prerequisite for more effective therapeutic strategies and consistent diagnostic markers. The recent research on epigenetic mechanisms has significantly enriched our knowledge about the regulatory characteristics involved in the initiation, progression, and metastasis of PDAC. This book chapter has focused on the most critical epigenetics mechanisms, including DNA methylation, histone modifications, and modulated expression of miRNAs that play a significant role in PDAC tumorigenesis, and could serve as future therapeutic targets. Currently, significant emphasis is still given on detecting somatic genetic alterations in PDAC. However, it seems also promising to investigate the underlying epigenetic mechanisms for completing the full puzzle of altered gene expression in PDAC. The epigenetics field has developed strongly and will continue to advance into a frontier field for PDAC research. Additionally, it is essential to highlight the features of epigenetic mechanisms of gene regulation—their reversibility. This feature provides a ground for specifically targeting the epigenetic changes contributing to PDAC.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 6

The Role of DNA Repair and the Epigenetic Markers Left after Repair in Neurologic Functions, Including Memory and Learning

Carol Bernstein and Harris Bernstein

Abstract

In eukaryotic cell nuclei, DNA is wrapped around and firmly associated with histone proteins, forming chromatin. When DNA is damaged, the chromatin structure needs to be loosened to allow repair enzymes to gain access to the damage. This requires modifying the histone proteins. These modifications, called epigenetic alterations, do not alter the base-pair sequence. Repair-associated epigenetic alterations are usually transient, removed when no longer needed for repair. However, some remain after repair. In the human brain, long-lasting novel epigenetic alterations appear to account for the persistence of addictions to such substances as alcohol, nicotine and cocaine. Certain neurodegenerative diseases are caused by inherited mutations in genes necessary for DNA repair. Deficient DNA repair in these diseases is associated with extensive epigenetic alterations that likely have a role in the disease phenotype. Persistent epigenetic alterations due to DNA repair processes, both histone modifications and methylations of DNA, can also have positive consequences. Stimulation of brain activity (e.g. learning and memory formation) is often accompanied by the generation of DNA damage in neuronal DNA, followed by repair associated with persistent epigenetic alterations. In particular, recent research has shown the need for non-homologous end joining and base excision repair in memory formation.

Keywords: DNA repair, epigenetic, histone acetylation, histone methylation, CpG island methylation, addiction, neurodegenerative disease, memory, learning, cognition

1. Introduction

Even in the earliest stages of evolution, damage to the genome was presumably a fundamental problem for life. Thus it is likely that organisms developed processes for repairing genome damages very early. Such repair processes are ordinarily restorative, designed to reestablish the original undamaged genome sequence. During the course of the evolution of lineages leading to mammals, DNA repair processes became more complex, and acquired additional capabilities. One such example is the employment of the DNA double-strand break repair process of non-homologous end joining in the generation of immunological diversity [1].

In chromatin, epigenetic alterations are an integral part of DNA repair processes [2]. Although most epigenetic alterations introduced during DNA repair are transient with restoration of the epigenetic pattern that existed prior to repair, some are long lasting. Epigenetic alterations can enhance or inhibit gene expression without changing the DNA base pair sequence. Examples of epigenetic alterations are hyper- or hypomethylation of cytosines in the DNA sequence, increased or decreased histone H3 and H4 acetylation by histone acetyltransferases or histone deacetylases, and increased or decreased histone methylation by histone methyltransferases or histone demethylases.

In humans, the oxygen demands of the brain are high, constituting about 20% of total body oxygen consumption, while the mass of the brain is only about 2% of body mass [3]. This results in elevated release of reactive oxygen species in the brain that, in turn, cause oxidative DNA damages. Because damages are prevalent, processes that repair DNA damages have a vital role in maintaining the health of brain neurons, and these DNA repair processes can cause epigenetic alterations.

When DNA repair processes are impaired or insufficient, the result can be improper (non-adaptive) epigenetic alterations. Such improper epigenetic alterations in neurons are likely an important underlying cause of certain addictions and neurodegenerative diseases. Several addictive agents cause increased DNA damage in neurons resulting in increased dependence on DNA repair. Addictions are associated with characteristic persistent patterns of epigenetic alterations in the brain. In several neurodegenerative diseases the neurological impairments are caused by inherited mutations in genes that encode proteins employed in DNA repair. These diseases are associated with particular patterns of epigenetic alterations in neurons. It is likely that the neurological impairments suffered by individuals during addiction or neurodegenerative disease are caused, at least in part, by epigenetic alterations resulting from insufficient or faulty DNA repair. That is, insufficient or faulty DNA repair may produce epigenetic alterations that have long-lasting negative consequences at the level of gene expression that manifest as neurological impairment.

Zovkic et al. [4] noted that learning and memory can be broadly defined as lasting alterations of a behavioral output produced in response to a transient environmental input. In order for a brief stimulus to cause a persistent change in behavior, neurons need to undergo some kind of molecular alterations that stabilize a memory into an enduring set of cellular marks. As reviewed by Bird in 2002 [5], in mammals, DNA methylation is adapted for specific cellular memory in development, even over successive cell divisions. This observation of cellular memory indicated that epigenetic mechanisms could provide a molecular basis for neuronal memory formation and maintenance in non-replicating neurons [4]. In addition to DNA methylation/demethylation, it is now known that other mechanisms such as chromatin histone acetylation and histone methylation can also cause persistent epigenetic changes [6].

In the sections below, we review evidence for the following ideas. Neuronal activity causes DNA damages, and repair processes are required to deal with these damages. Such repair processes involve epigenetic alterations, some of which are long lasting. Individuals, addicted to abuse of certain substances that cause DNA damage, have long-lasting epigenetic alterations in brain neurons that appear to be related to the dependency. Also, inherited inability to adequately repair DNA damages can cause epigenetic alterations in neurons associated with neurodegenerative disease. However, long-lasting epigenetic alterations can also be adaptively beneficial. Cognitive functions such as memory and learning in response to external stimuli appear to depend, at least in part, on persistent epigenetic alterations arising during DNA repair processes.

2. Epigenetic alterations required for DNA repair

DNA is condensed in the nucleus of the cell in a highly organized and compact manner, referred to as chromatin (reviewed by Walker and Nestler [7] and Ding et al. [8]). In chromatin, the DNA is packaged with histone proteins to form nucleosomes. DNA repair proteins are recruited and interact with DNA in response to DNA damage. However, the architecture of nucleosomes and the organization of chromatin can present barriers to DNA damage recognition and repair. Epigenetic modifiers play an important role in regulating nucleosome and chromatin structure to facilitate DNA repair. Epigenetic alterations relax certain regions of chromatin to allow access to DNA repair enzymes and also condense certain regions to repress transcription in order to facilitate repair. When repair is complete, epigenetic modifications are largely returned to the state before damage occurred. These roles of epigenetic modifiers in DNA repair have been described as the "access-repair-restore" model [9].

2.1 Histone acetylation

The basic unit of chromatin, the nucleosome, is composed of 147 DNA base pairs wrapped around a histone octamer consisting of two copies of each of the following proteins: H2A, H2B, H3, and H4. The histones also have histone tail extensions, constituting up to 30% by mass of the histones (**Figure 1**). Each histone protein can undergo post translational modifications in which molecules, such as an acetyl group or one (or up to three) methyl group(s), are covalently added to (or removed from)

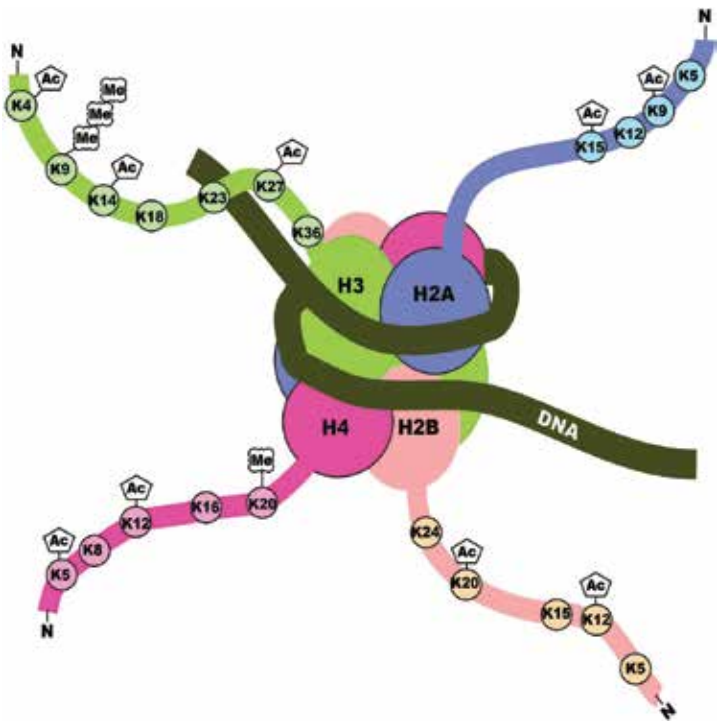

Figure 1.
A nucleosome showing 4 pairs of histones (H2A, H2B, H3, and H4), each pair with the same color. The amino-terminal (N-terminal) tails of one of each pair of histones is shown, labeled with the positions of lysine amino acids (labeled "K" in the single letter code for lysine) that are subject to acetylation or methylation. The number at each lysine indicates its position counting from the amino end of the protein chain. Acetylations (indicated by Ac) and methylations (indicated by Me) are shown in some positions susceptible to these alterations. The DNA, wound around the histone core, is indicated by the dark line.

lysine residues of their amino terminal (N-terminal) tail. The single letter K designates lysine. If an acetyl group is added to a lysine located as the 4th amino acid from the N-terminal tail end of histone 3, this is designated H3K4Ac. These modifications not only alter the structure of the nucleosome but also change the interaction of DNA with the associated histones, thus allowing entry of DNA repair enzymes into chromatin and permitting histones to be moved, if needed, to allow for repair [2]. The epigenetic modifications, if they remain after DNA repair, also can increase or decrease the likelihood of transcription of a given gene near the site of the repaired DNA damage [10].

Acetylation of histone lysines promotes chromatin relaxation to facilitate DNA repair [2]. It is also generally associated with a permissive transcriptional state. By negating the positive charge associated with the lysine residues on histone tails, acetylation promotes an "open" chromatin state.

2.2 Histone methylation

Histone lysine methylation is associated with either activation or repression of gene expression depending on which residues are methylated and whether one, two or three methyl groups are added at that position [11]. For instance enrichment of H3K4Me1 or H3K4Me3 at specific gene regions is correlated positively with increased transcription levels, whereas enrichment of H3K9Me2, H3K9Me3 or H3K27Me3 is negatively correlated with transcription. In response to DNA double-strand breaks, histone methyl transferases are recruited to sites of damage where they catalyze trimethylation of H3K9 and H3K27, thereby repressing transcription in order to facilitate DNA repair [12].

2.3 DNA methylation

An important epigenetic regulator in addition to histone modification is DNA methylation. DNA methylation often occurs with the addition of a methyl group to the DNA sequence cytosine-phosphate-guanine (CpG) at the C5 position (5mC). DNA methylation at gene promoters is generally associated with repression, while methylation within genes has been associated with active transcription [7]. DNA methyl transferase 1 (DNMT1) binds to sites of oxidative damage formed in GC-rich regions of the genome and promotes formation and recruitment of a large epigenetic silencing complex. Localization of these epigenetic modifiers to sites of oxidative damage in promoter CpG islands results in increased DNA methylation.

2.4 Noncoding RNA

Non-coding RNAs provide an additional type of epigenetic regulation. As one example, microRNAs are short sequences of RNA (about 22 bases) that exert a repressive role on gene expression by binding a target sequence on specific mRNAs and blocking translation or inducing degradation. The typical microRNA has about 400 specific target mRNAs. In one report, specific microRNAs collaborated with histone deacetylases and cooperatively regulated several relevant target genes [13].

2.5 Epigenetic alterations allow DNA repair

Figure 2 illustrates some of the actions of epigenetic alterations. Histone acetyltransferases add acetyl groups to histone tails to open chromatin structure to make DNA damages accessible to repair enzymes. If the acetyl groups remain after DNA repair, this allows genes in the area of DNA repair to be switched on. Histone deacetylases remove acetyl groups from histone tails to complete DNA repair and return chromatin to its condensed state existing before DNA repair. Improper actions of histone deacetylases can inappropriately switch genes off.

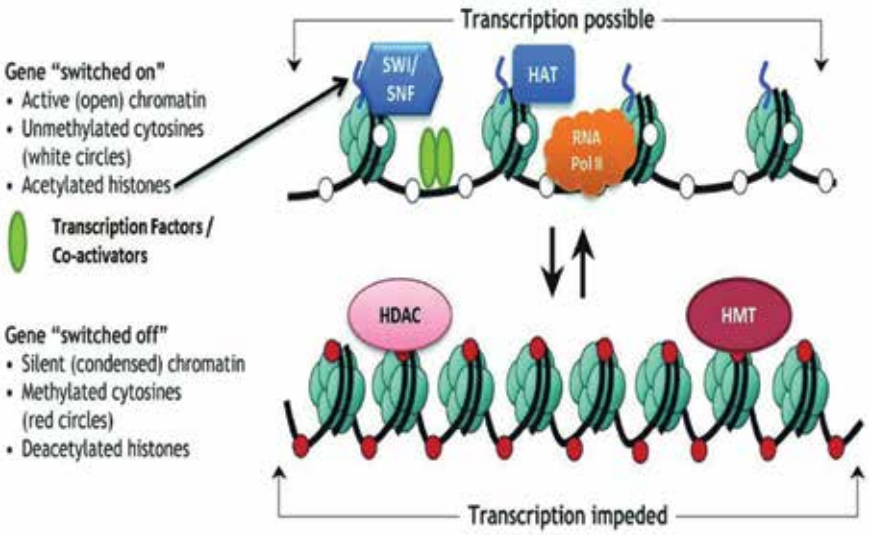

Figure 2.
A gene located in chromatin, with its DNA wrapped around histones. Open chromatin (top image) has been relaxed by acetylation of histone tails. Condensed chromatin (lower image) has been tightened by removal of acetyl groups and addition of methylation of histone tails. Symbols include histone acetyltransferase (HAT), histone deacetylase (HDAC), and histone methyltransferase (HMT). SWI/SNF (not an epigenetic alteration) is a nucleosome-remodeling complex that stimulates gene expression if the epigenetic factors (methylation on cytosines in DNA; acetylations and methylations of lysines in histone tails) allow transcription [14].

In this figure, histone methyltransferase (HMT) is shown as switching a gene off. However, some histone methylations serve to activate genes [11].

DNA gene promoters without methylated cytosines are indicated (in **Figure 2**) as allowing transcription (upper image), and DNA gene promoters with methylated cytosines (in CpG sites) are indicated as impeding transcription. The green vertical ovals represent transcription factors. They are not epigenetic factors, but also regulate gene transcription.

3. Epigenetic remnants (scars) after DNA repair

Dabin et al. [15], in an extensive review, noted that after repair of various types of DNA damages there are a number of types of epigenetic alterations that could potentially remain as scars. These epigenetic alterations include (1) changes in DNA methylation, (2) incorporation into nucleosomes of new histones with a pattern of acetylations or methylations that differ from that in the histones originally present before DNA damage, (3) incorporation into nucleosomes of histone variants such as histone H2AZ, (4) altered acetylation or methylation of histone tails, and even (5) altered histone density at repair sites. Several illustrative reports showing such scars after DNA repair are described below.

3.1 Homologous recombinational repair (HRR) of double-strand breaks leaves epigenetic alterations

Homologous recombinational repair (HRR) modified the DNA methylation pattern of a repaired DNA double-strand break in a green fluorescent protein (GFP) gene inserted into the HeLa cell genome [16]. In different subclones isolated after HRR repair events, the repair created either more highly methylated or less highly methylated cytosines in the GFP gene DNA. HRR also altered local histone H3 methylation,

forming H3K9Me2 or H3K9Me3 at repair locations. However, H3K9Me2/3 was selectively retained after HRR only in recombined genes with increased DNA methylation.

During a 2-week period after repair, some transcription-associated demethylation of the repaired DNA was promoted by base excision repair enzymes [16]. Subsequently, the repaired genes displayed stable but diverse methylation profiles. These profiles governed the levels of expression in each clone. These epigenetic alterations (scars) were stable over time and were recovered with the same frequency after 3 years of continuous culture.

3.2 Double-strand break repair by non-homologous end joining leaves epigenetic alterations

DNA repair by non-homologous end joining induces alterations in DNA cytosine methylation and these alterations are a source of permanent epigenetic changes [17]. In a HeLa cell line containing a green fluorescent protein (GFP) based reporter gene, a double-strand break in the gene followed by non-homologous end joining repair created two populations of cells, those with increased DNA methylation in the GFP gene (identified by a dim green color) and those with decreased DNA methylation in the GFP gene (identified by a bright green color). The degree of methylation for each population changed somewhat over the subsequent 4 days, but then remained stable for 24 days. Even though the HeLa cells were undergoing replication, the epigenetic changes produced stable high expressing or low expressing clones.

3.3 Base excision repair is associated with epigenetic alterations

The major forms of oxidative DNA damage are non-bulky lesions such as 8-oxo-2′-deoxyguanosine and thymine glycol that are repaired predominantly by base excision repair. After oxidative DNA damage was increased in HCT116 cells in culture, histone alterations were found in genes with CpG island-containing gene promoters and these histone alterations caused decreased transcription [18]. The histone alterations introduced by increased oxidative damage included reduction in H3K4Me3 and H4K16Ac and an increase in H3K27Me3. DNA methylation was also increased, but primarily in promoters of genes which normally have low basal expression [18].

3.4 Histone replacements during DNA repair

For many types of DNA damage, histones must be removed and replaced during the repair of the damaged DNA [19]. Disruption of nucleosomes in human cells after introduction of double-strand breaks or UV damage occurs with a drop in histone H2B levels and a selective loss of histones H2A and H2B, but not of H3 or H4 at the site of the damage [19]. After DNA repair, new histones (in addition to some pre-existing histones) are deposited at the site of repair. The new histones lack the histone post-translational modifications that existed before the repair. The presence of the differently modified new histones can specifically mark the domain as a site of repair, and remain as a scar [19]. The failure to recycle all of the pre-existing histone marks results in alterations in gene expression [15].

4. Addiction

One of the principal features of addiction is its persistence. The persistent behavioral changes appear to be due to long-lasting changes, resulting from epigenetic alterations affecting gene expression, within particular regions of the brain [20].

4.1 Alcohol

Alcohol can be addictive. About 7% of the US population are alcoholics, with alcohol use disorder [21]. Many negative physiologic consequences of alcoholism are reversible during abstinence. Long-term chronic alcoholics suffer a variety of cognitive deficiencies [22]. Multiyear abstinence resolves many neurocognitive deficits. One exception is lingering deficits in spatial processing [23]. In addition, some frequent long-term consequences are not reversible during abstinence. Alcohol craving (compulsive need to consume alcohol) is usually present long-term among alcoholics [24]. Among 461 individuals who sought help for alcohol problems, follow-up was provided for up to 16 years [25]. By 16 years, 54% of those who tried to remain abstinent without professional help had relapsed, and 39% of those who tried to remain abstinent with help (such as Alcoholics Anonymous) had relapsed.

Long-term, stable consequences of chronic alcohol abuse are thought to be due to stable changes of gene expression resulting from epigenetic alterations within particular regions of the brain [26–28]. For example, in rats exposed to alcohol for up to 5 days, there was an increase in histone 3 lysine 9 acetylation in the pronociceptin promoter in the brain amygdala complex. This acetylation is an activating mark for pronociceptin. The nociceptin/nociceptin opioid receptor system is involved in the reinforcing or conditioning effects of alcohol [29].

4.2 Cigarette smoking

Cigarette smokers (about 21% of the US population in 2013) [30] are usually addicted to nicotine [31]. This is a strong addiction. The proportion of smokers who reported having seriously tried to quit and who managed to quit for 6 months or more was less than 10% [32].

After 7 days of nicotine treatment of mice, the post-translational modifications consisting of acetylation of both histone H3 and histone H4 was increased at the *FosB* promoter in the nucleus accumbens of the brain, causing a 61% increase in FosB expression [33]. This also increases expression of the splice variant *Delta FosB*. In the nucleus accumbens of the brain, Delta FosB functions as a "sustained molecular switch" and "master control protein" in the development of an addiction [34, 35]. Similarly, after 15 days of nicotine treatment of rats, the post-translational modification consisting of threefold increased acetylation of histone H4 occurs at the promoter of the dopamine D1 receptor gene in the prefrontal cortex of the rats. This caused increased dopamine release in the prefrontal cortex reward-related brain region, and such increased dopamine release is recognized as an important factor for addiction [36].

4.3 Cocaine

Cocaine addiction occurs in about 0.5% of the US population. In humans treated for cocaine addiction, the relapse rate after 5 years was 25% [37]. Repeated cocaine administration in mice induces post-translational modifications including hyperacetylation of histone 3 (H3) or histone 4 (H4) at 1696 genes in one brain reward region, the nucleus accumbens, and deacetylation at 206 genes [7, 38]. At least 45 genes, shown in previous studies to be upregulated in the brain nucleus accumbens of mice after chronic cocaine exposure, were found to be associated with post-translational hyperacetylation of histone H3 or histone H4. Many of these individual genes are directly related to aspects of addiction associated with cocaine exposure [38].

4.4 Addictive substances can cause DNA damage

In rodent models, many addictive substances cause DNA damage in the brain. For example, alcohol, through its metabolic product acetaldehyde, induces double-strand breaks in DNA in the mouse brain [39].

Nicotine from cigarette smoke also very likely causes DNA damage in the brain. Nicotine reaches the brain 10–20 seconds after a puff of smoke. The level of nicotine in the brain is 75–80% as high as in the blood or the liver [40]. E-cigarette smoke is composed primarily of nicotine vapors. Nicotine from E-cigarettes, applied to mice (with the dose and duration equivalent in human terms to light E-cigarette smoking for 10 years), caused DNA damages including mutagenic O^6-methyl-deoxyguanosines and γ-hydroxy-1,N_2-propano-deoxyguanosines in the lung, bladder, and heart [41]. These same damages are likely to occur as well in neurons upon exposure to nicotine.

Cocaine [42] and methamphetamine [43, 44] each also cause DNA damage in the brain.

After repair at the sites of DNA damages caused by drugs of addiction, the epigenome may not return entirely to their pre-damage states. Some of the methylations of DNA and/or the acetylations or methylations of histones at the sites of DNA repair may remain and thus become epigenetic scars on chromatin [15]. Such epigenetic scars likely contribute to the persistent epigenetic alterations found in addiction.

5. Neurodegenerative diseases with deficient DNA repair

DNA repair processes in mammalian cells normally involve extensive chromatin remodeling. This remodeling involves epigenetic modifications of chromatin that are usually transient, but may persist. When a protein necessary for proper DNA repair is mutationally defective, epigenetic alterations that deviate from a normal functional pattern can be introduced. In a number of neurodegenerative diseases, such epigenetic alterations appear to significantly underlie the disease phenotype.

We describe below four neurodegenerative diseases, ataxia telangiectasia, Huntington's disease, Aicardi-Goutières syndrome and Cockayne syndrome that have inherited genetic deficiencies due to mutations in genes necessary for DNA repair. We briefly summarize for each disease, the notable neurodegenerative features of the disease, the DNA repair processes that are defective, and the accompanying epigenetic alterations that likely have a role in the etiology of the disease. On the basis of the evidence reviewed, it appears that the proper functioning of the nervous system depends on DNA repair processes that not only restore damaged DNA sequence information, but also promote normal gene expression through the maintenance of an appropriate pattern of epigenetic markers.

5.1 Ataxia telangiectasia (AT)

AT is a multisystem disease characterized by neurodegeneration in the central nervous system. Certain regions of the brain including the cerebellum, are adversely affected in AT resulting in difficulty with movement and coordination. There is also an association with microcephaly. AT is inherited as an autosomal recessive trait, and is caused by mutation of the gene *AT mutated* (*ATM*) that encodes a serine/threonine protein kinase. The wild-type ATM protein has a key role in the DNA damage response. ATM is part of a molecular complex that signals the presence of oxidative DNA damage, including double-strand breaks, and facilitates subsequent repair [45].

ATM protein is employed in chromatin remodeling and in epigenetic alterations that are required for repairing DNA double-strand breaks [45]. ATM mutation causes defects in epigenetic regulation that likely contribute to the rapid postnatal degeneration of the cerebellum that underlies the progressive ataxia observed in AT [45]. AT is associated with histone acetylation alterations, including significant decreases in histone H3 and H4 acetylation [46]. ATM regulates neuron specific epigenetic alterations involving histone deacetylase-4 [45]. In ATM mutant neurons, misallocation of histone deacetylase-4 represses transcription of genes important in neuronal function and synaptic maintenance [45].

5.2 Huntington's disease (HD)

HD typically occurs in midlife. The symptoms include progressive movement disorder, cognitive dysfunction and psychiatric impairment. HD is inherited in an autosomal dominant manner. HD results from an unstable expansion of CAG repeat sequences in exon 1 of the *huntingtin* gene (*HTT*). Several lines of evidence link the HTT protein to repair of DNA damage [47]. HTT is a scaffolding protein that directly participates in oxidative DNA damage repair [48]. The ATM protein recruits HTT to sites of DNA damage. HTT co-localizes with, and acts as a scaffold for, proteins of the DNA damage response pathway in response to oxidative stress. The fibroblasts of HD patients with expanded CAG repeats have deficient oxidative damage repair [48].

Impaired DNA repair in HD also appears to cause deleterious epigenetic alterations that are linked to transcriptional dysregulation. Individuals with HD experience accelerated epigenetic aging of the brain, particularly in the frontal lobe, cingulate gyrus and the parietal lobe. This process is associated with substantial changes in brain DNA methylation levels [49]. Also post-translational modifications of histone proteins are significantly altered in HD patients as well as in HD cellular and animal models [50].

5.3 Aicardi-Goutières syndrome (AGS)

AGS is characterized by early onset, often in early infancy. Features of AGS include neurological dysfunction, psychomotor retardation, seizures, and microcephaly [51]. AGS is an inherited disease and most cases are inherited in an autosomal recessive pattern. AGS arises from mutations in genes encoding proteins TREX 1 (AGS1), RNase H2 (AGS2, 3 and 4) and SAMHD1 (AGS5) [51]. The incorporation of ribonucleotide triphosphates (rNTPs) into DNA is perhaps the most common type of endogenous DNA damage encountered in proliferating cells [52]. Removal of rNTPs incorporated into DNA is referred to as rNTP excision repair. Key players in rNTP excision repair are TREX1 and RNase H2 [52]. RNase H2 is the predominant nuclear enzyme to hydrolyze the RNA strand of RNA/DNA hybrids [53].

TREX1, *RNASEH2* and *SAMHD1* mutations in AGS cells cause common molecular abnormalities including increased levels of RNA:DNA hybrid species and genome-wide DNA hypomethylation, a substantial epigenetic perturbance [51]. AGS2 and AGS4 mutant cells display about a 20% reduction in genomic methylation levels overall, and this reduction is spread along the length of entire chromosomes impacting nearly all compartments including genic, intergenic unique and repeat regions [51].

5.4. Cockayne syndrome (CS)

Due to impaired neurological development, individuals with CS are characteristically mentally retarded and have microcephaly. CS is caused by mutations in the *CSA* and *CSB* genes. CS is inherited as an autosomal recessive trait. Transcription of DNA can be inhibited by DNA damage, and restoration of transcription requires removal

of blocking damages by a sub-pathway of nucleotide excision repair that specifically removes transcription-blocking DNA damages. This sub-pathway is referred to as transcription-coupled DNA repair (TCR). In mammals, TCR depends on the CSA and CSB proteins. More than 70% of CS syndrome patients have a mutation in the *CSB* gene. CSA and CSB proteins regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase at sites of DNA damage [54, 55].

Among the proteins recruited by CSA and CSB are nucleotide excision repair proteins and histone acetyltransferase, an enzyme that catalyzes chromatin remodeling and epigenetic alteration [54]. CSB can slide histones along DNA and histone chaperone proteins that accept and donate histones can greatly facilitate this process [55]. Nucleosome remodeling by CSB is important for TCR, and inability to efficiently mobilize nucleosomes appears to contribute to the underlying mechanism of CS [55]. The chromatin remodeling activity of CSB appears to create an epigenetic landscape that permits more efficient DNA repair or facilitates transcription resumption after repair is completed [56].

5.5 Perspective on the role of DNA repair in neurodegeneration

The neurodegenerative diseases AT, HD, AGS and CS are due to mutation in genes that encode proteins employed in DNA repair. Inadequate DNA repair can lead directly to cell death and neuron depletion that may be reflected in microcephaly, as is seen in AT, AGS and CS. The defects in DNA repair also cause disruptions in the pattern of epigenetic alteration required for normal neuronal function. These epigenetic alterations likely underlie characteristic features of the disease phenotype. Thus it appears that important functions of the nervous system, including those involved in various aspects of cognition and motor function, depend on the role of intact DNA repair processes in maintaining normal patterns of epigenetic markers.

5.6 Other neurodegenerative diseases deficient in DNA repair

In addition to the four neurodegenerative diseases discussed above, there is also evidence for defective DNA repair in the neurodegenerative diseases amyotrophic lateral sclerosis [57], fragile X syndrome [58], Friedrich's ataxia [59], spinocerebellar ataxia type 1 [60], trichothiodystrophy [61], and xeroderma pigmentosum [62].

Amyotrophic lateral sclerosis is causally linked to mutations in the gene *FUS* [57]. ALS patients with *FUS* mutations have increased neuronal DNA damage. FUS protein functions in the DNA damage response including recruitment to double-strand breaks and homologous recombinational DNA repair. FUS protein also directly interacts with histone deacetylase 1 in response to DNA damage, and this interaction is necessary for efficient DNA repair [57].

Fragile X syndrome is a common form of inherited mental retardation. The fragile X mental retardation protein FMRP is a chromatin-binding protein that functions in the DNA damage response, likely in DNA repair [58]. Fragile X syndrome is caused by loss of expression of the *FMR1* gene, most often due to an expansion of a CGG repeat in the first exon of *FMR1*. The repeat expansion results in abnormal methylation of the promoter region which leads to transcriptional silencing of the *FMR1* gene [63].

Friedreich ataxia, a progressive neurodegenerative disease, is caused by deficient frataxin protein resulting from downregulation of the *FXN* gene. Frataxin is employed in the repair of DNA double-strand breaks [59]. Most individuals with Friedrich ataxia have a homozygous mutation consisting of a GAA trinucleotide repeat expansion within the first intron of the FXN gene. This expansion itself may lead to downregulation of the *FXN* gene. In addition, there is a repressive

heterochromatin effect around the *FXN* gene caused by the expanded GAA repeats, consisting of high levels histone methylation of H3K9 and H3K27 [64].

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease characterized by progressive motor incoordination. SCA1 results from mutation in the *ATXN1* gene that leads to a pathogenic glutamine-repeat expansion in the protein ataxin-1 (ATXN1). The multifunctional protein TERA/VCP/p97 acts in DNA damage repair. Glutamine-repeat expansion mutant proteins such as mutant ATXN1 impair accumulation and function of TERA/VCP/p67 leading to an increase in unrepaired DNA double-strand breaks [60]. Also mutant *ATXN1* represses gene transcription by decreasing histone acetylation [65].

Trichothiodystrophy (TTD) is an autosomal recessive disorder with a range of clinical neurodevelopmental features and often photosensitivity. All photosensitive TTD individuals have a mutation in the *XPB*, *XPD* or *TTDA* genes that encode subunits of the dual functional repair/transcription factor IIH (TFIIH) [61]. These individuals deficient for TFIIH are defective in nucleotide excision repair, a process that repairs transcription-blocking DNA damages, including UV induced DNA damages, thus explaining their photosensitivity. Induction of DNA damage in cells with *XPB* or *XPD* mutations that cause TTD results in reduced transient DNA strand breaks that are intermediates during DNA repair [66]. Also methylation of histone H3 (H3K9Me3) was reduced in an evaluated model promoter region [66].

Xeroderma pigmentosum (XP) is an autosomal recessive genetic disorder. XP has characteristic neurological manifestations, but the most prominent feature of the condition is sensitivity to sunlight resulting in a high predisposition to UV-induced skin cancer. Seven different complementation groups (genes) *XPA*, *XPB*, *XPC*, *XPD*, *XPE*, *XPF* and *XPG* encode proteins employed in nucleotide excision repair, a process that repairs bulky DNA damages including damages caused by UV-light [62]. XPF and XPG proteins are endonucleases that also trigger chromatin looping and DNA demethylation that promote accurate expression of activated genes [67].

6. Mental activity is associated with DNA damage and repair in the brain

An easy type of DNA damage to measure is the double-strand break. When a double-strand break occurs there is a rapid effect on particular histones near the break. A variant histone, H2AX, is sometimes present in histone cores, and it constitutes about 2–25% of the H2A histones in mammalian chromatin [68]. After a double-strand break, H2AX histones near the break are phosphorylated by the kinases ATM, ATR and DNA-PK [69], allowing formation of H2AX phosphorylated on serine 139 near the break. This histone is then designated γH2AX. γH2AX can be detected as soon as 20 seconds after irradiation of cells (with DNA double-strand break formation), and half maximum accumulation of γH2AX occurs in 1 minute [68]. Chromatin with phosphorylated γH2AX extends to about a million base pairs on each side of a DNA double-strand break [68]. It is easy to detect γH2AX by immunohistochemistry, and these large segments of chromatin with γH2AX are called γH2AX foci.

Learning and new memories occur when mice explore a new, strange environment. This is a low level stimulation. Exploration of a novel environment increased the number of neurons with double-strand breaks in neuronal DNA as measured by γH2AX foci [70]. This occurs in different brain regions but particularly in the dentate gyrus, which is involved in spatial learning and memory. Within 24 hours of break formation, DNA repair occurs with removal of the breaks [70]. When double-strand breaks in this situation were also measured by the comet assay (another simple assay), roughly 30–40% of dentate gyrus nuclei had comet tails indicating double-strand breaks in the nuclear DNA [70].

6.1 Visual stimulation

Another neuronal activity also caused double-strand breaks. Exposure of anesthetized mice to visual stimuli activated the primary visual cortex (V1) of the brain. One eye was exposed to visual stimuli for 15 minutes, while the other was shielded from light. One hour after the visual stimulation began, the number of cells with γH2AX foci in the stimulated contralateral V1 was roughly twice as high as that in the unstimulated ipsilateral V1 [70].

6.2 Optogenetic stimulation

Optogenetic stimulation of a mouse striatum brain region also caused DNA double-strand breaks [70]. Transgenic mice expressing Cre-recombinase in medium spiny neurons of the dorsomedial striatum were used. The Cre-recombinase gene inserted into DNA of the striatum neurons in these mice provides a topoisomerase I like mechanism to carry out site-specific recombination events. Using this system, a viral vector was infused into the striatum, carrying a genetic segment coding for a light sensitive ChR2 protein. The ChR2 gene frequently recombined into the mouse dorsomedial striatum DNA. A glass fiber was then implanted close to the viral injection site. Two weeks later, awake mice were stimulated by light through the glass fiber. This caused neuronal activity in the dorsomedial striatum, resulting in behavioral ipsiversive rotations in mice (mice turning in a circle). The mice were then terminated and the mouse brains examined. The illuminated striata contained many more cells with γH2AX foci than the non-illuminated contralateral striata [70].

6.3 Non-homologous end joining (NHEJ) repair required for long-term memory retention

One form of long-term memory, through associative learning, is contextual fear conditioning [71]. This fear conditioning occurs, for instance, when a rodent is placed in a novel environment (a new context) and is then subjected to an electric shock (e.g. a footshock). This produces robust fear learning, shown by a strong fear response, when the rodent is placed in that context again. Contextual fear conditioning occurs very rapidly (can occur with a single event) and has a lasting effect.

Madabhushi et al. [72] subjected wild-type C57BL/6 mice to a training paradigm for contextual fear conditioning, following which they prepared hippocampal lysates and measured γH2AX levels (as a measure of double-strand breaks in DNA). Elevated γH2AX levels were detectable in hippocampal lysates within 15 minutes after exposure to the fear-conditioning paradigm.

NHEJ, which repairs double-strand breaks in DNA, appears to be needed specifically for consolidation of memory into long-term memory. Contextual fear conditioning in mice increased NHEJ repair activity in the hippocampus brain region measured at 10 and 60 minutes after training [73]. The hippocampus is important in forming memories [74].

When NHEJ repair was active, memories were demonstrated in fear-conditioned mice at 6 and 24 hours after training. Ara-C (cytosine arabinoside) interferes with DNA synthesis. Injecting animals systemically with ara-C 1 hour before exposing them to the conditioning inhibited NHEJ repair [68]. If NHEJ repair was blocked before fear conditioning, memories of fear conditioning were substantially diminished at the 6- and 24-hour time periods tested. Thus it appears that NHEJ repair is required for memory formation. Other cognitive elements were not blocked by ara-C. Mice given ara-C and then subjected to contextual fear conditioning maintained their short-term memory (tested at 30 seconds after training) and exploratory

behavior in an open field 24 hours after training. Treatment with ara-C also did not cause general malaise, motor in-coordination, sedation, or anxiety.

7. Long-term memory depends on epigenetic alterations

7.1 Contextual fear memory conditioning causes changes in DNA methylation in brain neurons

Halder et al. [75], in a mouse study, evaluated differently expressed genes and short differentially methylated regions in neurons of the anterior cingulate cortex, a brain region important for associative memory acquisition and maintenance of long-term memory. In the anterior cingulate cortex at 1 hour after contextual fear conditioning, there were 6250 differentially methylated genes with 46,395 differently methylated short regions (700 base pair regions). (Frequently, multiple short differentially methylated regions occurred in a differentially methylated gene.) At 4 weeks after training 1223 differentially methylated genes and 5018 differently methylated short regions persisted. In addition, at 4 weeks after training they found 1700 differentially expressed genes in the anterior cingulate cortex. These findings suggest that long-term memory (4 weeks) is associated with differential methylation of DNA and altered expression of genes.

Halder et al. [75] also evaluated differentially methylated regions and differently expressed genes in the hippocampal CA1 region, a region that is crucial for short-term memory formation during contextual fear conditioning. They found that, in contrast to the anterior cingulate cortex, in the hippocampus there were 1619 differentially methylated regions after 1 hour, but these changes did not persist, and almost none were present after 4 weeks.

Also studying the hippocampus, Duke et al. [76], working with rats, found that at 24 hours after contextual fear conditioning there were more than 5000 differentially methylated regions (500 base pair short regions), but less than 20 differentially methylated regions after context change alone. Hypermethylated differentially methylated regions overlapping differentially expressed genes were associated with decreased gene expression, consistent with the concept that cytosine methylation is often a mechanism for suppressing transcription. Also at 24 hours after contextual fear conditioning, there were more than 2000 differentially methylated regions that were associated with 1048 genes having down-regulated expression and 564 genes having up-regulated expression (usually known to be associated with hypomethylated regions). At 24 hours after training, 9.17% of the genes in the rat genome of hippocampus neurons were differentially methylated. Gene Ontology term analysis was performed, and differentially expressed gene enrichment analysis revealed that many of the genes involved in synaptic functions after fear conditioning were up-regulated.

7.2 The role of base excision repair in memory consolidation

In both the studies of Halder et al. [75] and Duke [76], above, there were on the order of a thousand demethylations of cytosines in neuron genomes during memory consolidation in the brain after contextual fear conditioning. The two likely processes of demethylating cytosine each depend on base excision repair, as shown in **Figure 3**. These processes were reviewed by Bayraktar and Kreutz [77]. There is considerable evidence for the left hand process illustrated in **Figure 3**. In this process there are two or more fast oxidations by one of the ten-eleven translocation methylcytosine dioxygenases (TET1, TET2, TET3), first altering 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and then producing 5-formylcytosine (5fC) followed by

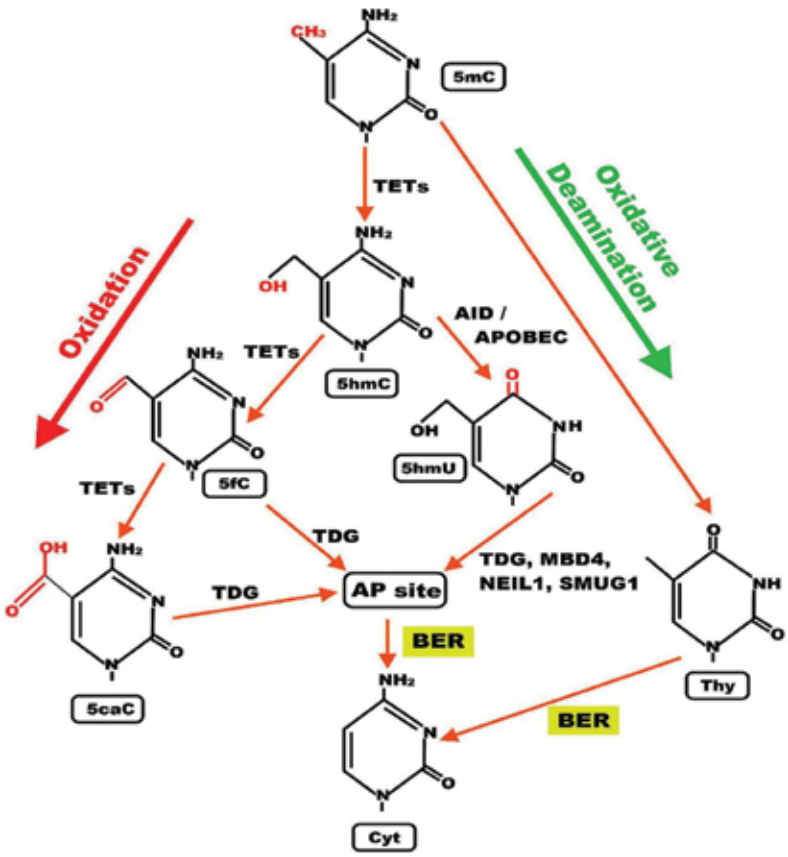

Figure 3.
Demethylation of 5-methylcytosine (5mC) to cytosine (Cyt) in DNA depends on base excision repair (BER) as the final step. In initial steps, the ten-eleven translocation methylcytosine dioxygenase family of enzymes (TET1, TET2, and TET3) each may catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and further steps form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC can be excised from DNA by thymine DNA glycosylase (TDG) to form an apyrimidinic site (AP site). AID/APOBEC is a family of cytidine deaminases that can oxidatively deaminate 5mC to 5-hydroxymethyl uracil (5hmU) or 5mC to thymine (Thy). 5hmU can be excised from DNA by TDG, methyl-CpG-binding domain protein 4 (MBD4), endonuclease VIII-like 1 (NEIL1) or single-strand selective monofunctional uracil DNA glycosylase (SMUG1).

5-carboxylcytosine (5caC). Both 5fC and 5caC can be excised by thymine DNA glycosylase (TDG), generating an apyrimidinic (AP) site, which is repaired by base excision repair to place cytosine (cyt) in the DNA opposite guanine. However there is some indication that a cytidine deaminase (AID/APOBEC) enzyme can carry out oxidative deamination of 5mC to 5-hydroxymethyluracil, which is then excised by one of the four enzymes shown, to form an AP site. Alternatively, a cytidine deaminase (AID/APOBEC) may carry out an oxidative deamination of 5mC by to thymine, and the mispair of thymine with guanine is then repaired by base excision repair to generate cytosine paired with guanine in DNA.

Zhang et al. [78] generated homozygous mutant mice deficient in TET1 catalytic activity. These mice were viable and fertile, with no discernible morphological or growth abnormality. The Tet1 deficient mice would be expected to have reduced ability to convert 5mC to cytosine by the TET/base excision repair-dependent pathway. When examined in neural progenitor cells, 478 genes showed elevated promoter DNA methylation levels compared to the wild-type control, while only 32 genes had lower DNA methylation. There was a link between the altered DNA methylation pattern and transcriptional activity. In the neural progenitor cells of TET1 mutant

mice 1267 genes were down-regulated with respect to transcription and 498 were up-regulated compared to wild-type. In particular, with TET1 mutant mice, 39 genes were found to be both hyper-methylated and down-regulated in neural progenitor cells isolated from the *dentate gyrus* (part of the brain hippocampus). Four-month-old wild-type and TET1 knockout mice were tested in the Morris water maze. The TET1 deficient mice, with reduced ability to use a pathway dependent on base excision repair, showed impairment in spatial learning and short-term memory.

8. Perspective on the role of DNA repair in cognitive functions

The evidence discussed above in Section 6 clearly indicated that neuronal activity causes DNA double-strand breaks, especially in early response genes after neuronal stimulation. NHEJ repair is required to repair these breaks, and NHEJ repair is required for long-term memory formation. As discussed in Section 7, long-term memory formation depends on large numbers of epigenetic alterations including methylations and demethylations of cytosine in DNA. Although it is known that repair of double-strand breaks by NHEJ repair can leave epigenetic alterations (scars) (including alterations in the pattern of cytosine methylation) after the repair occurs, it is not known whether the NHEJ repair "scars" are a major portion of these epigenetic alterations. About a thousand demethylations occur during long-term memory formation in rats and mice. Base excision repair is central to demethylation of 5mC to cytosine. A deficiency in the TET/base excision repair pathway causes diminished epigenetic demethylations of DNA as well as alterations in memory.

Overall, memory and learning depend on epigenetic alterations. Two forms of DNA repair, NHEJ repair and base excision repair, have essential roles in cognitive functions, and at least base excision repair has a direct role in regulating one major type of epigenetic alteration, the demethylation of 5mC to cytosine in DNA during memory formation.

9. Conclusion

In eukaryotic cell nuclei, DNA is associated with histone proteins in highly organized and compact structures to form chromatin. When the DNA is damaged, repair enzymes need to gain access to the damage, and this requires modification of the compact structure. These modifications, termed epigenetic alterations, include acetylation of histones, methylation of histones and methylation of CpG sequences in DNA. Such epigenetic alterations can allow access of repair enzymes to sites of DNA damage while not disturbing the DNA base-pair sequence.

DNA repair processes are characteristically initiated rapidly and completed in a short period of minutes to hours, but epigenetic alterations introduced by such repair may be retained after repair is completed. A type of epigenetic alteration that can last after repair of a double-strand break is the DNA methylation of CpG islands in gene promoters. Such epigenetic alterations can silence gene expression. Also, several types of oxidative DNA damage are removed by base excision repair. Base excision repair is accompanied by epigenetic alterations of histones that are associated with genes containing CpG islands in their promoters. These epigenetic alterations can cause decreased transcription of the genes.

The persistent behavioral changes that are a prominent feature of addictions appear to be the result of epigenetic alterations that affect gene expression in particular regions of the brain. Specific epigenetic alterations have been found to be associated with addiction to alcohol, nicotine and cocaine. The epigenetic alterations that occur in those particular regions of the brain are considered to be

involved with each of the addictions. Nicotine and cocaine, and alcohol through its metabolic product acetaldehyde, cause DNA damage in the brain. Such DNA damage is subject to DNA repair processes that likely cause at least a portion of the long lasting epigenetic alterations found in the brains of addicted individuals.

In humans and other mammals inherited mutations in genes necessary for DNA repair can cause neurodegenerative diseases. Examples of such diseases are ataxia telangiectasia, Huntington's disease, Aicardi-Goutières syndrome and Cockayne syndrome. The deficiencies in DNA repair in these diseases cause disruptions in the pattern of epigenetic alterations required for normal neuronal function. These epigenetic alterations likely underlie key features of the neurodegenerative disease phenotypes.

Learning and new memories occur when mice explore a new, strange environment. Exploration of a novel environment increases the number of neurons with double-strand breaks in neuronal DNA, particularly in the dentate gyrus, which is involved in spatial learning and memory. Another neuronal activity, visual stimulation, was found to cause DNA double-strand breaks. Direct stimulation of the striatum region of the brain also caused DNA double-strand breaks. Memory retention of context associated electric shock events in mice involved induction of double-strand breaks and their repair by the process of non-homologous end joining in the hippocampus, a region of the brain known to be important in forming memories. Inhibition of non-homologous end joining substantially diminished memory retention.

The anterior cingulate cortex is a brain region important for long-term memory formation. Long-term memory (4 weeks in mice) subsequent to a contextual conditioning experience was found to be associated with substantial retention of a differential DNA methylation and gene expression pattern in the anterior cingulate cortex. In addition, differential DNA methylation in the hippocampus appears to be associated with short-term memory formation. Together, long-term and short-term memory formations are associated with on the order of a thousand demethylations of cytosines in neuron genomes during memory consolidation. Demethylation of 5-methylcytosine to cytosine in DNA depends on base excision repair.

In general, the evidence indicates that, in mammals, DNA repair processes can cause epigenetic alterations in chromatin, some of which are long lasting. These epigenetic alterations can have negative consequences on neurological function such as in certain addictions and neurodegenerative diseases. In addition, epigenetic alterations resulting from DNA repair processes, such as non-homologous end joining and base excision repair, appear to have a positive role in facilitating adaptive cognitive capabilities that include memory and learning.

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Chapter 7

Regulation of Oxidized Base Repair in Human Chromatin by Posttranslational Modification

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Abstract

Base excision repair (BER) is the major pathway for the repair of oxidized bases and apurinic/apyrimidinic (abasic; AP) sites produced by reaction with reactive oxygen/nitrogen species (ROS/RNS). These metabolites are generated spontaneously by endogenous cellular processes and also by environmental agents. Because most of these lesions are promutagenic, linked to diverse disease-associated somatic mutations, as well as heritable single nucleotide polymorphisms (SNPs) in the normal human population, their prompt repair is warranted. Impairment of repair leading to mutation, a hallmark of cancer, underscores the essentiality of BER for maintaining genome integrity in humans and other mammals. In mammals, repair of oxidized bases and other BER substrates is initiated by DNA glycosylases (DGs), which excise the damaged bases and cleave the DNA strands at the resulting AP sites, followed by sequential end processing, gap-filling DNA synthesis, and ligation. *In vitro* BER performed with naked DNA substrates has been extensively studied, which delineates its basic mechanistic steps and subpathways. However, recent interest is directed to unraveling BER in cell chromatin, including its regulation via posttranslational modifications (PTMs), which occurs possibly in concert with nucleosome remodeling. Emerging reports on various PTMs of BER enzymes indicate that the PTMs, while dispensable for the enzymatic activity, regulate overall repair by modulating interactions with other repair proteins and chromatin factors, assembly of BER complexes, as well as turnover of the proteins, and may ultimately dictate the cellular phenotype. Here, we discuss recent advances in the BER field by reviewing the PTMs and how they regulate BER in chromatin.

Keywords: oxidative stress, base oxidation, base excision repair, posttranslational modifications, acetylation, phosphorylation, SUMOylation, methylation, chromatin

1. Introduction

DNA, the genetic repository of all cellular functions, is packaged with histones into chromatin consisting of nucleosome units. One hundred forty-seven base pair (bp) segments in DNA wrap ~1.65 times in a left-handed superhelical turn around a histone octamer consisting of two histone H2A-H2B dimers and a H3-H4 tetramer, which form the nucleosome core; the adjacent nucleosomes are separated by some

50 bp unfolded, linker DNA bound to histone H1 or H5. Organization of DNA into chromatin enables the compaction required to accommodate large eukaryotic genomes inside the cell nucleus. This compaction renders DNA inaccessible to any DNA transaction machinery. Replication and transcription are tightly coordinated with specific interactions of their complexes with DNA [1, 2].

The integrity of DNA is under constant threat, naturally from endogenous sources, as well as by environmental factors in the form of a chemical addition, an alteration in the nitrogen base structure, thereby creating an abnormal nucleotide, or a break in one or both strands of DNA [3–8]. Cellular metabolic processes including mitochondrial respiration and hydrolytic reactions generate reactive molecules, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and alkylating agents. Some chemical bonds in DNA are susceptible to spontaneous hydrolysis. About 70,000 lesions are generated per cell, per day in humans. Single-strand breaks (SSBs), as well as a plethora of oxidized bases, are formed during oxidative genome damage. In addition, deamination, depurination, depyrimidination, double-strand breaks (DSBs), propano-, etheno-, and malondialdehyde-derived DNA adducts, base propenals, and alkylated bases are also formed endogenously. Environmental factors such as UV rays, ionizing radiation (IR), heat, and chemicals from tobacco smoke and industrial sources pose additional risks to DNA.

2. Oxidative genome damage and oxidized bases

For aerobic organisms, oxygen acts like a double-edged sword; while it is absolutely essential for life, it is also a threat to the life, recognized as the "Oxygen Paradox" [9–11]. ROS, which include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and the hydroxyl radical ($^{\bullet}OH$), along with RNS, for example, peroxynitrite ($ONOO^-$) react with all biological molecules including DNA. The hydroxyl radical having the highest reduction potential is mainly generated from Fenton reaction between reduced redox active metal ions (Fe^{2+}, Cu^+) and H_2O_2 [12], as well as by the IR-induced radiolysis of water [13]. A wide variety of cellular antioxidant defense mechanisms including both redox-buffering enzymatic and nonenzymatic systems have evolved, for example, superoxide dismutases, catalases, glutathione peroxidases, peroxiredoxins, and glutaredoxins; these counteract the detrimental effect of oxidative stress to the biological molecules, and an imbalance in their homeostasis leads to increased damage to the biomolecules [14].

A plethora of oxidized base lesions are generated mostly from guanine (G) in DNA, which has the lowest redox potential among the natural bases. Other lesions including 2-deoxyribose modifications, SSBs, DSBs, and protein-DNA cross-links are also ROS reaction products in DNA [10, 14–17]. Nearly 100 such lesions have been identified; however, because of the lack of sensitivity of the techniques used to identify the lesions and inherent instability of some of them, the total number formed in the genome under a pro-oxidant environment is likely to be much higher [18].

The most commonly formed oxidized base lesion is 7,8-dihydro-8-oxoguanine (8-oxoguanine, 8-oxoG), which was discovered by Kasai and Nishimura in 1983 and coined as 8-hydroxyguanine [19–21]. All the nucleobases are also ionized by IR and by high intensity 266-ns laser photolysis. The DNA bases undergo one-electron oxidation (one electron ionization potential of G<A<C~T). 8-oxoG is generated at a much higher level (>5-fold) than the combined level of other one-electron base oxidation products. Singlet oxygen ($^1O_2$), the major ROS in UVA-mediated oxidation of DNA, specifically targets G and 2-deoxyribose moiety [22–24]. Other major oxidized base lesions are 5-hydroxy-6-hydrothymine, thymine glycol (TG), cytosine glycol (CG), 5-hydroxycytosine (5-OHC), uracil glycol (UG), 5-hydroxyuracil

DNA bases	Base oxidation products			
Thymine	5,6-dihydroxy-5,6-dihydrothymine (thymine 5,6-glycol)	5-hydroxy-5-methylhydantoin	5-hydroxymethyluracil	5-formyluracil
R=H Cytosine R=CH_3 5-methyl cytosine	5-hydroxycytosine	5,6-dihydroxy-5,6-dihydrouracil	5-hydroxyhydantoin	5-hydroxymethylcytosine
	5-formylcytosine	5-carboxylcytosine	1-carbamoyl-3,4-dihydroxy-2-oxoimidazolidine	
Guanine	7,8-dihydro-8-oxoguanine	2,6-diamino-4-hydroxy-5-formamidopyrimidine	2,2,4-triamino-5(2*H*)-oxazolone	
Adenine	7,8-dihydro-8-oxoadenine	4,6-diamino-5-formamidopyrimidine	Inosine	

Table 1.
Common oxidized bases detected in DNA.

(5-OHU), 8-hydroxyadenine, and 2-hydroxyadenine [14, 17]. Hypochlorous acid (HOCl), generated by myeloperoxidase in neutrophils during inflammation, chlorinates both DNA and RNA bases [25, 26], and the main products are 5-chlorocytosine, 8-chloroadenine, and 8-chloroguanine. A summary of commonly formed oxidized bases detected in cellular DNA is shown in **Table 1** [16]. Apart from ROS-induced generation of oxidized bases, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) are formed enzymatically during transcriptional reprogramming involving oxidative demethylation of 5-methyl C (5mC), mostly localized in promoter CpG islands, induced by TET dioxygenases [27, 28]. However, enzymatically generated 5-methyl C oxidation products are produced >2-fold higher than that from direct oxidative damage to DNA [29, 30]. Additionally, tandem base lesions are produced by radicals generated from $^{\bullet}OH$ or one-electron oxidation reactions. Examples include the addition of either 5-(uracilyl)methyl radicals or 6-hydroxy-5,6-dihydrocytosin-5-yl radicals to 5′-adjacent guanine moieties in the DNA of cells exposed to H_2O_2 [31, 32] and formation of a guanine-thymine cross-link upon initial formation of guanine radical cation [33, 34]. One-electron oxidation also leads to DNA-protein cross-links. UVA irradiation of 6-thioguanine-containing DNA forms DNA-protein cross-links in human cells [33, 35].

3. Fate of oxidized bases and accumulation of mutations

ROS-induced oxidized base lesions and AP sites if left unrepaired are replicated by replicative or DNA translesion synthesis (TLS) polymerases [36]. Their misreplication generates mutations, a hallmark of cancer genomes, which account for two-thirds of single base pair substitutions [37–40]. Furthermore, single nucleotide polymorphisms (SNPs), observed in normal human genomes, also likely result from such spontaneous single base pair substitutions. U and 5-OHU, the spontaneous and ROS-induced oxidative deamination product of C, respectively, preferably pair with A during replication, resulting in GC → AT transition mutation; 8-oxoG,

the predominant oxidized base lesion mispairs with A, leading to GC → TA transversion mutation [41, 42]. In response to continuous assault by both endogenous and environmental factors, cellular defense mechanisms including diverse DNA repair pathways have evolved in all organisms to correct these base modifications and maintain genomic integrity.

4. Base excision repair of oxidized bases

Base excision repair (BER) is responsible for repairing most oxidized base lesions, AP sites, and DNA SSBs. The basic mechanism of BER first elucidated in *Escherichia coli* is broadly conserved across all organisms, as highlighted in several reviews [43–46]. BER requiring only four or five enzymes in the basic reaction steps is initiated with excision of the damaged base by a monofunctional DNA glycosylase (DG), for example, uracil-DNA glycosylase (UDG) or 3-methyladenine-DNA glycosylase, generating an abasic apurinic/apyrimidinic (AP) site due to hydrolysis of the N-glycosidic bond of the damaged base. The AP endonuclease (APE1 in mammalian cells) cleaves the resulting AP site in the second step and generates 3′ OH and 5′ deoxyribose phosphate (dRP) termini. The DNA polymerase in the third step fills in the single nucleotide gap. In mammalian cells, DNA polymerase β (Pol β) also has intrinsic dRP lyase activity, which cleaves the dRP residue and generates 5′ phosphate; the resulting nick after incorporation of the correct base is sealed by DNA ligase III (Lig III) complexed with XRCC1 in the final step.

The BER initiating DGs for oxidized bases, on the other hand, are bifunctional with intrinsic AP lyase activity. The bifunctional oxidized base-specific DGs further process the AP site via β or βδ lyase reaction. The Nth family of DGs, OGG1, and NTH1, via β eliminations generates 3′ phospho α,β-unsaturated aldehyde (3′ PUA; formally named 3′ phospho 4-hydroxylpentenal) and 5′ phosphate at the strand break. NTH1 prefers oxidized pyrimidines as substrates, and 8-oxoG and ring opened guanine, that is, formamidopyrimidine (Fapy-G), are preferred substrates for OGG1. The Fpg/Nei family DGs NEIL1, NEIL2, NEIL3, discovered by us and others [47–51] catalyze βδ elimination and remove the deoxyribose residue to produce a 3′ phosphate and 5′ phosphate at the strand break. NEILs prefer modified pyrimidine substrates, NEIL1 having preference for ring-opened purines, for example, Fapy-A and Fapy-G. The activity and substrate specificity of NEILs depend on the DNA structure, and NEILs have significant 5-OHU excision activity with single-stranded or bubble, forked DNA. In contrast, OGG1 and NTH1 prefer double-stranded DNA substrates. Usually, the base excision and lyase reactions act in a concerted sequence. However, due to weak lyase activity of OGG1, intact AP sites are the major product after OGG1-catalyzed cleavage of 8-oxoG [52, 53]. All these bifunctional DGs have broad and overlapping substrate range and possess backup activity for many base lesions. This accounts for the fact that only few DGs have been discovered so far for much larger number of oxidized bases and for the nonessentiality of individual DGs.

The 3′ phosphate generated by the NEILs by βδ elimination is a poor substrate for mammalian APE1 and is processed by polynucleotide kinase phosphatase (PNKP) [54–57]. Thus, for oxidized bases, the DGs actually define the subsequent steps. APE1 is responsible for processing the β elimination product of OGG1 and NTH1, whereas PNKP is required for generating 3′-OH termini from 3′ phosphate, a βδ elimination product of NEILs. Furthermore, AP sites and 3′ PUA generated by other DNA glycosylases can also be processed through a NEIL-PNKP-dependent pathway [53, 57]. This alternative repair route provides the functional redundancy in mammalian BER for genome safeguarding against a plethora of endogenous and induced oxidative damages.

BER, in the simplistic model, generates a 1-nucleotide gap after excision of the damaged base and has been termed single nucleotide BER (SN-BER) or short-patch BER (SP-BER). In contrast, long-patch BER (LP-BER) involves repair synthesis of two to eight deoxynucleotides. The 5′ blocking group after oxidation of AP sites cannot be removed by Pol β via its dRP lyase activity. Instead it is removed by 5′-flap endonuclease 1 (FEN-1), which is normally required for removing the 5′ RNA primers from Okazaki fragments during DNA replication. Thus, the subsequent steps of LP-BER are identical to that of DNA replication, utilizing DNA replication machinery, involving DNA polymerases δ/ε (Pol δ/ε) and DNA ligase I (Lig I). These enzymes including FEN-1 are recruited by the sliding clamp PCNA, loaded by replication factor-C (RFC), as in replication [58]. Thus, the choice of LP-BER vs. SN-BER depends on the 5′-terminus at the base cleavage site. With unaltered aldehyde group in deoxyribose, Pol β could carry out SN-BER by excising the 5′-dRP. LP-BER becomes necessary for repairing the oxidized AP sites, which cannot be processed by the 5′ end cleaning lyase activity of Pol β. The nuclear replicative Pol δ/ε lack dRP lyase activity and thus repair synthesis by these enzymes have to follow the LP-BER subpathway. Because Pol β-depleted cells are resistant to oxidative stress, Pol δ/ε can substitute for DNA Pol β and carry out the preferred LP-BER. The BER subpathways are schematically shown in **Figure 1**, adapted from [44].

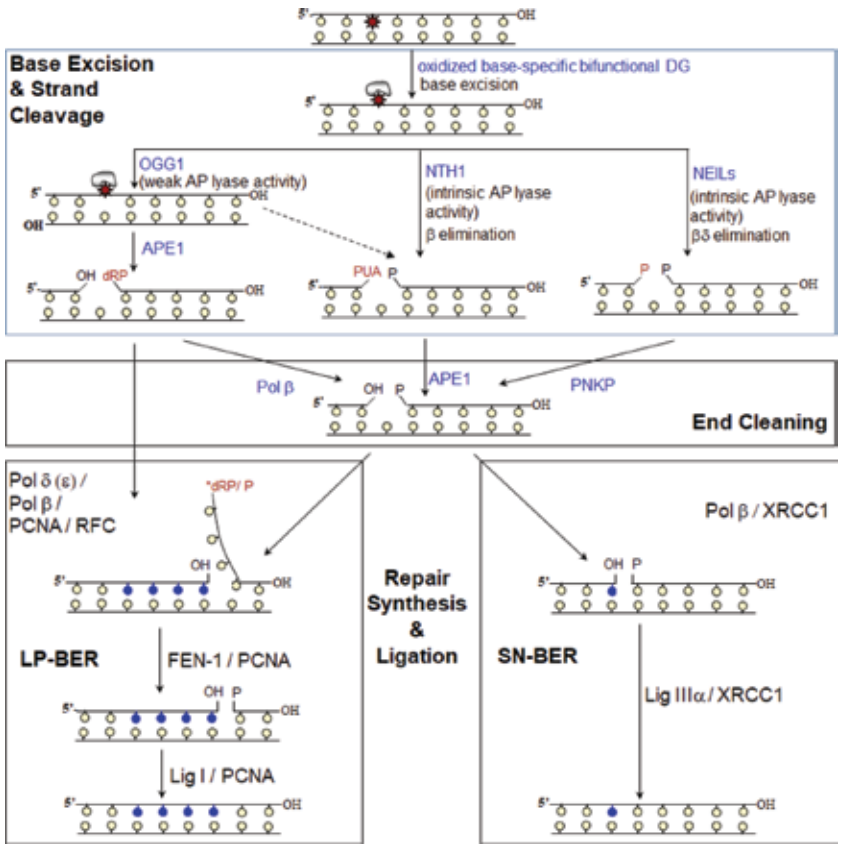

Figure 1.
A schematic representation of oxidized base-specific BER subpathways. The damaged base is represented as ✸. BER is initiated by the DGs: OGG1, NTH1, NEILs, and converge to common steps for end cleaning, followed by repair synthesis and ligation. See text for details.

5. Prereplicative BER of oxidized bases

The genomic integrity is particularly vulnerable during replication. Transient single-stranded (ss) DNA serving as a template during DNA replication after unwinding of the duplex genome is particularly vulnerable to ROS, which induces oxidized bases, sugar fragments, as well as strand breaks. Most oxidized bases do not stall replicative DNA polymerases, but they mispair during replication, thereby causing mutations. In contrast, bulky lesions, which stall replicative polymerases, block replisomes so as to allow repair. However, blocked replication may also lead to fork collapse, causing significant alteration in genomic stability. Furthermore, oxidized deoxynucleotides may be incorporated into the progeny strand during replication. If left unrepaired, these mutations could accumulate in progeny cells, a recipe for pathologies linked to genomic instability, including cancer, accelerated aging, and degenerative brain diseases [59, 60]. Repair of oxidative lesions, which are generated at much higher abundance than the bulky adducts in the replicating genome, is thus critical to maintain genomic fidelity. Mammalian cells have developed multiple ways to faithfully repair such base damages via prereplicative repair in the template strand and postreplicative repair in the progeny strand, immediately after replicative synthesis. Both the pathways involve an intricate collaboration of specific repair machinery with the replication proteins, likely via formation of dynamic "preformed" "repair-replication complexes" at the replication fork [61, 62].

Repair of most mutagenic base lesions except 8-oxoG, for example, 5-OHU, TG, 5-OHC, Fapy-A, 8-oxoA, and UG must be carried out prior to replication in order to prevent mutation fixation. How such lesions, which do not block replicative Pol δ, are flagged for prereplicative repair without causing DSBs was unclear. Our recent study showed that the mammalian DG NEIL1 binds to the oxidized lesion sites in ss DNA substrates *in vitro* to facilitate fork regression and participates in prereplicative repair of the damaged base in the reannealed duplex DNA [61, 62]. We compared the function of NEIL1 in stalling the replication fork at the damage sites for the prereplicative repair to the function of a "cow catcher" attached to the front of early steam locomotives that served to push aside animals or debris from the track ahead of the train's traversal, in a simplistic analogy to this exquisitely orchestrated process [63]. The key features of this "cow catcher" model are the ability of NEIL1 to recognize base lesions in ss DNA templates and its nonproductive binding to lesions in ss DNA, which, while preventing lethal DSB formation, causes the stalling of the replication fork. Subsequent fork reversal allows base lesion repair in the reannealed duplex. High expression and activation of NEIL1 in replicating cells, together with its stable physical and functional association with proteins in the DNA replication complex [48, 64–66], are consistent with this surveillance role of NEIL1. The human genome during each cell division may be at higher risk for oxidative damage whose repair would prevent accumulation of mutations in the daughter cells. Thus NEIL1's prereplicative BER function appears to be critical for preventing mutations and maintaining genome fidelity during cell division.

6. Posttranslational modifications of BER proteins

In vitro BER studies, carried out during the last couple of decades, are straightforward, mainly documenting functions of the repair proteins; however, in the complex cellular environment, the pathways are tightly regulated by interactions among the partner proteins in multiprotein complexes, which in turn also dictates the stability of the complexes. The stability and subcellular localization of these proteins are

regulated by site-specific posttranslational modifications (PTMs), primarily involving acetylation, methylation, phosphorylation, SUMOylation, ubiquitination, and PARylation. Thus PTMs are at the root of major regulatory processes, by bestowing novel biochemical properties to the modified proteins, including changes in enzymatic activity, subcellular localization, interaction partners, protein stability, and DNA binding. Although purified recombinant BER proteins without any PTMs are proficient in their enzymatic activities, *in cellulo* BER is significantly affected by these PTMs. In this section, we discuss all the major PTMs of BER proteins identified so far.

The hallmark of mammalian DGs and early BER proteins is the presence of nonconserved, intrinsically disordered appendages at the N or C terminus, which are absent in their bacterial orthologs. Some examples are the N-terminal extension in human NTH1 absent in the *E. coli* Nth, C-terminal extension in human NEIL1 which is lacking in *E. coli* Nei, N-terminal extension in human APE1 lacking in *E. coli* Xth [44, 65, 67, 68]. Although the unfolded sequence generally exists at the N or C terminus, this could also exist internally as in Human NEIL2, where it may serve as a linker of the two domains. Analogous to the situation of histones H3 and H4, where mostly all PTMs occur in the disordered N-terminal tail [69, 70], PTMs in many early BER proteins are clustered in their disordered domains. See **Table 2** for the major BER PTMs known so far.

6.1 Acetylation

Acetylation of histones was discovered back in 1963 after the Nobel prize-winning discovery of acetyl CoA [71–74], and acetylation of histones at the ε-amino group of Lys residues in their disordered N-terminal region was shown to suppress their abilities to inhibit transcription [75]. Following these pioneering discoveries that linked histone acetylation to chromatin decondensation and transcriptional activation [76–78], diverse acetylation modifiers were identified and characterized. These include various histone acetyltransferases (HATs) such as E1a-binding protein p300 (p300), CREB-binding protein (CBP), ortholog of yeast transcription regulator Gcn5, TAF(II)250 subunit of transcription factor IID, several members of the MYST family (MOZ, YBF2/SAS3, SAS2, and TIP60) and p300/CBP associated factor (PCAF). Histone deacetylases (HDACs) were subsequently discovered as "erasers," which include distinct members, HDACs1–11 and SIRTs in different transcriptional repressor complexes SIN3, NURD, etc., which regulate acetylation/deacetylation cycle in cells [79–81]. These discoveries set the stage for epigenetic regulation of gene expression. Simultaneously, the concept of "reader" proteins [80, 82] that specifically recognize acetylated Lys residues through their bromodomains was introduced in addition to the "writers" (HATs) and "erasers" (HDACs). Although the first discovered nonhistone protein acetylation dated back in 1997 for the tumor suppressor TP53 [83], the overwhelming numbers of nonhistone protein acetylation, particularly in large macromolecular complexes involved in chromatin remodeling, DNA repair, cell cycle, etc., were appreciated much later, after 2006, from mass spectrometric-based proteomic approaches, and provided the global scenario of "cellular acetylome" [81, 84–86].

6.2 Phosphorylation

Although enzymatic phosphorylation of proteins was discovered in 1954 [87], phosphorylated protein was known much earlier, based on identification of phosphate in vitellin [88], followed by detection of phosphoserine in this protein [89]. During the 1950s, ATP was discovered to be required for phosphorylation when the phosphate group was found to be covalently attached to specific serine/

Functional class	BER protein	PTM and identified site	BER activity	Protein stability	Reference
DNA glycosylases	Uracil DNA glycosylase (UNG)	Phosphorylation; T6, S23, T60, S64, T126	+	–	[127, 128, 213]
		SUMOylation		+	[214]
		Ubiquitination		–	[127, 215, 216]
	Single-strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1)	Ubiquitination		–	[215, 216]
	Methyl CpG-binding domain protein 4, DNA glycosylase (MBD4)	Phosphorylation; S156,S262	+		[217]
	Thymine DNA glycosylase (TDG)	Acetylation; K94, K95, K98	–		[129, 130]
		Phosphorylation; S93, S96, S99	+		[129, 130]
		SUMOylation; K330 K341	– +		[131, 218–221]
		Ubiquitination		–	[222, 223]
	MutY DNA glycosylase homolog (MYH)	Phosphorylation; S524	+		[224, 225]
		Ubiquitination; C-terminal K between aa 475–535		–	[226]
	8-Oxo guanine DNA glycosylase 1 (OGG1)	Acetylation; K338, K341	+		[203]
		Phosphorylation; S326	+		[227, 228]
		Ubiquitination	–	–	[229]
	Nei-like DNA glycosylase 1 (NEIL1)	Acetylation; K296, K297, K298	+	+	[161]
		Phosphorylation; S61, S207, Y263, S269, S306			[230–232]
	Nei-like DNA glycosylase 2 NEIL2	Acetylation; K49, K153	–		[233]
	N-methylpurine DNA glycosylase (MPG)	Acetylation	+		[234]
		Phosphorylation; S172	+		[235]

Functional class	BER protein	PTM and identified site	BER activity	Protein stability	Reference
End processors	Apurinic/ apyrimidinic endonuclease 1 (APE1)	Acetylation; K6, K7, K27, K31, K32, K35	+	+	[135, 136, 236–238]
		Phosphorylation; T233	–		[239, 240]
		Ubiquitination; K6, K7, K24, K25, K27, K31, K32, K35		–	[137, 138, 241]
	Polynucleotide kinase phosphatase (PNKP)	Phosphorylation; S114, S126	+	+	[139–141]
		Ubiquitination; K414, K417, K484		–	[139]
	Flap endonuclease-1 (FEN-1)	Acetylation; K354, K355, K377, K380	–		[242]
		Phosphorylation; S187	–	–	[144, 145]
		Methylation; R192	+	+	[146]
		SUMOylation; K168		–	[143]
DNA polymerases	DNA polymerase β (Pol β)	Acetylation; K72	–		[147]
		Methylation; R137, R83, R152	+		[148, 149]
		Ubiquitination; K41, K61, K81		–	[150, 151]
	DNA polymerases δ (Pol δ)	Phosphorylation; S458 of p68 subunit	–		[243]
DNA ligases	DNA ligase IIIα (Lig IIIα)	Phosphorylation; S123			[244]
		Ubiquitination		–	[150, 245]
Accessory proteins	X-Ray repair cross-complementing 1 (XRCC1)	Phosphorylation; S518, T519, T523, C-terminal linker, T284, S371	+	+	[246–252]
		SUMOylation			[152, 253]
		Ubiquitination; BRCA1 C terminus (BRCT II) motif on the C-terminal end		–	[150, 245, 252]
	Poly(ADP-ribose) polymerase 1 (PARP-1)	Acetylation; K498, K505, K508, K521, K524			[254]
		Phosphorylation; S372, T373	+		[255]
		SUMOylation; K203, K482, and K486			[132, 133]
		Ubiquitination		–	[132, 245, 256]

Table 2.
PTMs of BER proteins.

threonine residues [90, 91]. Subsequently, various kinases that phosphorylate serine/threonine and later tyrosine residues were characterized for their ability to modulate protein functions [91–93]. As with acetylation, phosphorylation induces conformational changes in the protein that stimulates its enzymatic activity and modulates protein-protein interactions [92, 94, 95]. Although the initial studies in protein phosphorylation were focused on cellular communications and signal transduction pathways, eventually the critical role of protein kinases and the relevance of phosphorylation/dephosphorylation events in DNA damage response (DDR) are extensively acknowledged, and mass spectrometry-based global screening approaches enabled identification of diverse phosphorylation targets [96, 97].

6.3 Ubiquitination and SUMOylation

Proteins are also posttranslationally modified via isopeptide bond formation with small proteins, which leads to nonlinear polypeptides [98, 99]. Ubiquitin is the first-discovered and well-characterized member of this growing family of small peptide modifiers, which covalently modify diverse proteins involved in chromatin organization, gene expression, signal transduction, DDR, DNA repair, and protein degradation [100–102]. Ubiquitin signals are generated by an enzymatic cascade involving E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. Ubiquitination is a highly dynamic process with deubiquitinases (DUBs) involved in this signaling, and growing evidence indicates the involvement of ubiquitination/deubiquitination in BER, as shown in **Table 2**.

Small ubiquitin-related modifier (SUMO), containing 100 amino acid (aa) residues protein, is ubiquitin-like polypeptide, which is conjugated to substrates in a manner similar to ubiquitination [102, 103]. The SUMO paralogs are synthesized as precursor proteins that are cleaved by a family of SUMO isopeptidases [104]. Mature SUMO is subsequently activated by a heterodimeric E1-activating enzyme Aos1/Uba2 (SAE1/SAE2) forming a thioester bond between its catalytic cysteine and the C-terminal carboxyl group of mature SUMO. Then SUMO is transferred to the catalytic cysteine of the E2-conjugating enzyme Ubc9. In contrast to the ubiquitin system where dozens of E2 enzymes have been identified, Ubc9 is the only known SUMO E2 conjugating enzyme. Finally, an isopeptide bond is formed between SUMO and the substrate by E3 ligases. A consensus SUMO acceptor site has been identified consisting of the sequence ΨKXE, where Ψ is a large hydrophobic amino acid and K is the site of SUMO conjugation [105]. There are at least four SUMO paralogs in humans, SUMO1, SUMO2, SUMO3, and SUMO4, which have more than 1000 protein targets. SUMOylation is highly dynamic and can be reversed by the action of deSUMOylating enzymes (SENPs). SUMOylation regulates protein-protein interactions involving SUMO-interacting motifs (SIMs), and it targets a group of proteins in the same pathway to facilitate association of multiprotein complexes for transcription, nuclear transport, chromatin assembly and modification, chromosome segregation, DNA damage repair, replication, and cell signaling [106, 107].

6.4 PARylation

Poly ADP-ribosylation (PARylation), a crucial PTM that appears rapidly at DNA damage sites, is catalyzed by poly(ADP-ribose) polymerases (PARPs). The human PARP family contains 17 members among which only PARP1, 2, and 3 are involved in DDR [108–111]. PARPs covalently attach the ADP-ribose unit via an ester bond to the carboxyl group of glutamate or aspartate and sometimes also attach to cysteine or lysine of the target proteins [112–114]. PARPs successively

transfer ADP-ribose units from NAD^+ to produce PAR chains containing up to 200 ADP-ribose units; however, in many cases, only single mono ADP-ribose moiety is transferred to the target proteins. Strand breaks in DNA activate PARP1, the founding and predominant member of the PARP family; the primary substrate of PARP1 is itself. Many proteins in the DDR pathways as well as the damage processing enzymes interact with PARP1 and/or are PARylated [112, 115]. In cells, PARylation/dePARylation is tightly and dynamically regulated; the PAR polymers are degraded by PAR glycohydrolase (PARG), possessing both exoglycosidic and endoglycosidic activities, and release free ADP-ribose moieties [116–118]. ADP-ribosyl-acceptor hydrolase (ARH) also exhibits PAR-degrading activity, although it has only exoglycosidase activity [119, 120]. Retention of PAR chains in cells triggers apoptotic cell death [121]. Although PARP1 interacts with the SSBR sensor XRCC1, as well as with other BER/SSBR proteins, and enables early recruitment of XRCC1 to the DNA lesions [122–126], there is no convincing evidence for PARylation of BER/SSBR proteins.

6.5 Cross-talks between different PTMs, their regulation, and effect on BER

Proteins employ diverse PTMs sequentially or concurrently to expand their repertoire of functions, thereby impacting global cellular signaling. The best example is the disordered N-terminal tail of histone H3, which has multiple sites for acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation [69, 70]. These PTMS could act synergistically or via reciprocal exclusion to modulate chromatin organization, thus affecting the transcriptome. The same Lys residues (K9, K27) in H3 are targets for both acetylation (marker of active chromatin) and methylation; however, monomethylation of these residues are markers of active chromatin, while di- and trimethylation are associated with repression. Recent evidence on BER enzymes, summarized below, suggests that specific modification at one site can dramatically influence another modification at a different site, which may critically impact BER activity.

Cyclin-dependent kinase (CDK)-mediated phosphorylation of UNG2 (nuclear UDG) in S phase signals its ubiquitination-dependent degradation, and CDK-inhibitor roscovitine prevents such degradation [127, 128]. This suggests that phosphorylation-induced conformational change in UNG2 is a prerequisite for ubiquitination.

In the case of TDG, acetylation inhibits its repair activity by two distinct mechanisms. TDG acetylation at K94, K95, and K98 by p300/CBP suppresses BER by preventing APE1 recruitment to the damage site [129]. Protein kinase C (PKC)-mediated phosphorylation at S93, S96, and S99, close to the acetylation sites, may promote repair by sterically blocking repair-inhibitory acetylation of adjacent lysine residues [130]. On the other hand, SUMOylation at K341 inhibits TDG's interaction with CBP, preventing its acetylation and thereby promoting BER [131].

PARP1, SUMOylated at K203 and K486, is a target for ubiquitination and degradation, which is believed to be the mechanism for its turnover [132]. In contrast, PARP1's SUMOylation at K482 does not degrade the protein, rather stimulates PARylation of chromatin-associated proteins [133]. On the other hand, acetylation of PARP1, which stimulates its transactivation function, is inhibited by K486 SUMOylation. Thus, K486 SUMOylation restrains PARP1's transactivation function [134].

While acetylation of APE1 enhances its stability in chromatin and enzymatic activity [135, 136], CDK5-mediated phosphorylation enhances its ubiquitination and degradation [137, 138]. Thus, it is possible that phosphorylation and acetylation are mutually exclusive, acetylation stabilizing the protein, and phosphorylation guiding to its degradation.

In the case of PNKP, ATM-dependent phosphorylation was shown to prevent ubiquitination and hence its degradation. Thus, in response to oxidative stress, ATM phosphorylates and stabilizes PNKP in order to activate a coordinated DDR pathway [139–141]. Furthermore, PNKP interacts with the deubiquitination enzyme ataxin-3 (ATXN3), which enhances its stability and phosphatase activity [142].

Phosphorylation of FEN-1 by CDK1 at S187 was shown to promote SUMOylation at K168, which enhanced its polyubiquitination-dependent degradation [143]. Phosphorylation inhibits FEN-1's flap endonuclease activity [144, 145], which cross-talks with methylation, a lesser studied PTM of BER proteins. Methylation by arginine methyltransferase 5 at R192 prevents this phosphorylation and thus is proposed to be essential for the repair activities of FEN-1 [146]. Thus, in response to oxidative stress in cycling cells, methylation of FEN-1 could be a critical requirement for LP-BER.

Acetylation of Pol β at K72 inhibits its dRP lyase activity [147], and this could account for acetylation-induced inhibition of enzymatic activity and switch from SN-BER to LP-BER. Methylation of Pol β at R137 has no effect on dRP lyase or DNA polymerase activities but inhibits its interaction with PCNA [148] and could thus be predicted to inhibit LP-BER. In contrast, R83 and R152 methylation enhanced Pol β's DNA binding and increased processivity [149]. Cellular Pol β level appears to be maintained by two ubiquitin E3 ligases, Mule and CHIP. DNA Pol β is monoubiquitinated by Mule, which in turn is recognized and polyubiquitinated by CHIP in undamaged cells. In response to oxidative stress, it is deubiquitinated, thus ensuring its stability and oxidized base damage repair [150, 151].

A recent study shows how PARylation stimulates SUMOylation [152]. In response to DNA strand breaks induced by alkylating agent methylmethanesulfonate (MMS), PARP1 is activated and synthesizes PAR chains; this promotes recruitment of SUMO E3 TOPORS to XRCC1, which facilitates XRCC1 SUMOylation. XRCC1 SUMOylation recruits Pol β at the damaged sites and thus ensures completion of BER.

7. Does chromatin organization affect BER? Understanding BER at the chromatin context

BER, as studied *in vitro* with naked DNA substrates, involves sequential enzymatic steps in which each enzyme utilizes the product of the previous step as the substrate. This observation inspired the prevailing dogma that the sequential steps in BER involves the hand-off process where the product of one step is handed over to the enzyme in the next step [153, 154]. Later steps generate intermediate product lesions that are more toxic than the original lesions. The BER intermediates such as AP sites and SSBs, which are highly mutagenic, interfere with replication and transcription, and hence the entire BER steps must be coordinated once the repair is initiated [155–158]. Cumulating evidence suggests that the BER proteins act in concert beyond simply recognizing and acting upon the product of the previous step, by being present at the site of the original lesion [43, 52, 61, 62, 64, 65, 125, 159, 160]. This is the basis for the emerging paradigm of "preformed BER complexes," named, "BERosomes" in mammalian cells. Being an integral part of complexes, it may be easier for the BER intermediates to be handed over to the next enzyme, which likely undergoes allosteric changes after binding to its substrate. Recent studies in our and collaborators' labs suggest that these "BERosomes" are constitutively chromatin-bound to ensure prompt repair in the event of any threat [62, 135, 161]. Simultaneously, recent interests in the BER field have evolved toward deciphering the role of different chromatin factors and the underlying chromatin remodeling in oxidized base repair.

Several *in vitro* studies showed reduced BER activity with reconstituted core nucleosome particles, where every step during repair of diverse lesions was found to be inhibited by histones [162–170]. Overall BER efficiency is strongly inhibited by the presence of nucleosomes, which interfere with the interaction between the repair proteins and their substrate lesions, thereby compromising physical interaction and catalysis. Because oxidized bases perturb the DNA structure only mildly [170], whether chromatin remodeling occurs during BER was questionable. But, as BER efficiently occurs in cells, the results from these *in vitro* experiments imply that chromatin rearrangement occurs at oxidized DNA damage sites *in cells*, as was shown in the case of repair of DSBs, UV ray-mediated damages, and mismatched base pairs [171–173].

An inverse correlation exists in cells between BER and chromatin compaction. ROS induces assembly of BER complexes preferentially on open chromatin regions [174], as we have also observed that the BER complexes are constitutively present on actively transcribing sequences [175]. Interestingly, BER is involved during active CpG demethylation in promoters, mediated by TET dioxygenase(s) during transcriptional activation [176–180]. The TET proteins oxidize 5mC to 5hmC, 5fC, and 5caC; 5fC and 5caC are the TDG substrates. Thus, this coordination between CpG DNA demethylation, an epigenetic process essential for chromatin decondensation during transcriptional activation, and base damage repair supports our notion that "open-chromatin prefers BER activity across the genomic landscape" and highlights a regulatory link between epigenetics, chromatin remodeling, and BER.

Various ATP-dependent chromatin remodeling (ACR) complexes, which play significant roles in protein/DNA and protein/protein interactions in chromatin and regulate transcription, DNA repair processes such as DSB repair (DSBR), nucleotide excision repair (NER), and cross-link repair, also affect BER. ACR complexes utilize the energy of ATP hydrolysis to restructure nucleosomes on chromatin [181–183], thereby affecting gene expression profile and DNA repair. Four structurally related, but functionally distinct, ACR complex families were identified: SWI/SNF (switching defective/sucrose nonfermenting; most extensively studied), ISWI (imitation switch), CHD (chromodomain, helicase, DNA binding), and INO80 (inositol requiring 80). *In vitro* BER studies with reconstituted nucleosomes showed enhanced repair activity in the presence of purified SWI/SNF or ISW1/ISW2 complexes [184–186]. There are some indirect evidences of ACR during BER in yeast and mammalian cells. Depletion of STH1 (ATPase subunit of RSC, a member of SWI/SNF family) causes genome-wide BER inhibition and thus emphasizes a link between chromatin organization and BER [187]. In a recent study, depletion of ALC1/CHD1L, another member of SWI/SNF, compromises chromatin relaxation, associated with BER inhibition and increased sensitivity to MMS and H_2O_2 in chicken cells [188]. On the contrary, INO80 deficiency in MMS-sensitive yeast cells has no effect on genome-wide BER [189]. K56 acetylation in histone H3 is increased in chromatin of both yeast and mammalian cells following MMS treatment, which generates alkylated base substrates for BER, [190, 191]. H3K56Ac was also found to be enriched at DSBR sites and responsible for SWI/SNF complex recruitment during transcription [192]. Thus, it would be interesting to examine if any specific PTM(s) would target ACR after oxidized base damage and illuminate the phenomenon of ACR during BER. In any event, additional studies are required to test if ACR plays a role in enabling BER in condensed chromatin. It would be also of interest to explore if the BER proteins possess inherent chromatin remodeling activities, similar to the NER proteins, which have SWI/SNF domains [193–195]. Though no known BER proteins have SWI/SNF domains, the XRCC1-Lig IIIα complex could disrupt nucleosomes *in vitro* and enable BER completion [166].

Poly-ADP-ribosylation of histones by PARP1 after genome damage adds negative charge on histones and disrupts histone-DNA interactions, thereby promoting chromatin decondensation and enhancing interaction between the proteins involved in DNA transactions and DNA [111, 196–198]. This could increase DNA accessibility to the BER proteins. Although PARP1's role in regulating transcription is well established, this would link chromatin remodeling to BER.

Nucleosomes pose obstruction to all DNA transactions and are likely disassembled to allow DNA replication, repair, and transcription, followed by their reassembly, which utilizes both parental histones and newly synthesized histones. Such replication-coupled nucleosome assembly in the S phase or replication-independent, transcription-coupled assembly throughout the cell cycle involves histone chaperones functioning at multiple steps of nucleosome formation [172, 199, 200]. Replication-coupled nucleosome assembly is aided by the chromatin assembly factor (CAF-1) and Rtt106 with the help of antisilencing function 1A (ASF1A) protein. Histone cell cycle regulator (HIRA) protein, along with Daxx, mediates replication-independent nucleosome assembly. While exploring chromatin-bound BER complexes, we serendipitously discovered CHAF1A (the largest subunit of CAF-1, along with other subunits CHAF1B and RBBP4), ASF1A, and various H3/H4 variants in the immunoprecipitation complex of NEIL1 or acetylated NEIL1 (201; unpublished). This underscores the importance of the diverse chromatin components in preformed "BERosomes," which could regulate oxidize base repair in chromatin. We showed that ROS-induced oxidized base lesions caused transient dissociation of CHAF1A, ASF1A, and histones from the BER complexes and were restored back after repair completion. The repair activities of NEIL1 and OGG1, as well as complete cellular BER, were found to be inhibited by CAF-1, as well as the CHAF1A monomer [201]. So, we propose a hypothesis of temporal regulation of BER by the histone chaperones, whose dissociation from BER complexes is essential to initiate BER [201]. This has been illustrated in **Figure 2**.

Recently, we discovered acetylation of NEIL1 at the disordered C-terminal K296-K298 by p300, which enhances its activity, and found that acetylated NEIL1 (AcNEIL1) could be detected only in the chromatin fraction and not in the soluble nuclear fraction [161]. Although the nonacetylable NEIL1 3KRmutant (Lys296–298 substituted with Arg) translocates to the nucleus and binds to chromatin, presumably due to retention of positive charges as in the WT enzyme, it forms less stable BER complexes with the histones, histone chaperones, and downstream BER proteins. Thus, as proposed earlier [65], the positive charge cluster in the disordered C-terminal region is required for NEIL1's nonspecific DNA binding, after which acetylation occurs on the chromatin. Hydrophobic interaction of NEIL1 after acetylation-mediated charge neutralization probably stabilizes NEIL1's complexes with nucleosome components and downstream BER proteins. Consequently, cells with acetylable NEIL1 exhibit enhanced BER efficiency and are less sensitive to oxidative stress. It is thus likely that unmodified NEIL1 binds to chromatin nonspecifically, and acetylation specifically at the promoter regions of actively transcribing genes by enhanced p300 activity actually stabilizes NEIL1's (and possibly other DG's) BERosomes on these preferred chromatin regions (**Figure 3**), which warrants further investigation.

In a separate study, while investigating how APE1 repairs AP sites in cells, our collaborator's lab found that acetylated APE1 (AcAPE1), like AcNEIL1, is exclusively and stably chromatin-bound throughout the cell cycle [135]. APE1 undergoes acetylation after binding to AP sites in chromatin, which enhances its enzymatic activity. In the absence of APE1 acetylation, cells accumulated AP sites and exhibited higher sensitivity to DNA damaging agents. We predict that other BER proteins OGG1 and MPG, whose repair activity is enhanced by acetylation, are similarly stabilized in chromatin-bound state.

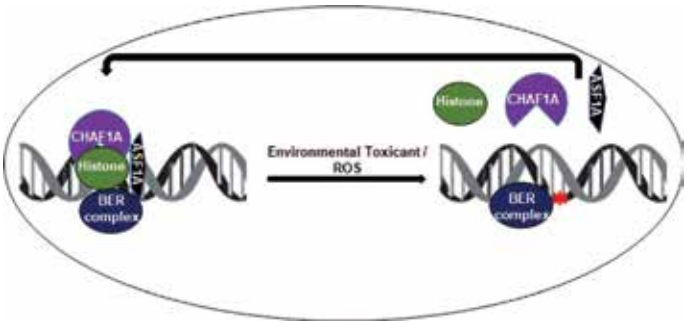

Figure 2.
A schematic showing chromatin-bound BER complexes with histones and histone chaperones. ROS-induced damage causes transient dissociation of histones and histone chaperones to initiate BER, which are restored back after repair completion.

Figure 3.
An illustrative view of "open" chromatin regions, containing bound "BERosomes" with histones, histone chaperones, PARPs, TETs, etc., for preferential repair of these transcriptionally active regions.

8. Future perspectives

The genome-wide impact of various PTMs in the cross-talks among BER proteins, which dictates the overall repair efficiency, thus preserving genomic integrity against genotoxic insults from both endogenous and external oxidative stress, has not been investigated. In this NextGen era, holistic, whole-genome scanning approaches, although a daunting challenge, make it likely to map individual PTMs of BER proteins, the kinetics of their formation and removal, and their correlation with both intrinsic and ROS-induced BER efficiency across the genomic landscape. Because histone PTMs have been well established in chromatin remodeling, it is also important to explore how specific histone PTMs interfere with the BER PTMs.

The Access-Repair-Restore model [182, 202] provides an accepted view of DNA repair in chromatin, where chromatin remodeling is essential for the DNA repair machineries to get access to the damaged DNA. For BER, it is still not clear how chromatin remodeling and the associated histone PTMs initiate BER. The BER complexes constitutively bind to "open" chromatin regions, and chromatin remodeling could assist specific enzyme-substrate binding and enzyme catalysis needed to initiate and propagate BER. Moreover, although chromatin remodeling has been found to enable BER, the enhanced repair activity may be simply due to ROS-induced stimulation of BER genes' expression or their specific PTM (acetylation), as has been shown by us [203–205], along with enhanced substrate binding in "open" chromatin. This may underestimate the contribution of ACR complexes at oxidized base lesion sites to enhance BER. Alternatively, in cells, chromatin remodeling-stimulated BER could be linked to replication and transcription, similar to transcription-coupled NER, which always occurs on "open" chromatin [206]. Indeed, repair of oxidized bases preferentially occurs in the transcribed strand [175], which could be assisted by Cockayne syndrome protein B (CSB), a NER factor, in transcription-coupled but NER-independent fashion [207]. Because BER/SSBR proteins such as PARP1 and APE1 are emerging as potential

therapeutic targets [208–212], understanding if and how chromatin remodeling impacts BER activity is crucial to manipulating BER for effective modulation of repair activity in cancer cells. This would provide better efficacy and specificity in cancer therapy.

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Conflict of interest statement

The authors declare that there are no conflicts of interest associated with this study.

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Section 4

DNA Repair and Cellular Processes

Chapter 8

The Role of DNA Repair in Cellular Aging Process

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Abstract

Aging is defined as the time-dependent decline of functional properties. One common denominator of aging is mitochondrial dysfunction and accumulation of genetic damage throughout life. In fact, the imperfect maintenance of nuclear and mitochondrial DNA likely represents a critical contributor of aging. Each day, the integrity and stability of DNA are challenged by exogenous physical, chemical, or biological agents, as well as by endogenous processes, including DNA replication mistakes, spontaneous hydrolytic reactions, and reactive oxygen species. In this way, DNA repair systems have evolved a complex network that is collectively able of dealing with most of the damages inflicted. However, their efficiency may decrease with age and, therefore, influence the rate of aging. Thus, the purpose of this work is to summarize the recent knowledge in cellular aging process and its link with DNA repair systems, with a particular emphasis on the molecular mechanisms associated.

Keywords: DNA damage, DNA repair, BER, NER, MMR, HR, NHEJ

1. Introduction

Aging is a complex biological process that results in a progressive loss of physiological integrity. Overall, aging is a consequence of accumulation of cellular damage and is characterized by nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, cellular senescence, mitochondrial dysfunction, loss of proteostasis, deregulated nutrient sensing, stem cell exhaustion, and altered intercellular communication (**Figure 1**) [1]. Although aging may involve damage to various cellular constituents, there is evidence suggesting that DNA constitutes the key target in this process [2]; consequently, genomic instability is the main factor of aging [3–5]. Genome instability has been implicated as a cause of aging since unrepaired DNA damage, DNA mutations, and epimutations accumulate in an age-related manner [3]. In the same way, the notion that multi-system premature aging syndromes are mainly caused by defects in genome maintenance or affect genome function highlights the role of genome integrity in aging [6]. Meanwhile, normal aging is accompanied by telomere shortening with cell division due to the "end-replication problem" and telomere end processing. Currently, there is a wide body of evidence associating reduction in the length of telomeres with failure of cell division and senescence of normal cells, and oxidative stress and inflammation can contribute to the rate of attrition of telomere length [7]. Age-related changes involve

alterations in DNA methylation patterns and posttranslational modification of histones such as increased histone H4K16 acetylation [8], H4K20 trimethylation [9], or H3K4 trimethylation [10], as well as decreased H3K9 methylation [11] or H3K27 trimethylation [12]. At the same time, with aging there is also a global heterochromatin loss and redistribution [13], thus affecting the expression of several genes, mainly those involved in DNA repair, cellular proliferation, differentiation, and cell-cycle regulation, and therefore triggering the emergence of other hallmarks of aging [14, 15]. Cellular senescence is a process that has become an important contributor in aging since it imposes a permanent proliferative arrest of cells in response to various stressors such as DNA damage and telomere loss [16]. Furthermore, as cells and organisms age, mitochondria suffer a decline in their integrity and function, tending to diminish the efficacy of the respiratory chain and thus reducing ATP generation, increasing electron leakage and ROS production that can damage DNA, proteins, and lipids, among other important biomolecules [17]. Proteostasis involves mechanisms for correct folding proteins and mechanisms for the degradation of proteins, which act in a coordinated fashion to prevent the accumulation of damaged components and assuring the continuous renewal of intracellular proteins. There is evidence that aging is associated with perturbed proteostasis, thus favoring the development of several diseases [18]. Recent data have shown that anabolic signaling accelerates aging; in agreement with this, caloric-restricted diet decreases nutrient signaling and as a result, a long life span is promoted since DNA repair systems are improved; on the other hand, protein homeostasis decreases ROS production and delays cellular senescence [19]. Decline in the regenerative potential of tissues is one of the most obvious characteristics of aging, where stem cell exhaustion is also important and explained by a decreased cell-cycle activity. Interestingly, this correlates with the accumulation of DNA damage, telomere shortening, and overexpression of cell-cycle inhibitory proteins such as p16INK4a, increasing the relevancy of DNA repair systems [20]. Finally, aging also involves changes at the level of intercellular communication, where neurohormonal signaling tends to be deregulated together with composition of the peri- and extracellular environment

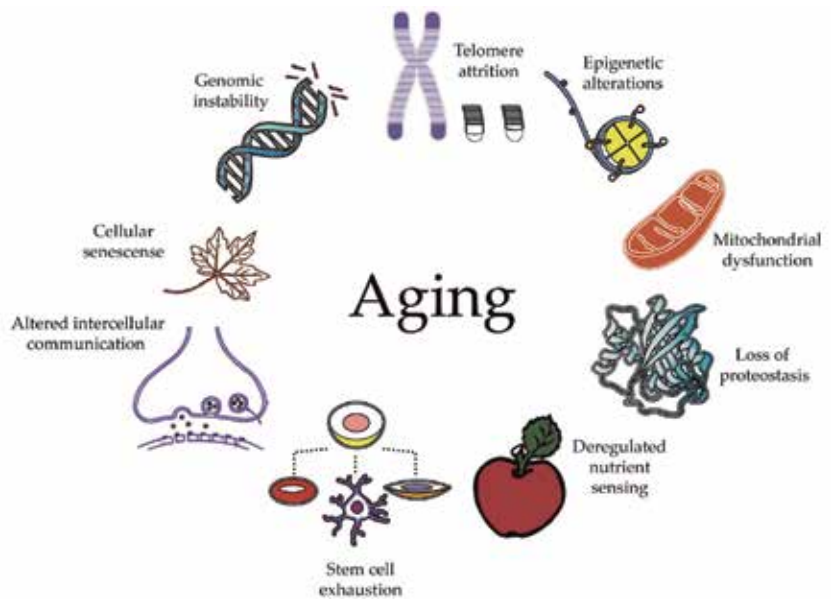

Figure 1.
The hallmarks of aging. The figure illustrates nine hallmarks previously described [1] and where age-related changes in DNA repair systems have important roles to promote the development of this phenotype.

and immune system, specially increasing inflammatory reactions and declining immunosurveillance against pathogens and premalignant cells [21]. In this way, our work focuses on describing the molecular bases that associate DNA damage and the cell aging process, with a special emphasis in DNA repair systems.

2. Age-related changes in DNA repair

Each day, the integrity and stability of DNA are challenged by exogenous physical, chemical, or biological agents, as well as by endogenous processes, including DNA replication mistakes, spontaneous hydrolytic reactions, and reactive oxygen species (ROS). Thus, depending on the source of damage, DNA can be affected in different ways, including nucleotide alterations, bulky adducts, single-strand breaks (SSB), and double-strand breaks (DSB). To combat threats posed by DNA damage, cells have evolved complex and finely regulated mechanisms collectively referred to as DNA damage response (DDR) which detects DNA lesions, signals their presence, and promotes their repair [22–24]. However, according with the genome maintenance hypothesis of aging, DNA repair can itself be subject to age-related changes and deterioration, allowing accumulation of damages (**Figure 2**). The wide diversity of DNA-lesion types requires multiple, largely distinct DNA repair mechanisms that differ in their components, whereas some lesions are subject to direct protein-mediated reversal, most are repaired by a sequence of catalytic events mediated by multiple proteins [22]. Thus, cells with defects in key proteins involved in DDR have been shown an accelerated aging phenotype caused by the accumulation of mutations and epimutations that eventually cause malfunction of the cells, senescence, or apoptosis [25].

2.1 Response to DNA single-strand breaks (SSBs)

2.1.1 Base excision repair (BER)

BER pathway corrects DNA damage from oxidation, deamination, alkylation, and other small DNA alterations that do not distort the overall structure of double helix. In general, BER is initiated by a DNA glycosylase that recognizes and

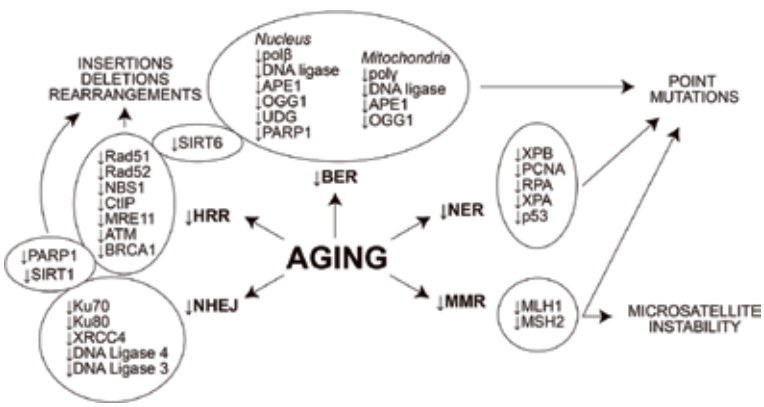

Figure 2.
Age-related changes in DNA repair and their consequences. Aging involves deterioration of DNA repair systems allowing the damages to accumulate and eventually cause a malfunction of the cells. In general, all age-related changes in DNA repair pathways promote genomic instability in different ways. Decline in efficiency and fidelity of BER and NER leads to point mutations, whereas inefficient MMR leads to microsatellite instability and point mutations. Meanwhile, deficiencies in NHEJ and HRR result in deletions and genomic rearrangements.

removes the damaged base, leaving an abasic (apurinic/apyrimidinic; AP) site that is subsequently processed by an AP endonuclease (APE), an exonuclease, a DNA polymerase, a ligase, and many other ancillary factors in a short-patch repair or long-patch repair [26]. Notably, several pieces of evidence indicate that the efficacy of BER may negatively change with age, and it has a significant impact in longevity together with homologous recombination repair (HRR) [27]. Age-related changes in the BER mechanism have been studied mainly in neuronal extracts where it constitutes the main repair pathway. In this way, an overall deficiency in several factors has been observed [28], where DNA polymerase β (pol β) together with DNA ligase [29] and APE1 activities [30, 31] seem to be the most limiting factors. Interestingly, an age-dependent attenuation in the transcriptional activation of pol β and APE1 was observed in response to DNA damage [32] together with APE1 accumulation in the nucleus and mitochondria [33]. Aging has also been shown to have a significant effect on cleavage efficacy of tetrahydrofuran:A, U:G mispair, U:A base pair, thymine glycol:A, and 8-oxo-7,8-dihydroguanine:C [34]. Thus, senescent human fibroblasts as well as leukocytes from old donors showed higher basal level of AP sites than young donors. However, after a challenge with the oxidizing agent H_2O_2 or the alkylating agent methyl methanesulfonate (MMS), the number of AP sites increased quickly in young cells, whereas in senescent and older cells, they were observed to grow slowly with a concomitant loss of viability, suggesting a decrease in DNA glycosylase activity, mainly in OGG1 8-oxoguanine and 3-methyladenine DNA glycosylases [35], although other reports have also mentioned a decrease in the UDG uracil-DNA glycosylase [28]. Because polyADP-ribosylation (PARylation) levels are linked to downstream mechanisms in DNA repair together with other cellular deficiencies as cell-cycle arrest, cell survival, cell death, and/or cell transformation, a decline in PARP1 activity is important since it has been linked with the age in humans and rats [36]. Further, a decrease in the interaction between the endonuclease VIII-like NEIL1 and PARP1 was observed in old mice when compared to young mice [37], which also could be associated with the decrease in PARP1 activity. Meanwhile, a significant decrease in the expression of SIRT6 has been reported to have a relevant role in BER because it regulates repair activity through a PARP1-dependent pathway [38]. Since sirtuins can function as metabolic sensors, they could also be related with a significative increase in pol β [39] and APE activities [30] under caloric restricted diets. Consequently, BER pathway showed to be deficient when repairing age-downregulated genes in comparison with genes that are not affected by age [40].

On the other hand, the mitochondrial free radical theory of aging states that free radicals generated in mitochondria are strongly related with the intrinsic aging process, mainly due to the accumulation of oxidative damage and derived mutations in mitochondrial DNA (mtDNA) mainly in D-loop region. mtDNA is more susceptible to oxidative damage than the nuclear genome, presumably because of the physical proximity of the source of ROS and lack of histones [41]. BER is the predominant and best understood DNA repair pathway in mitochondria involving at least four components, a DNA glycosylase, an AP endonuclease (or other mechanism for processing abasic sites), DNA polymerase γ (pol γ), and DNA ligase [42]. Recently, pol β was also detected in mitochondrial protein extracts, where it is required to provide enhanced mtDNA BER activity [43]. In a similar way to nuclear BER, in rat brain mitochondria, there is a marked age-dependent decline in mitochondrial BER activity, as indicated by a pol β, pol γ, ligase, APE1 endonuclease, and OGG1 glycosylase activities [44]. Interestingly, activity of mitochondrial OGG1 AE8-oxoguanine DNA glycosylase increases in mouse liver mitochondria according with the age [45]. However, a significant fraction of the OGG1 remains in the outer membrane and intermembrane space in an immature form, presumably because

its import into the mitochondrial matrix is impaired as a consequence of aging. In addition, a nearly identical phenomenon was observed with the mitochondrial uracil-DNA glycosylase [46].

2.1.2 Nucleotide excision repair (NER)

NER is the primary pathway for repairing a wide range bulky DNA lesions, including UV-induced photoproducts (cyclopyrimidine dimers [CPDs], 6–4 photoproducts [6-4PPs]), adducts formed by mutagens in the environment such as benzo[a]pyrene or some aromatic amines, some oxidative endogenous lesions such as cyclopurines, and adducts formed by cancer chemotherapeutic drugs such as cisplatin. NER can be initiated by two subpathways: global genome NER (GG-NER) where the participation of XPC-RAD23B is involved and the transcription-coupled NER (TC-NER) where RNA polymerase interacts with CSA, CSB, and XAB2. Both converge to complete the excision process requiring the core NER factors RPA, XPA, TFIIH, XPD, XPB, XPG, and ERCC1–XPF, among other auxiliary proteins [47]. NER activity decreases with aging possibly because there is a transcriptional downregulation of NER genes together with an altered protein function or processing and a decrease in energy production [48]. In this manner, it was previously observed that aged human skin [49] and fibroblasts [50] showed decreased levels of XPB, PCNA, RPA, XPA, and p53, and more importantly the UVB-induced pyrimidine dimers were removed in a slower manner than in younger counterparts [50]. Interestingly, the effect of age on the repair of UV-induced DNA damage varies for transcribed and nontranscribed DNA, decreasing considerably in unexpressed DNA [51, 52] but improving in both cases under calorie restricted diets [52]. Furthermore, UV-induced damage and repair in telomeres showed to be slower and less frequent than in other regions of the genome such as active genes [53]. Additionally, ERCC1 and XPF, which are considered as the rate-limiting members in NER, also showed an age-dependent decline in their relative expression levels [54]. Because XPC, XPB, and XPF appear to be dependent on the activation status of the IGF-1R, decreased levels of IGF-1R observed with aging also contributed with the decline of NER pathway [55]. Meanwhile, in an assay based in plasmid reactivation after UV damage, cells from older donors introduced an increased number of mutations in the transfected plasmid, which suggests that not only the repair is less efficient with age but also more mistakes are made [51].

2.1.3 Mismatch repair (MMR)

The mismatched nucleotides in the DNA can result from polymerase misincorporation errors, recombination between imperfectly matched sequences, chemical or physical damage to nucleotides, and deamination of 5-methylcytosine (5mC) mostly during replication. MMR pathway consists of four major heterodimeric complexes, MutL homolog (MutL)α, MutLβ, MutS homolog (MutS)α, and MutSβ. MutLα involves MLH1 and PMS2, whereas MutLβ consist of MLH1 and PMS1. Meanwhile, MutSα consists of MSH2 and MSH6, and MutSβ is constituted by MSH2 and MSH3. Thus, MutSα complex recognizes single mispaired bases, whereas MutSβ detects mispaired runs of 3–6 bases. MutSα or MutSβ recruits MutLα or MutLβ and forms a tetrameric complex that serves as a base for the recruitment of excision and repair machinery [56]. MMR removes mispaired bases preventing mutations [57], and defects in this pathway are strongly associated with a substantial destabilization of microsatellites, which are tandemly repeated sequences (from 1 to 6 bp), highly polymorphic, interspersed in the genome, and susceptible to slippage during replication [58]. Previously, a decline in MMR function and efficiency correlation with age

was observed [59, 60], especially in microsatellite sequences [61] where age-related methylation of the MLH1 [62, 63] and MSH2 [64] promoters could be associated to microsatellite instability (MSI). Interestingly, MLH1 shores showed a decrease in methylation with increasing age [65]. Shores are regions of the genome around CpG islands with lower GC content and with the ability to control gene expression.

2.2 Response to DNA double-strand breaks (DSBs)

2.2.1 Homologous recombination repair (HRR)

With aging there is an increase in DNA double-strand breaks [66]. However, it is unknown whether this increase is a consequence of accumulation of unrepaired DSBs or progressively delayed repair events, possibly as a reflection of an inherently limited capacity to process DSBs [67]. To repair this kind of DNA damage, HRR, considered a highly reliable pathway, allows the cell to access and copy information from the intact DNA sequence into the sister chromatid. Notably, HRR is restricted to late S to G2 phases when chromosomes are aligned [68]. RAD51 and other members of the RAD52 epistasis group as RAD50, MRE11, and XRS2 are needed for HRR. The efficiency of HRR is enhanced by mediator proteins that promote the loading of RAD51 onto ssDNA, RAD52 among them [69]. HR-mediated repair efficiency declines precipitously during cellular aging together with a decline of RAD51, RAD51C, RAD52, NBS1, CTIP, and MRE11 levels [66, 70]. Furthermore, in human and mice oocytes, a decrease in expression of BRCA1 and ATM [71] and an impaired recruitment of RAD51 to DNA damage sites during aging [72] were observed, which could force cells to utilize the error-prone NHEJ pathway. At the same time, in older mice a lower activity of the ATM kinase that results in less p53 phosphorylation was reported, thus affecting apoptosis, cell-cycle arrest, and senescence [73]. In addition to the above, the decrease in the levels of PARP1 [36] and SIRT6 [38] not only affects BER pathway but also has a relevant role in HRR since supplementation of recombinant SIRT6 was able to partly restore HR activity [70]. This could be related to a higher binding of DBC1 to PARP1 inhibiting its enzymatic activity as well as the change in NAD+ levels [74]. Decreased NAD+ levels observed with age also reduce activity of other sirtuins as SIRT1 and SIRT7 together with PARP1, reducing NHEJ and HRR pathways [75]. Although HRR is essential, its activity must be carefully controlled in order to maintain genomic integrity [76]. Previously, it has been demonstrated that frequency of recombinant cells is highly variable among tissues, from very low levels in the brain and stomach to very frequent in the pancreas and spleen. Additionally, de novo recombination events indeed accumulate in mice colonic somatic stem cells with age [77].

2.2.2 Nonhomologous end joined (NHEJ)

In human cells, NHEJ is the major pathway for the repair of DSBs, where two ends of DNA with little or no sequence homology are brought together and repaired. NHEJ can act throughout most of the cell cycle but predominantly in G1 phase [68]. NHEJ is divided into two subpathways: the classical NHEJ pathway (c-NHEJ), in which DNA-PKcs, Ku70/Ku80 heterodimers, Artemis, XRCC4, XLF, and DNA Ligase 4 are involved, and the alternative NHEJ pathway (alt-NHEJ), comprised of the repair factors PARP1 and DNA ligase 3 [78]. Both NHEJ pathways are associated with changes in DNA sequence, where c-NHEJ causes deletions and insertions, whereas alt-NHEJ propitiates the loss of genetic information between microhomologies on chromosomes [79]. NHEJ becomes inefficient and more error-prone during cellular senescence, thus favoring genomic instability and higher

incidence of cancer in the elderly [80, 81]. Furthermore, NHEJ-mediated VDJ recombination in B lymphocytes is impaired, reducing class switch recombination efficiency and contributing to reduced humoral repertoire and impaired immunity with aging [82]. Frequency of microhomology-mediated end joining (MMEJ) increases as a compensatory mechanism; however, at the same time, it favors that more mistakes are generated [81]. Ku 70 and 80 proteins decreased their expression at least twofold in two lines of senescent human fibroblast; at the same time, their localization was changed concentrating them in the nucleus when compared with young cells where they are present in both the nucleus and cytoplasm [83]. Cytoplasmic Ku proteins could serve as a reserve (pool) that is recruited to the nucleus upon DNA damage; therefore in senescent cells these proteins are unavailable to repair new lesions [25]. Additionally, binding activity of the Ku 70/80 heterodimers to broken DNA ends also declines with aging [66]. Notably, mice and cells deleted for either Ku70 or Ku80 exhibited not solely NHEJ disruption but also altered BER [84]. On the other hand, decreased expression of XRCC4, DNA ligase 4, and DNA ligase 3 has been observed, and this implicates that during the aging process, NHEJ becomes more inefficient and inaccurate, leaving more damage sites repaired with a loss of additional genetic information [72]. Interestingly, aging increases DNA-PK activity phosphorylating HSP90α and decreasing its chaperone function in AMPK, which is critical for mitochondrial biogenesis and energy metabolism [85]. Consistently, DNA ligase 4 and Ku80 gene promoters were frequently observed as hypermethylated in elderly people, which could be associated with the silencing expression of both genes [86]. However, as mentioned for other DNA repair mechanisms, caloric restriction diet improves NHEJ activity possibly through SIRT1 and FOXO activity [87].

3. Conclusions

Aging is a consequence of damage accumulation in different cellular constituents and where DNA damage is one of the most important. Every day there are thousands of insults that affect DNA, either due to endogenous factors (such as metabolism) or exogenous factor like contact with radiation sources or exposure to toxic substances; but only a minimal amount (less than 0.02%) accumulates as permanent damage, while the rest is totally repaired. However, if only one gene is not repaired and its function is important as that of a proto-oncogene, a tumor suppressor, or any DNA repair genes, this could lead to accumulation of mutations, and then DNA damage checkpoints can halt the cell cycle and induce cellular senescence or apoptosis, or well erroneous repair or replicative bypass of lesions can result in mutations and chromosomal aberrations leading the cells to transform into cancer cells.

Notably, DNA repair systems are able of dealing with most of the damages inflicted to DNA; however, their efficiency decrease with age, permitting that point mutations, insertions, deletions, and rearrangements, among others, occur more frequently and accumulate over time. This is due in part to the fact that critical proteins involved in DNA repair significantly decrease their expression in an age-related manner. In **Figure 2**, the main age-related changes reported over the different mechanisms of DNA repair together with their consequences that globally cause genomic instability and favor cellular senescence and cancer are summarized.

Overall, this area needs to be more exploited in order to improve our quality of life and prevent or delay the harmful effects of aging. Thus, the more knowledge we acquire about the natural cell aging process and its interrelation with the mechanisms of DNA repair, the closer we will be to develop drugs, therapies, or even vaccines that could help us to prolong our life.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Hepatocarcinoma Angiogenesis and DNA Damage Repair Response: An Update

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Abstract

Hepatocarcinoma is one of the most common lethal human malignant tumors, mainly because of active angiogenesis. This kind of high angiogenesis often accounts for early metastasis, rapid recurrence, and poor survival. Growing evidence has proved that hepatocarcinoma angiogenesis is closely associated with multiple risk factors, such as DNA damages resulting from hepatitis B and C virus infection, aflatoxin B1 exposure, ethanol intake, and obesity. Genetic alterations and genomic instability, probably resulting from low DNA damage repair response (DRR) and the following unrepaired DNA lesions, are also increasingly recognized as important risk factors of hepatocarcinoma angiogenesis. Dysregulation of DRRs and signaling to cell cycle checkpoints involving in DRR pathways may accelerate the accumulation of DNA damages and trigger the dysregulation of angiogenesis-related genes and the progression of hepatocarcinoma. In this review, we discussed DNA damages/DRRs and angiogenesis during hepatocarcinogenesis and their interactive regulations. Hopefully, the review will also remind the medical researchers and clinic doctors of further understanding and validating the values of DNA damages/DRRs in hepatocarcinoma angiogenesis.

Keywords: hepatocarcinoma, angiogenesis, DNA damage, DNA damage repair response

1. Introduction

Hepatocellular carcinoma, also termed as hepatocarcinoma, is one of the most common malignant tumors, with more than 500,000 new cases per year [1]. Until recently, it has been frequent to consider hepatocarcinoma as a tumor with low incidence in the western world but with high incidence in the eastern countries [1]. However, increasing data exhibit that the incidence of this tumor has increased in both western and eastern countries. Etiologically, several risk factors, including hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin B1 (AFB1), and alcohol, have been identified for increasing disease incidence worldwide [2]. Although molecular mechanisms of hepatocarcinoma caused by these risk factors have not still been clear, chronic and permanent liver damage and damage response may play a vital role. Macrocosmically, liver damage consists of a series of pathological changes, such as chronic hepatitis, liver cirrhosis, nodular hyperplasia, and

dysplasia [3]. Microcosmically, chronic DNA damage, including the formation of DNA adducts, DNA strand break and bulk, gene mutations, and genomic instability, is the most important type [4].

Because of early blood metastasis and high death rate of this malignancy, it has become the third most common cause of cancer-associated deaths worldwide. This death risk could be explained by high angiogenesis capacities of hepatocarcinoma [1, 2]. Increasing evidence has exhibited that hepatocarcinoma patients with high microvessel density (MVD) in tumor tissues would feature a poor prognosis, and angiogenesis has been regarded as an important marker predicting the risk of invasiveness and metastasis [5]. This chapter summarizes the latest findings in hepatocarcinoma angiogenesis, DNA damage, and damage repair response (DRR). We also try to shed light on the effects of DNA damage and dysregulation of DRR on tumor angiogenesis.

2. Angiogenesis and regulation in hepatocarcinoma

2.1 Angiogenesis process in hepatocarcinoma

Several previous reviews have summarized the angiogenesis in hepatocarcinoma [5–7]. In brief, angiogenesis is a kind of crucial biological function and survival potential for normal organism development, growth, and adaptation to new environment. The dynamic balance between increasing and decreasing potential of angiogenesis is essential in the different physiological and pathological conditions, such as injury cure, damage repair, inflammatory procession, tumor progression, blindness, and ischemia. Hepatocarcinoma angiogenesis was extensively studied via cell models, experimental animal models, and human tumor samples [5–7]. Accumulating data have proved that local hypoxia in tumor tissues and the change in genome resulting from genetic or environmental risk factors will lead to the secretion and synthetics of angiogenetic regulative factors and triggering angiogenesis [8–10]. In hepatocarcinoma tissues, the process of angiogenesis consists of the following several stages: sprouting, extracellular matrix component (ECMs) reconstruction, endothelial cell (EC) migration and proliferation, lumen formation, and stabilization of newborn vessels (**Figure 1**) [11].

The establishment of conditions allowing ECs proliferation and migration, which often results from local hypoxia, first facilitates endothelial sprouting and budding. During this stage, hypoxia induces the secretion and synthetics of angiogenetic factors, such as nitric oxide (NO), vascular endothelial growth factor (VEGF), CD31, angiopoietin-1, and so on [11]. The NO-induced vasodilation and VEGF-caused high permeability result in the extravasation of plasma components (including fibrinogen and fibrin). Together with ECMs, these plasma components lay down and form provisional scaffolds for migrating ECs. The basement membranes and ECMs (mainly consisting of collagen I and IV and laminin) are next degraded, and subsequently, ECs migrate into local sites and proliferate. Increasing proliferation of ECs in the local hypoxia tissues leads to the formation of nascent vessels with lumen. After that, nascent vessels are recruited and structurally stabilized under the conditions of physical forces and a series of molecules such as platelet-derived growth factor β (PDFG-β), angiopoietin-1, angiopoietin-2, VEGF, and transforming growth factor β1 (TGF-β1) [7, 11, 12].

Vessels in hepatocarcinoma differ from other liver diseases or normal vessels [5, 11, 13]. First, tumor vessels typically appear as irregular diameter and abnormal branching patterns [5]. Second, pericytes of vessels are often incompletely covered or lost; furthermore, their basement membranes are also incomplete [11]. Third, tumor vessels sometimes form irregular channels and the walls of these channels are

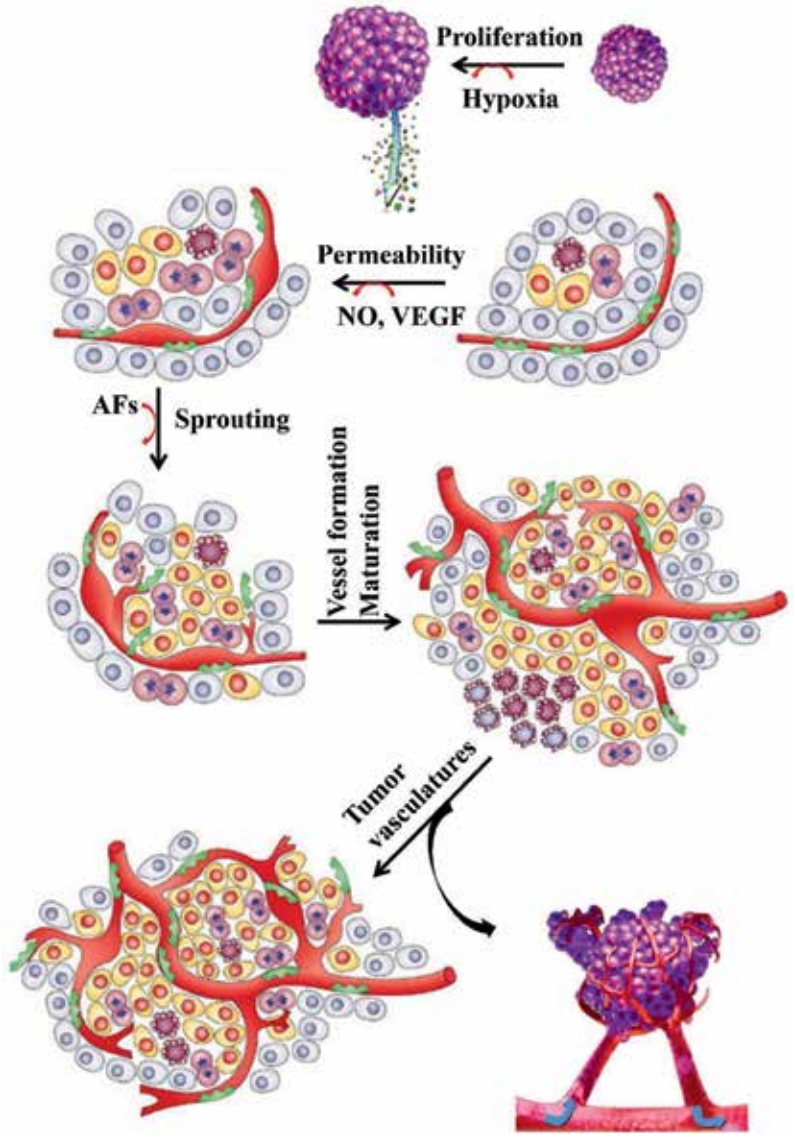

Figure 1.
Angiogenesis procession in hepatocarcinoma. The procession of angiogenesis consists of: (1) sprouting and budding; (2) ECM remodeling; (3) EC proliferation and migration; (4) lumen formation and three-D organization; and (5) stabilization of nascent vessels.

comprised of cancer cells. Moreover, the endothelial cells may be replaced by cancer cells partially or completely. Finally, angiogenesis in hepatocarcinoma not only appears abnormal architecture but also accompanies abnormal molecular expression and regulation [6, 14]. These characteristics result in abnormal structures and function for hepatocarcinoma; however, they can provide some important cues for early diagnosis and therapeutic strategies for cases with hepatocarcinoma.

2.2 Angiogenesis regulation in hepatocarcinoma

A series of angiogenic and antiangiogenic factors (**Tables 1** and **2**) regulate the angiogenesis process in hepatocarcinoma [5]. During the process of hepatocarcinoma angiogenesis, hypoxia and VEGF family play a vital role. Hypoxia in local

No.	Active factors	Effects	Process involved in hepatocarcinoma
AF01	NO	Stimulating vasodilation	Increasing vessel permeably
AF02	VEGF family members	(1) Increasing vascular permeability (2) inducing EC proliferation (3) Progressing leukocyte adhesion (4) Regulating neovascular lumen diameter	(1) Sprouting and budding (2) Vessel growth 3-D organization
AF03	VEGF-R	Integrate angiogenic and survival signals	Vessel growth
AF04	NRP-1	Integrate angiogenic and survival signals	Vessel growth
	Angiopoietins	Inducing EC proliferation	Vessel growth
	IL-4	Inducing EC proliferation	Vessel growth
	IL-8	Inducing EC proliferation	Vessel growth
	Hepatocyte growth factor	Inducing EC proliferation	Vessel growth
	Tissue factor	Inducing EC proliferation	Vessel growth
	Fibronectin	Progressing ECM remodeling	
AF05	Integrins avb3	(1) ECM receptors, intercellular communication (2) Mobilized during EC migration (3) Regulating neovascular lumen diameter	ECM remodeling and EC migration Newborn vessel stabilization
AF06	Integrins avb5	(1) ECM receptors, intercellular communication (2) Mobilized during EC migration (3) Regulating neovascular lumen diameter	ECM remodeling and EC migration Newborn vessel stabilization
AF07	Integrins a6b1	(1) ECM receptors, intercellular communication (2) Mobilized during EC migration (3) Regulating neovascular lumen diameter	ECM remodeling and EC migration Newborn vessel stabilization
AF08	uPA	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF09	Plasminogen activators	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF10	MMPs	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF11	Heparinases	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF12	chymases	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF13	Tryptases	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization
AF14	Cathepsins	(1) Remodeling ECM (2) Releasing and activating growth factors	ECM remodeling and EC migration Newborn vessel stabilization

No.	Active factors	Effects	Process involved in hepatocarcinoma
AF15	PlGF	Inducing EC proliferation	Vessel growth
AF16	aFGF	Inducing EC proliferation	Vessel growth
AF17	bFGF	Inducing EC proliferation	Vessel growth
	FGF-R1	Receptor for aFGF	Vessel growth
	FGF-R2	Receptor for bFGF	Vessel growth
AF18	HGF	Inducing EC proliferation	Vessel growth
	c-Met	Receptor for HGF	Vessel growth
AF19	TGF-a	Inducing EC proliferation	Vessel growth
AF20	TGF-b	Inducing EC proliferation	Vessel growth
	EGF-R	Receptor for TGF-a and TGF-b	Vessel growth
AF21	MCP-1 and other chemokines	Pleiotropic role in angiogenesis	Newborn vessel stabilization
AF22	MEF2C	Regulating neovascular lumen diameter	Newborn vessel stabilization
AF23	Ephrin's	Determining branching and arterial/venous specification	Newborn vessel stabilization
AF24	PDGF-B and receptors	Recruiting pericytes	Newborn vessel stabilization
AF25	Ang-1	(1) Stabilizing intercellular contacts (2) Inhibiting permeability	Newborn vessel stabilization
AF26	Ang-2	Ang-1 antagonist (destabilizes vessels; causes EC death)	Vessel regression
AF27	Tie-2	Receptor for Ang-1 and Ang-2	Newborn vessel stabilization
AF28	TGF- 1	(1) Promoting vessel maturation (2) stimulating ECM generation (3) Inducing differentiation of mesenchymal cells to pericytes	ECM remodeling and EC migration
AF29	Endoglin	(1) Promoting vessel maturation (2) stimulating ECM generation (3) Inducing differentiation of mesenchymal cells to pericytes	ECM remodeling and EC migration
AF30	Cyr61	(1) Stimulating directed migration of EC through an AVB integrin-dependent pathway (2) Acting as ECM modifiers (3) Promoting EC survival	ECM remodeling and EC migration
AF31	Fisp12	(1) Stimulating directed migration of EC through an AVB integrin-dependent pathway (2) Acting as ECM modifiers (3) Promoting EC survival	ECM remodeling and EC migration

Abbreviations: *VEGF, vascular endothelial growth factor; ECM, extracellular matrix component; EC, endothelial cell; PEDF, Pigment epithelium-derived factor; platelet and endothelial cell adhesion molecule 1; TIMPs, Tissue inhibitor of metalloproteases; IFN, interferon; MMPs, matrix metalloproteinases; Ang, angiopoietin; IL, interleukin; PIGF, placenta growth factor; HGF, hepatocyte growth factor; TGF, transforming growth factor; EGF, epidermal growth factor.*

Table 1.
Angiogenesis active regulative factors in hepatocarcinoma.

No.	Active factors	Effects	Process involved in hepatocarcinoma
IF01	Arrestin	Suppressing VEGF-regulating vessel growth	Vessel growth
IF02	Canstatin	(1) Interruption of stable cell-ECM connections (2) Inducing EC apoptosis	Vessel regression
IF03	Interleukin 12	Suppressing EC cell proliferation	Vessel growth
IF04	PEDF	Suppressing EC cell proliferation	Vessel growth
IF05	VE-cadherin	(1) Adhering junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF06	PECAM-1	(1) Adhering junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF07	Plakoglobin	(1) Adhering junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF08	b-Catenin	(1) Adhering junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF09	Claudins	(1) Tightening junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF10	Occludin	(1) Tightening junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF11	JAM-1	(1) Tightening junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF12	JAM-2	(1) Tightening junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF13	JAM-3	(1) Tightening junction molecules (2) Intercellular adhesion (3) Providing vessel tightness	Newborn vessel stabilization
IF14	Connexins	(1) Gap junction molecules (2) Facilitating intercellular communication	Newborn vessel stabilization
IF15	Integrins avb3	Suppressing VEGF- and Flk-1-mediated EC survival	Vessel growth
IF16	Integrins avb5	Suppressing VEGF- and Flk-1-mediated EC survival	Vessel growth
IF17	PAI-1	(1) Inhibiting ECM degradation by MMPs (2) Inhibiting EC proliferation	ECM remodeling and EC migration
IF18	TIMPs	(1) Inhibiting ECM degradation by MMPs (2) Inhibiting EC proliferation	ECM remodeling and EC migration
IF19	Angiostatin and related plasminogen fragments	Suppressing tumor angiogenesis	Vessel growth
IF20	Endostatin	Suppressing EC cell proliferation	Vessel growth

No.	Active factors	Effects	Process involved in hepatocarcinoma
IF21	Antithrombin III	Suppressing EC cell proliferation	Vessel growth
IF22	IFN-a	Suppressing EC cell proliferation	Vessel growth
IF23	IFN-b	Suppressing EC cell proliferation	Vessel growth
IF24	LIF	Suppressing EC cell proliferation	Vessel growth
IF25	PF4	Suppressing EC cell proliferation	Vessel growth
IF26	TSP-1	Inhibiting lumen formation	Vessel regression
IF27	Ang-1 (excess)	Making vessels too tight and inhibiting sprouting	Newborn vessel stabilization
IF28	Ang-2	Facilitating sprouting in the presence of VEGF	Vessel regression
IF29	sTie-2	Inhibitor for Ang-1 and Ang-2	Vessel regression
IF30	sFlt-1	Inhibitor for VEGF family	(1) Sprouting and budding (2) Vessel growth 3-D organization
IF31	Thrombospondin-1	Suppressing EC cell proliferation	Vessel growth
IF32	Thrombospondin-2	Suppressing EC cell proliferation	Vessel growth
IF33	Tumstatin	Suppressing EC cell proliferation	Vessel growth
IF34	Vasostatin	Suppressing EC cell proliferation	Vessel growth

Abbreviations: *VEGF, vascular endothelial growth factor; ECM, extracellular matrix component; EC, endothelial cell; PEDF, Pigment epithelium-derived factor; platelet and endothelial cell adhesion molecule 1; TIMPs, Tissue inhibitor of metalloproteases; IFN, interferon; MMPs, matrix metalloproteinases; Ang, angiopoietin.*

Table 2.
Angiogenesis inhibitive regulative factors in hepatocarcinoma.

tumor tissues, an important pathophysiological phenomenon caused by rapid growth of tumor, leads to the expression of hypoxia-inducible factor (HIF)-1α, which is a key inducible factor for angiogenesis in hypoxia tissues [7, 14]. On the one hand, HIF-1α can induce the expression of hypoxia-response-related genes like NO, VEGF, transforming growth factor (TGF) α and β, adrenomedullin (ADM), LDL-receptor-related protein 1 (LRP1), and leptin; on the other hand, local hypoxia status in tumor tissues also downregulates the expression of antiangiogenic factors such as thrombospondin-1 (TS1) and -2 (TS2) [15–17]. Additionally, growing literature has shown that lots of factors, including genetic or acquired alterations in the oncogenes (i.e., Ras, c-Jun, and Myc) and tumor suppressor genes (i.e., TP53), Hepatitis B Virus X (HBx) protein, chromobox 4, and DNA damage induced by chronic inflammation and AFB1 exposure, can increase the expression proangiogenic factors [18–23]. For example, HBx protein has a potential for increasing HIF-1α expression via promoting transcriptional and translational activity and therefore accelerating angiogenesis during carcinogenesis process of hepatocarcinoma [24]. Recent studies have reported that chromobox 4 (a known transcriptional regulator and also a SUMO E3 enzyme) can promote angiogenesis via stabilizing HIF-1 in hepatocarcinoma [18, 19]. VEGF (including its glycoprotein family members VEGF-A, -B, -C, and -D) is another important angiogenic factor that always upregulates in most cases with hepatocarcinoma [5]. The upregulation of VEGF in hepatocarcinoma is proved not only to increase tumor neovascularization but also to accelerate tumor growth via in vitro cell experiments and animal

models. The role of VEGF is mediated mainly by two receptors: VEGF-R1 (also called Flt-1) and VEGF-R2 (also termed as KDR/Flk-1). Both VEGF-R1 and VEGF-R2 have tyrosine kinase activity and are normally expressed in hepatic parenchyma cells including endothelial cells of portal and sinusoidal tracts [5, 6]. In hepatocarcinoma, both mRNA and protein amount of them are increasing noticeably in the tumor tissues compared to peri-tumor tissues [25]. Some other factors, such as angiopoietin 1 and 2, involve in the regulation of angiogenesis in hepatocarcinoma (**Tables 1** and **2**) [5, 6, 13]. Together, increasing angiogenic potential but decreasing antiangiogenic potential facilitates hepatocarcinoma angiogenesis.

2.3 Angiogenesis biomarkers in hepatocarcinoma

In the past decades, several biomarkers, such as VEGF, angiogenin, and MVD, have been selected for elucidating angiogenic potential of hepatocarcinoma. **Table 3** summarized the potential of these biomarkers for hepatocarcinoma angiogenesis and angiogenesis-related tumor biological actions. Among these biomarkers, VEGF is concerned especially because of its clinic significance. For example, a hospital-based clinic samples analyses (including 7 cases with liver low-grade dysplastic nodule [DN], 8 cases with liver high-grade DN, 11 cases with early hepatocarcinoma, 17 cases with small hepatocarcinoma, and 21 cases with advanced hepatocarcinoma) by Park et al. [26] showed that the amount of VEGF increased gradually from low-grade DN to early hepatocarcinoma. Furthermore, this increasing expression of VEGF is significantly associated with neoangiogenesis (marked by MVD with CD34 staining) and cancer cell proliferation. Collectively, we can conclude that increasing VEGF expression and MVD are positively associated with tumor vascularization and the following tumor progression and poor survival of tumor cases. Furthermore, increasing evidence has exhibited that serum levels of VEGF are not only parallel with the amount in tumor tissues but also can predict therapy response of patients with hepatocarcinoma [29–32]. Thus, VEGF may be useful for improving therapeutic strategies of hepatocarcinoma based on the angiogenesis thesis.

No.	Study design	Samples	Results	Ref#
1	Hospital-based sample study	LGDs (n = 7), HGDs (n = 8), eHCCs (n = 11), shocks (n = 17), and aHCCs (n = 21)	(1) VEGF expression increases gradually from LGD to eHCC. (2) The sHCCs has an increasing neoangiogenesis and cell proliferation compared to aHCCs. (3) The levels of VEGF expression are positively associated with MVD (marked by CD34 staining).	[26]
2	Hospital-based sample study	HCCs (n = 60)	Amount of VEGF in the serum of patients positively correlates with that in the tumor tissues.	[27]
3	Hospital-based sample study	HCs (n = 20), CHs (n = 36), LCs (n = 77), and HCCs (n = 86)	Plasm VEGF levels are increasing in patients with HCC compared to in non-HCCs and this increase will more noticeable in cases with metastasis HCCs.	[28]
4	Hospital-based sample study	HCs (n = 30), LCs (n = 26), and HCCs (n = 52)	Plasm VEGF levels are increasing in patients with HCC compared to in non-HCCs and this increase will shorten the survival of HCCs.	[29]
5	Prospective study	HCCs (n = 100)	Plasm VEGF levels of HCC cases are related to tumor stage, postoperation recurrence, and blood invasion.	[30]

No.	Study design	Samples	Results	Ref#
6	Hospital-based sample study	HC (n = 15) and HCCs (n = 98)	Serum VEGF is a significant biomarker for HCC survival (including OS and RFS).	[31]
7	Prospective study	HCCs (n = 80)	Serum VEGF levels were correlated with clinical data, tumor response to TACE and survival results.	[32]
8	Hospital-based sample study	HCCs (n = 48)	TACE treatment can upregulate expression and bFGF in HCC tissues possibly due to hypoxia and ischemia.	[33]
9	Hospital-based sample study	HCCs (n = 38)	TACE treatment can upregulate expression and bFGF in HCC tissues possibly due to hypoxia and ischemia.	[34]
10	Hospital-based sample study	HCCs (n = 41)	Angiogenin mRNA in serum and tumor tissues positively associating with MVD and poor prognosis of cases	[35]
11	Hospital-based sample study	HCCs (n = 90)	MMP-2, MMP-9 and VEGF expression is positively correlated to the prognosis of HCC patients.	[36]
12	Hospital-based sample study	HCCs (n = 30)	The serum levels of Ang-2, HGF, IL-8, PDGF-BB, and VEGF were correlated with poor effects of sorafenib treatment in patients with HCC.	[37]
13	Hospital-based sample study	CHs (n = 79) and HCCs (n = 89)	(1) TEMs are involved in HCC angiogenesis. (2) The frequency of circulating TEMs was significantly higher in HCC than non-HCC patients. (3) The TEMs have higher diagnostic value for HCC than AFP, PIVKA-II and ANG-2.	[38]
14	Animal model	/	Mobilized EPCs participate in tumor angiogenesis of HCC	[39]

Abbreviations: *LGDs, patients with low-grade dysplasia; HGD, patients with high-grade dysplasia; eHCCs, patients with early hepatocellular carcinoma; HCC, hepatocellular carcinoma; sHCCs, patients with small HCC; aHCCs, patients with advanced HCC; HCCs, patients with HCC; HCs, healthy controls; LCs, patients with liver cirrhosis; VEGF, vascular endothelial growth factor; MVD, microvessel density; OS, overall survival; RFS, tumor reoccurrence-free survival; TACE, transarterial chemoembolization; bFGF, basic fibroblast growth factor; EPCs, bone marrow-derived endothelial progenitor cells; TEMs, TIE2-expressing monocytes/macrophages; Ang-2, angiopoietin-2; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL-8, interleukin-8.*

Table 3.
The potential of biomarkers for hepatocarcinoma angiogenesis and angiogenesis-related tumor biological actions.

3. DNA damage and DRR in hepatocarcinoma

3.1 DNA damage induced by risk factors for hepatocarcinoma

Multiple risk factors, including HBV and HCV infection, AFB1 exposure, ethanol consumption, and obesity, have been reported to correlate with hepatocarcinogenesis (**Figure 2**) [4]. These risk factors can induce multiple types of DNA damage, such as DNA single-stand break (SSB), double-strand break (DSB), base damage, DNA-adduct formation, oxidation damage, gene mutation, chromosomal aberration, and genomic instability [4]. Results from epidemiological and experimental studies show that viral-DNA damage relationship is characterized by:

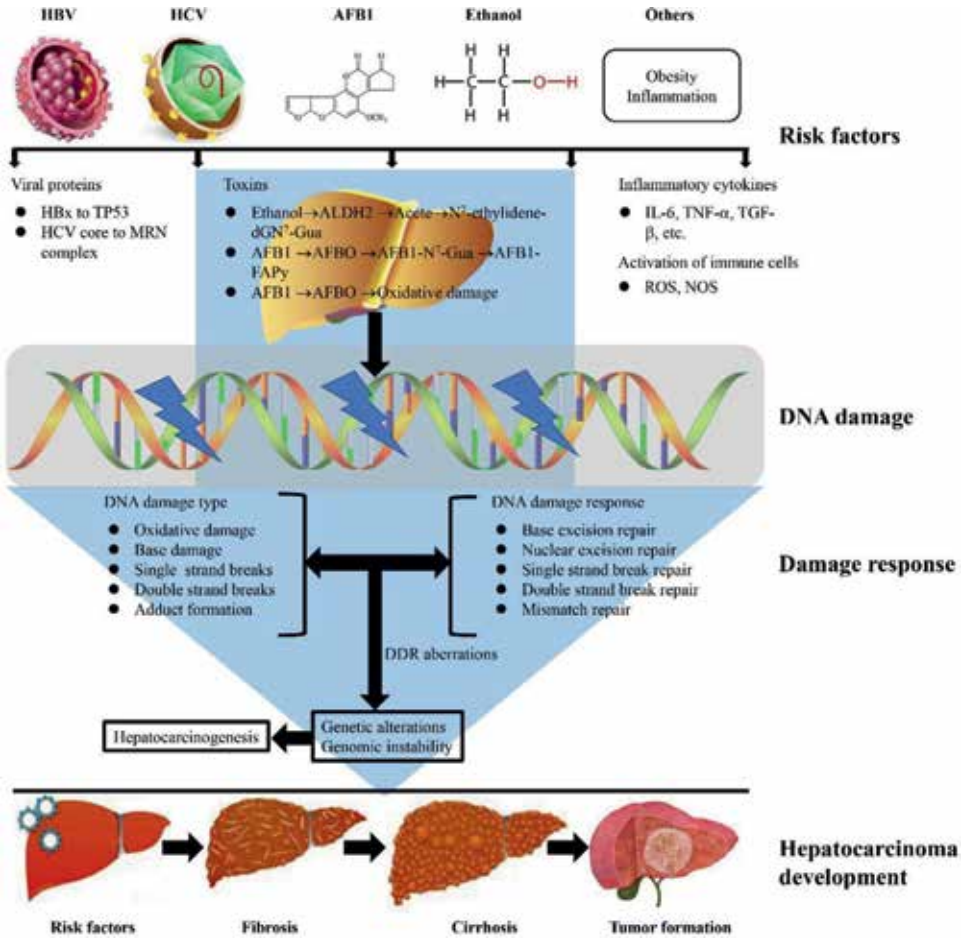

Figure 2.
Risk factors-induced DNA damage and damage repair response during hepatocarcinoma. **Abbreviations***: HBV, hepatitis B virus; HCV, hepatitis C virus; AFB1, aflatoxin B1; AFBO, AFB1-8,9-epoxide; IL, interleukin; TGF, transforming growth factor.*

(1) the integration of viral gene (such as HBx gene) into the genome of liver cells and resulting genomic instability of host cells [21, 24, 40, 41]; (2) TP53 mutation conducted by HBx integration resulting in abnormal cell response, including DNA repair, cell proliferation and cycle, and apoptosis potential [22]; (3) HCV core interfering the formation of Mre11/Rad50/Nbs1 (MRN) complex through the bind with Nbs1 [5]; (4) the inhibition of such DNA repair proteins as Ataxia telangiectasia mutated kinase (ATM) [42, 43]; and (5) inducing dysregulation of signal pathways, including Wnt/β-catenin pathway, sex steroid pathway, p38MAPK pathway, PI3K/Akt pathway, transforming growth factor β (TGFβ) pathway, NF-κB pathway, and so on [11].

For AFB1-induced DNA damage, adducts formation and gene mutations are concerned especially [44]. AFB1 is a known I-type chemical hepatocarcinogen produced mainly by *A. parasiticus* and *A. flavus* and a suspected risk factor for hepatocarcinoma in some dependent areas such as Sub-Saharan area, the southeast region of Asia, and the coast of southeast China. Results from prospective epidemiological and animal studies have exhibited that AFB1-induced DNA damage plays a vital role in the process of hepatocarcinoma caused by AFB1 exposure [40, 45]. Studies of AFB1 metabolism have further proved that cytochrome P450 (CYP) enzymes

in hepatocytes can facilitate AFB1 into its epoxy compound, also termed as AFB1-8,9-epoxide (AFBe). AFBe can covalently bind to genomic DNA and ultimately induce multiple types of DNA damage [46, 47]. Increasing evidence exhibits that AFB1 can multiplicatively interact with HBV and/or HCV infection during hepatocarcinogenesis, and that, this multiplicative interaction may be associated with more noticeable DNA damage induced by both AFB1 exposure and HBV/HCV infection [23]. Epidemiological studies based on the case-control design with a large sample have proved that patients with chronic virus hepatitis (including B and C type) will feature increasing hepatocarcinoma risk under the conditions of high AFB1 exposure [46]. Furthermore, patients with high AFB1 often companies with chronic virus infection and faces higher frequency of gene mutation like TP53 and ras [47]. Interestingly, the mutation at the codon 249 of TP53 gene, namely G:C > T:A mutation resulting in the change of arginine to serine, has been identified as a relatively specifically change and named AFB1-induced hot-spot mutation [44]. This mutation may lead to the dysfunction of TP53 protein and abnormal cell actions like promoting cell growth, inhibiting cell apoptosis, and inhibiting transcription mediated by TP53 [40].

Other risk factors like alcohol intake also cause malignant transformation of hepatocytes. Chronic ethanol intake will significantly increase hepatocarcinoma risk (about five times) if more than 80 g/day × 10 years. Actually, less than this amount of uptake also increases cancer risk in spite of nonsignificance [48]. Although mechanisms are not still clear, increasing data have shown that chronic hepatic injury, abnormal regeneration, and cirrhosis may act some role in hepatocarcinogenesis [4]. Pathological and molecular biological studies display that acetaldehyde, an important metabolic product of ethanol, can bind to DNA and form DNA adducts. The DNA adduct formation caused could trigger replication errors and/or mutations in tumor suppressor genes and/or oncogene [4]. Additionally, oxidative DNA damage is more noticeable in tissues with hepatocarcinoma than peri-tumor tissues [40, 46]. However, it is unclear whether acetaldehyde-DNA adducts and oxidative damages are true carcinogens and how they trigger hepatocarcinogenesis [4, 49]. Therefore, future studies on DNA damage are needed to better validate these risk factors and detailed molecular mechanisms.

3.2 DRR in hepatocarcinoma

DNA damage will trigger DRR pathways, a kind of prompt signal event which can harmonize whether cells obtain cycle arrest for DNA repair or induce death for eliminating cells with severe DNA damage and genomic instability [4]. In human, cells develop several types of surveillance mechanisms consisting of SSB repair (SSBR), DSB repair (DSBR), base excision repair (BER), base mismatch repair (MMR), and nucleotide excision repair (NER) (**Figure 2**) [4, 40]. Among these DNA repair pathways, BER, MMR, and NER can repair base damage such as base mismatches, AFB1-DNA adducts, DNA pyrimidine dimers, and DNA damage induced by irradiation and anticancer drugs. SSBR can repair SSB that is a severe DNA damage, if not repaired quickly, will disrupt genic transcription and replication and ultimately results in lethal DNA damage [40]. DSBR pathway involves in homologous recombination (HR), single-strand annealing (SSA), and nonhomologous end joining (NHEJ). HR pathway can repair DSBs through an accurate repair method using the undamaged homologous chromosome or sister-chromatid as DNA repair temple; whereas NHEJ and SSA pathways are nonhomologous repair methods and usually lead to essential mutagenesis, so far

DRR pathway gene/ proteins	DRR pathway	Abnormal of DRR	Effects on hepatocarcinoma	Ref#
hOGG1	BER	Ser to Cys at codon 326	Increased hepatocarcinoma risk	[51]
XRCC1	BER and SSBR	Arg to His at codon 280 Arg to Gln at codon 399 Arg to Trp at codon 194	(1) Increasing individuals' susceptibility to HBV infection (2) Increasing individuals' susceptibility to hepatocarcinoma (3) Increasing amount of AFB1-DNA adducts in liver tissues (4) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (5) Increasing the frequency of TP53M (6) Increasing MVD	[52–56]
XRCC3	DSBR	Thr to Met at codon 241 rs1799796 A > G	(1) Increasing individuals' susceptibility to hepatocarcinoma (2) Increasing amount of AFB1-DNA adducts in liver tissues (3) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (4) Increasing the frequency of TP53M (5) Associating with hepatocarcinoma clinicopathological features (6) Increasing MVD	[57–59]
XRCC4	DSBR	rs28383151 G > A Ala to Ser at codon 247	(1) Increasing individuals' susceptibility to hepatocarcinoma (2) Increasing amount of AFB1-DNA adducts in liver tissues (3) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (4) Increasing the frequency of TP53M (5) Associating with hepatocarcinoma clinicopathological features (6) Increasing MVD	[20, 21, 60–63]
XRCC5	DSBR	rs16855458 C > A rs9288516 T > A XRCC5 expression	(1) Increasing individuals' susceptibility to HBV infection (2) Increasing individuals' susceptibility to hepatocarcinoma (3) Associating with biological actions of hepatocarcinoma cells, such as increasing XRCC5 expression inhibiting cancer cells proliferation (4) Functioning as a tumor suppressor by inducing S-phase arrest in a TP53-dependent pathway	[64–69]
XRCC6	DSBR	XRCC6 expression	(1) Increasing individuals' susceptibility to hepatocarcinoma (2) Decreasing Toll-like receptor 4 (TLR4) against hepatocarcinogenesis (3) Increasing DNA damage, and promoting programmed cell death in TLR4-deficient livers (4) Early diagnostic value for hepatocarcinoma	[70–73]

DRR pathway gene/ proteins	DRR pathway	Abnormal of DRR	Effects on hepatocarcinoma	Ref#
XRCC7	DSBR	rs7003908 T > G	(1) Increasing individuals' susceptibility to AFB1 exposure (2) Increasing individuals' susceptibility to hepatocarcinoma (3) Increasing amount of AFB1-DNA adducts in liver tissues (4) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (5) Increasing the frequency of TP53M (6) Interacting with AFB1 exposure during hepatocarcinogenesis (7) Increasing MVD	[21, 74, 75]
DNA-PKcs	DSBR	Amount in liver tissues	Implying hepatocarcinoma-specificity	[76]
TP53	DRR pathway	Genic mutations such as TP53M, Arg to His at codon 273, Arg to His at codon 175, Cys to Tyr at codon 135, and Arg to Trp at codon 248	1. Implying individuals' AFB1 exposure 2. Associating with hepatocarcinoma risk 3. Increasing individuals' susceptibility to hepatocarcinoma 4. Decreasing DRR potential and increasing DNA damage	[40, 45, 77, 78]
XPC	NER	XPC expression Lys to Gln at codon 939	(1) Increasing individuals' susceptibility to hepatocarcinoma (2) Increasing amount of AFB1-DNA adducts in liver tissues (3) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (4) Increasing the frequency of TP53M and decreasing DRR potential (5) Associating with hepatocarcinoma clinicopathological features (6) Increasing XPC expression (7) Increasing MVD	[21, 79–81]
XPD	NER	Lys to Gln at codon 751	(1) Increasing individuals' susceptibility to hepatocarcinoma (2) Increasing amount of AFB1-DNA adducts in liver tissues (3) Increasing amount of adducts (including AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs (4) Increasing the frequency of TP53M and decreasing DRR potential (5) Interacting with gender during hepatocarcinoma (6) Increasing MVD	[21, 82]
Rad50	NER	Rad50 hook domain	Strongly influencing Mre11 complex-dependent DRR signaling, tissue homeostasis, and tumorigenesis	[83]
Nbs1	NER	Rs1805794 C > G Mutations in Nbs1	(1) Increasing hepatocarcinoma risk (2) Associating with TP53 inactivation	[84–87]
PARP-1	BER	DRR potential	(1) Modifying biological actions of hepatocarcinoma cells (2) A novel promising diagnostic marker for hepatocarcinoma	[88–90]

DRR pathway gene/ proteins	DRR pathway	Abnormal of DRR	Effects on hepatocarcinoma	Ref#
Rad10	NER	rs11615 C > T ERCC1–4533 G > A ERCC1–8092 C > A	(1) Increasing hepatocarcinoma risk (2) The amount of ERCC1 expression in tissues with hepatocarcinoma decreases cancer cells' sensitivity on anti-cancer drugs (3) Predicting the outcome of hepatocarcinoma patients receiving TACE treatment	[91–93]
ATM	HR and ENEJ	Ser to Ala at codon 1981 Ser to Ala at codon 1893 Ser to Ala at codon 367 Ser to Ala at codon 2996 Autophosphorylation at codon 1981 Ser	(1) The functional deficiency in radioresistant DNA synthesis and substrate phosphorylation such as TP53, Chk2, Nbs1, and SMCI (2) Increasing cells' sensitivity to risk factors and risk factors-induced DNA damage such as adduct formation and chromosome aberrations (3)The functional dysregulation for G2/M checkpoint (4) Extending activations of DNA damage signaling pathways to reach S phase arrest in hepatocarcinoma cells (5) Leading to ATM unable to be released from other ATM molecules, and increasing gene mutation risk	[94–100]

Abbreviations: *hOGG1, human oxoguanine glycosylase 1; XRCC1, X-ray repair cross complementing 1; BER, base excision repair; SSBR, single-strand break repair; HBV, hepatitis B virus; XRCC3, X-ray repair cross complementing 3; AFB1, aflatoxin B1; DSBR, double-strand break repair; WBC, white blood cell; TP53M, hot-spot mutation at codon 249 of TP53 gene; DNA-PKcs, DNA-activated protein kinase catalytic subunit; XRCC4, X-ray repair cross complementing 4; XRCC5, X-ray repair cross complementing 5; XRCC6, X-ray repair cross complementing 6; XRCC7, X-ray repair cross complementing 7; XPC, xeroderma pigmentosum, complementation group C; XPD, xeroderma pigmentosum, complementation group D; NER, nucleotide excision repair; PARP-1, poly(ADP-ribose) polymerase 1; ATM, Ataxia telangiectasia mutated kinase.*

Table 4.
The association between abnormal DRR potential and hepatocarcinogenesis.

as to induce chromosomal aberrations, abnormal cell cycle, and/or uncontrolled cell proliferation [50]. During DRR pathways, DNA repair genes play a central role [4]. Dysregulation of DRR caused by DNA repair genic mutations or low DNA repair capacity will increase hepatocarcinoma risk. **Table 4** summarized the effects of abnormal DRR in hepatocarcinogenesis. This evidence shows that dysregulation of DRR resulting from mutations in DNA repair genes and corresponding dysfunctions may promote hepatocarcinogenesis through the following pathways: (1) increasing individuals' susceptibility to risk factors such as hepatitis virus infection and AFB1 exposure [40, 60, 101]; (2) increasing individuals' susceptibility to cancer [45]; (3) increasing amount of carcinogens-DNA adducts in liver tissues [40]; (4) increasing amount of adducts (such as AFB1-DNA and AFB1-albumin adducts) in the peripheral WBCs and affecting immune reaction [61]; (5) increasing the frequency of tumor suppressor genes or oncogenes like Ras and TP53M [40, 47, 52, 61, 79]; and (6) interacting with risk factors during hepatocarcinogenesis [23]. Thus, the potential of DRR pathways should play an important function for hepatocarcinogenesis.

4. Hepatocarcinoma angiogenesis induced by DRR

Risk factors induced DNA damages and dysregulated DRRs are regarded as molecular events [4]. In human, risk factors for hepatocarcinoma can manifest acute and chronic DNA damage. Acute and noticeable DNA damages often lead to severe chromosome aberration and even cell death, whereas chronic DNA damages are the earliest molecular change in hepatocytes and ultimately result in hepatocarcinoma [40]. In the past decades, angiogenesis induced by dysregulation of DRR pathways may act as a vital role in the process of hepatocarcinoma. Evidence from epidemiological and clinicopathological studies has shown that higher potential of angiogenesis is in the liver of patients with chronic DNA damage and low DRR capacity [40, 102–105]. For example, Pastukh et al. [102] investigated the association between recruitment of DNA repair enzymes involving in BER pathway and VEGF expression via a chromatin immunoprecipitation technique. They found that hypoxia-induced reactive oxygen species (ROS) stress caused promoter base modifications targeted to hypoxic response elements (HREs) and increased VEGF expression. During this modification, 8-oxoguanine (8-oxodG, an oxidative DNA damage product) in VEGF promoter was temporally correlated with binding of human 8-oxodG glycosylase 1 (hOGG1, a BER repair enzyme), HIF-1α, redox effector factor-1, endonuclease one, and breaks in DNA strands. If 8-oxodG was decreased in the promoter region of VEGF, VEGF expression would downregulate [102]. Recent molecular epidemiological studies have further proved that genetic variants in hOGG1 genes increase hepatocarcinoma risk and modify the prognosis of this malignancy [103–105]. Collectively, these data suggest that increasing ROS like 8-oxodG resulting from low DRR capacity may promote angiogenesis.

Studies from high HBV and HCV infection and high AFB1 exposure area also display that the degrees of DNA damages are positively associated with MVD in tumor tissues from hepatocarcinoma [20, 55, 75, 79, 82]. For example, Lu et al. [20] investigated the effects of XRCC4 expression in tumor tissues on clinicopathological features and prognosis of hepatocarcinoma and found that decreasing XRCC4 expression was related to low DRR capacity, causing the formation of DNA adducts and TP53M. The dysregulation of XRCC4 may promote tumor proliferation and increase MVD. Several other studies further show that the low DRR capacity resulting from significant mutations in coding region of DNA repair genes (such as XRCC4, XRCC1, XPC, XPD, and XRCC7) increases MVD (**Table 4**) [21, 40, 52, 55, 59, 61, 62, 79, 80, 82]. Results from Lu et al. [20] and our studies [61, 62] showed that genetic alterations in the coding regions of XRCC4 gene (including Ala to Ser at codon 247 and Thr to Ala at codon 56) can decrease levels of XRCC4 protein expression and cause increasing amount of AFB1-DNA adducts and mutative frequency of TP53 gene in tissues with hepatocarcinoma. They also found that the amount of AFB1-induced DNA adducts, including 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB1 (AFB1-N^7-Gua) and formamidopyridine AFB1 adduct (AFB1-FAPy), was positively associated with the number of microvessels (a biomarker for angiogenesis). Results from our studies [79, 106, 107] furthermore displayed that three low DNA repair markers related to AFB1, including tumor risk, TP53M frequency, and AFB1-FAPy adduct amount, were significantly correlated with the number of microvessels in liver tissues. These individuals with high AFB1-FAPy adduct level in liver tissues had an increasing risk of high MVD than those low adduct level (OR = 1.68, 95% CI = 1.45–2.87) [106]. Liu et al. [108] and Wang et al. [109] further proved that the upregulation of microRNA-429 and microRNA-24 expression in tissues with hepatocarcinoma not only increased the amount of AFB1-DNA adducts

and the number of microvessels but also grew tumor metastasis risk via vessels and shorted patients' survival. Recent evidence has shown that microRNA-24/microRNA-429 can modify the capacity of DDR via controlling Nbs1 (a regulator of DRR) [110, 111] and angiogenesis via regulating the crosstalk between the pro-contractile transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP) signal (inducing a quiescent 'contractile' phenotype) and the pro-synthetic platelet-derived growth factor (PDGF) signal (causing a proliferative 'synthetic' phenotype) [112, 113]. This suggests that microRNA-24/microRNA-429 may play an important regulative role between DRR capacity and angiogenesis. Taken together, this evidence proves that low DRR-induced MVD augmentation is regulated by the amount of DNA damage.

Evidence from in vitro and in vivo studies further shows that dysregulation of DRRs and signaling to cell cycle checkpoints (CCCs) may modify hepatocarcinoma angiogenesis. CCCs involving in DRRs mainly encompass G1/S and G2/M checkpoint [114]. During G1/S checkpoint, both ATR and ATM act as central activators for DRR via inducing the phosphorylation of p53 protein which can activate p21 (a Cdk inhibitor). ATM/TP53/P21 pathway also plays an important function controlling G2/M procession [114]. The dysregulation of these factors and signal pathways can change the status of angiogenesis [115–119]. For example, Qin et al. [115] found that E2F1, an important cell cycle regulator, can modify angiogenesis via controlling VEGF expression by p53-dependent way. In this control model, deficient phenotype of E2F1 will result in VEGF overexpression, while its positive phenotype decreases VEGF expression [115]. Factors controlling cell shape and cytosol can regulate the cycle of vessel endothelial cells and angiogenesis [116, 117]. In mice model with the deficiency of BCL-2 (an important regulatory factor in DDRs), cells featured increasing DNA damage [118]; the inhibition of BCL-2 will result in the arrest of cells in S phrase and suppression of tumor angiogenesis [119]. In an integrated genomic study (including 5 hepatocarcinoma patients with hepatitis D visus [HDV] and 7 HDV-positive cirrhosis cases), Diaz et al. [120] investigated the association between HDV-related hepatocarcinoma and potential signal pathways involved in DNA damage and repair and cell cycle and found significant interactions of DDR/cell cycle-related genes, such as BRCA1, BARD1, CDK1, CDKN2C, CCNA2, CCNB1, CCNE2, GSK3B, H2AFX, MSH2, NPM1, PRKDC, and TOP2A. Results from the t-SNP (*t*-distributed stochastic neighbor embedding analyses) further exhibited that HUS1, BRCA1, BARD1, GADD45, DNA-damage-induced 14-3-3σ, and MSH2 gene involving in DRRs valuably scored with regulatory genes (such as ATM, TP53, NO, and epidermal growth factor), which involve in G2/M checkpoint and angiogenesis [120]. The dysregulation of HUS1 and corresponding genotoxin-activated checkpoint complex (also termed as Rad9-Rad1-Hus1complex) will cause abnormal DRR capacity and cell cycle in response to DNA damage and promote the alteration of hematogenous metastatic phenotype for hepatocarcinoma [121, 122]. The genetic alterations and abnormal expression of BRCA1 and GADD45 (two important regulatory factors in DRR and apoptosis pathways) in hepatocytes can also change TP53-dependent CCCs and VEGF expression [123, 124]. Altogether, these studies have proved that the dysregulation of DDRs can cause the abnormal regulation of CCCs and change the status of hepatocarcinoma angiogenesis.

Detailed molecular mechanisms of DRR dysregulation promoting hepatocarcinoma angiogenesis have still not been fully understood. Several possible pathways may play some important roles. First, DNA damage agents induce NO synthase and increase the expression of VEGF and HGF [125, 126]. Second, DNA damage agents like AFB1 cause the mutations of such genes as TP53, ras, and DNA repair genes. Activation of oncogenes and inactivation of tumor suppression genes and

DNA repair genes lead to uncontrolled expression of genes involving in angiogenesis such as VEGF and Ang-1/2 [5, 6]. Third, genetic alterations in DRR pathways may alter the microenvironment of tumor and promote angiogenesis [127–129]. Fourth, the abnormal DRRs may accelerate the accumulation of DNA damages and trigger the dysregulation of angiogenesis-related genes and the progression of hepatocarcinoma. Finally, some metabolic products (such as AFBO) or nucleotide sequences (HBx) of DNA agents can bind to genomic DNA of hepatocytes and may increase the activation of VEGF HREs [22, 40, 41, 45]. Taken together, under the conditions of low DRR capacity and/or chronic risk factors, DNA damages will accumulate in hepatocytes and ultimately induce hepatocarcinogenesis and tumor angiogenesis.

5. Summary and further direction

Abnormal angiogenesis and DNA damages/DRRs are two important pathophysiological events in the process of hepatocarcinogenesis. Recently, it has become a growing evidence of DNA damage and repair and angiogenesis in hepatocarcinogenesis. Low DRR capacity resulting genetic or obtained alterations may lead to the accumulation of DNA damages and induce angiogenesis and ultimately promote hepatocarcinoma development. The main challenge for this field is the explanations of molecular basis and regulative signal pathways of DNA damages/DRRs interacting with angiogenesis during hepatocarcinogenesis. A better understanding of hypervascular feature and corresponding mechanisms of hepatocarcinoma on the basis of DNA damage/DRR pathway may be helpful for the medical researchers and clinic doctors exploring and validating hepatocarcinogenesis but also for them designing safe and efficient antiangiogenic drugs.

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Abbreviations

AFB1	aflatoxin B1
Ang-2	angiopoietin-2
ATM	ataxia telangiectasia mutated kinase
BER	base excision repair
bFGF	basic fibroblast growth factor
DNA-PKcs	DNA-activated protein kinase catalytic subunit
DSBR	double-strand break repair
DRR	DNA damage repair response
G-CSF	granulocyte colony-stimulating factor
HBV	hepatitis B virus
HCV	hepatitis C virus
MVD	microvessel density
NER	nucleotide excision repair
HGF	hepatocyte growth factor
hOGG1	human oxoguanine glycosylase 1
IL-8	interleukin-8
PARP-1	poly(ADP-ribose) polymerase 1
SSBR	single-strand break repair
TEMs	TIE2-expressing monocytes/macrophages
TP53M	hot-spot mutation at codon 249 of TP53 gene
VEGF	vascular endothelial growth factor
XRCC1	X-ray repair cross complementing 1
XRCC3	X-ray repair cross complementing 3
XRCC4	X-ray repair cross complementing 4
XRCC5	X-ray repair cross complementing 5
XRCC6	X-ray repair cross complementing 6
XRCC7	X-ray repair cross complementing 7
XPC	xeroderma pigmentosum, complementation group C
XPD	xeroderma pigmentosum, complementation group D

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Chapter 10

Natural Drugs in DNA Repair

Thulasi G. Pillai, Cherupally K. Krishnan Nair and P. Uma Devi

Abstract

Natural products have been used in medicine right from the ancient civilisation. Natural products are used in many types of diseases, together with chemotherapy and radiotherapy. Many products are used against cancer. Many diseases are genetically derived. The drugs which have the capacity to act at genome level gains significant importance in any disease scenario. The genetic information essential for the identity and function of eukaryotic cells exist in DNA and during the lifetime of the cell DNA can be repeatedly damaged due to different factors. The stability and the fidelity of the replication process are meant to be the most remarkable features of the genetic material. The stability can be affected at any time. Compound which can enhance the DNA repair are applicable in many disease condition. Our study was focussed on the DNA repair enhancing property of a glucan from the macro fungi *Ganoderma lucidum*. Comet assay and chromosomal aberrations in mouse bone marrow were used as end points of study. Glucan was found to have DNA repair enhancing property in human lymphocytes.

Keywords: natural products, *Ganoderma lucidum*, glucan, mushroom, DNA repair

1. Introduction

The word 'Natural' has gained tremendous importance in the twenty-first century. Products obtained from nature are known to be natural. The Father of Medicine, Hippocrates has quoted that 'Let your food be our medicine and medicine be our food'. The incorporation of medicinal herbs and extract as food has been practiced long ago. In the present scenario, herbals are seen as potential medicine for a variety of diseases often viewed to super cede the pharmacological efficacy of allopathic drugs [1]. Natural products has become an extremely valuable commodity for the world today. The developing countries miss the modern medicine as they cannot afford it. Natural drugs were already there is use in Chinese medicine, Indian Ayurveda, Arabic Unani medicine and various other indigenous medicine. The two most important classics describing about more than 700 botanicals along with their classification, pharmacological and therapeutic properties are Charak Samhita and Sushrut Samhita (100–500 BC) [2, 3]. Recent reports have substantiated the general belief that traditional medicine is affordable as compared to modern medicine [4]. Natural products play a major role as 'drugs' and as 'lead structures' for the development of synthetic molecules [5]. Ancient people were fully aware of rich potential of herbs for curing different types of ailments. The twentieth century made invaluable contributions to the domain of medical sciences. The discovery of the fascinating molecule, DNA double helix and completion of human genome project were marvellous achievements that had no parallel.

Different modalities of DNA repair mechanisms are offered by natural drugs in mammalian system like base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), SSB repair, which includes BER and DNA-PK-mediated ligation; DSB repair, which includes NHEJ and HR; inter-strand cross-link repair and DPCs (DNA-protein cross links) repair. The drugs act even as a biological catalyst where the rate of the repair process is enhanced [6]. An important cell pathology determinant is the rate of DNA repair. Shortened lifespan and increased cancer incidence has been observed in experimental animals with genetic deficiencies in DNA repair. Mice deficient in the dominant NHEJ pathway and in telomere maintenance mechanisms get lymphoma and infections more often, and consequently have shorter lifespans than wild-type mice [7]. Mice with deficient key repair mechanisms and DNA helices unwinding transcription protein have premature onset of aging-related diseases and shortening of lifespan [8]. Few natural products with DNA protective activity are phenolic compounds, essential oils, alkaloids, caratenoids, glutathione and glucans. Polyphenols and phenolic compounds have the capacity to donate electrons and scavenge free radicals [9, 10].

Phenolic compounds have the capacity to donate electrons and directly scavenge free radicals [9, 10]. The extracts of *Geranium sanguineum* are rich with polyphenol compounds are found to exhibit anti-mutagenic and free radical scavenging capacities [11, 12]. Essential oils, are antioxidants. The essential oil from ginger is a natural antioxidant [13]. Alkaloids, are antioxidants. Carotenoids are lipophilic compounds. Lycopene present in tomatoes and other red fruits like red carrots, red bell peppers, watermelons, and papayas has good antioxidant capacity [14]. Glutathione is a free radical scavenger by either reacting directly with free radical molecules or by acting as proton donor for protection of active molecules as DNA [15]. Glucan is an important carbohydrate from plants, bacteria and fungi. It is discussed in detail here due to their diverse activity. Somehow the antioxidant activity is related to DNA repair mechanism as most of the compound which can repair DNA damage are found possess antioxidant capacity.

Macro fungi are distinguished as important natural resources with therapeutic potential. Studies were conducted on the glucan isolated from the medicinal mushroom and the macrofungi, *Ganoderma lucidum*. *Ganoderma* is popularly known as 'The mushroom of longevity and immortality'1. *Ganoderma lucidum*, commonly known as reishi, a mushroom like fungus which grows on logs or tree stumps is one of the most popular medicinal mushrooms in China, Japan and the United States (**Figure 1**). It has a shiny, hard, asymmetrical cap that ranges in colour from yellow to black. Species of the genus *Ganoderma* P. Karst (Ganodermatales) are important wood decaying mushrooms occurring throughout the world, mainly on tropical trees. Over 250 species of this mushrooms are known. The fruiting

Figure 1.
Ganoderma lucidum growing in wild.

bodies of *Ganoderma lucidum* contain a variety of chemical substances. The polysaccharides of *G. lucidum* are the other major source of its biological activity and therapeutic use. This mushroom has attracted great attention owing to its antitumor and hypoglycemic activities [16]. Many fungal polysaccharides have been reported to be active in humans. More than 180 chemical substances have been isolated from *Ganoderma*, which include polysaccharides, triterpenes, nucleosides, ergosterols, fatty acids, proteins, peptides and trace elements. *Ganoderma* has been extensively used as mushroom of immortality in China and other Asian countries. *Ganoderma* has been reported to have numerous pharmacological effects including immunomodulating, anti-inflammatory, analgesic, anticancer, anti-lipidemic and hepatoprotective antihypertensive effects [8, 17]. It is widely accepted that pharmacological effects of *Ganoderma* depends on its colour, on the stage of development and the environment in which it grows. The fruiting bodies of *Ganoderma lucidum*, commonly known as reishi have long been prescribed in Chinese medicine as a tonic and sedative [18]. In Chinese folklore reishi has been regarded as a panacea for all types of diseases, perhaps owing to its demonstrated efficacy as a popular medicine. *Ganoderma* is also used in treating conditions of the nervous system. The ability of bioactive polysaccharides and polysaccharide-bound proteins to modulate immune cells can be due to the structural diversity and variability of these macromolecules. The bioactive glucanes and proteoglucans isolated from medicinal mushrooms are the most promising class of immunoceutics. Unlike proteins and nucleic acids, polysaccharides contain repetitive structural features which are polymers of monosaccharide residues joined to each other by glycosidic linkages. Glucan appear to be beneficial to humans with impaired immune systems, and those suffering from infectious diseases and cancer, as well as in helping patient recovery from chemotherapy and radiotherapy.

The basic mechanism of DNA replication, recombination and DNA repair are conserved throughout evolution. The complementarity of strands of DNA and the double stranded nature of DNA plays the major role in all the process. Damage to DNA by physical, chemical and biological factors influences the extraordinary accuracy of the entire process. At each cell division a handful of error is introduced per billion bp. Treatment modalities for cancer like chemo and radiotherapy affect DNA in many ways. Drugs of natural origin are capable of increasing the rate of DNA repair. The chapter will focus on the natural drugs and their influence on DNA repair mechanism. In the hierarchy of targets of reproductive death, DNA must be surely placed at the top, though membrane damage should be considered as the second important target with eukaryotic cells which contain their DNA in the nucleus, little lethal damage is observed as long as the radiation is absorbed only by the outer membrane and cytoplasm. There is a drastic increase as soon as the ionizing radiation reaches the nucleus and hence DNA. The DNA damages produced by ionizing radiation can be intra- or inter-strand cross linking and single and double strand breaks (**Figures 2** and **3**). The cellular reactions include halt in cell cycle, advancement at cell cycle checkpoints and the stimulation of DNA repair. An unrepaired or misrepaired DNA damage can result in genetic or genomic variability, changes in cellular individuality and role, cell death, and in multi-cellular organisms, neoplastic transformation.

Humanities use of mushrooms extends as early to 5000 B.C. About 2000 species of edible mushrooms are known all over the world. The total production of the edible mushroom is about 3.75 million tonnes. However they are rich source of high quality protein, vitamins and minerals. The average protein content is 10–40% on dry weight basis and low in fat content. Extracts and powders of mushrooms (mycelia and sporocarps) in the form of sugar coated tablets are being marketed on commercial scale for treatment of diseases such as diabetes, cancer, etc. Medicinal macro fungi modulate immune system and possess antitumor, antimicrobial,

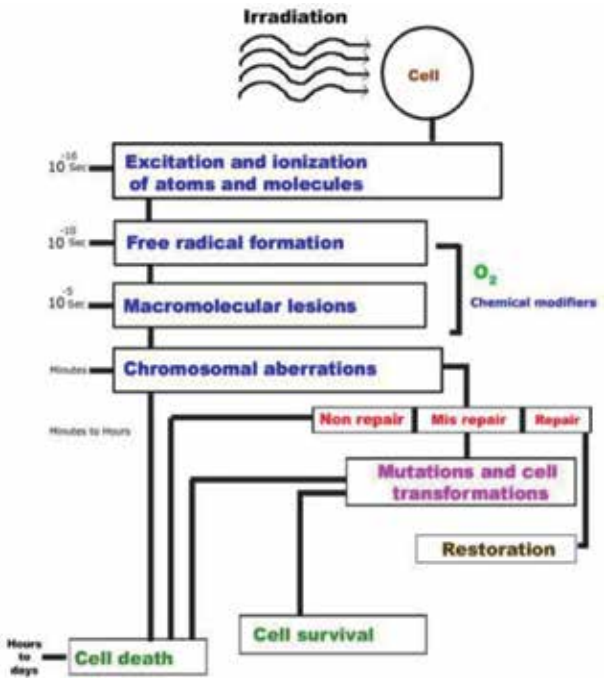

Figure 2.
Effect of radiation on cell.

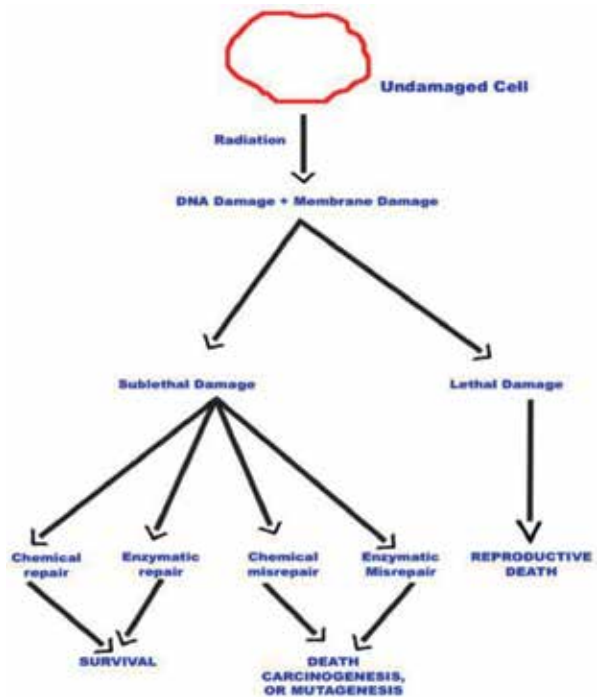

Figure 3.
Different types of damages in cell after radiation exposure.

anti-inflammatory activities. Attempts are done to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments [19]. More than 100 medicinal mushrooms have been identified.

Ganoderma lucidum, commonly known as reishi, the mushroom of immortality is one of the most popular medicinal mushrooms. The fruiting bodies of *Ganoderma lucidum* contain a variety of chemical substances including polysaccharides, triterpenes, nucleosides, ergosterols, fatty acids, proteins, peptides and trace elements. The polysaccharides of *G. lucidum* are the other major source of its biological activity and therapeutic use. This mushroom has attracted great attention owing to its antitumor and hypoglycemic activities [6]. Many fungal glucan have been reported to be active in humans.

2. Materials and methods

2.1 Animals

Swiss albino mice, were kept for a week under environmentally controlled conditions with access to standard food and water. Recommendations of the ethical Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) instituted by the Animal Welfare Division of the Government of India were followed.

2.2 Irradiation

Gamma cell facility of Bhabha Atomic Research Centre, Trombay was used for irradiation. Whole body irradiation to mice was given to unanesthetized animals, which were kept in well-ventilated Perspex boxes and was exposed at a dose rate of 1 Gy/min. *Ex vivo* irradiation of human peripheral leukocytes was done in Junior Theratron unit with a dose rate of approximately 0.4 Gy/min. Chemicals were obtained from Sigma Chemicals (St. Louis, Missouri) and purchased from Merck India Ltd., Mumbai.

3. Methods

3.1 Isolation of glucan

Ganoderma lucidum were collected from Southern parts of India. The polysaccharides were isolated from the fruiting bodies by the method of Mizuno [20]. Purification of the compound was done by ion-exchange chromatography. Qualitative confirmation was done by anthrone [21] and phenol sulphuric acid reagent [22]. Further characterization of the compound was done by IR and NMR, mass spectra, gel filtration and acid hydrolysis.

3.2 Comet assay

Comet assay was performed by the method of Singh with modifications [23]. DNA damage in blood leukocytes was estimated. Ten microliters of heparinised whole blood, is mixed with 200 μl of low melting point agarose at 37°C and layered on frosted slides pre-coated with 200 μl high melting point agarose. The slides were pre-chilled in lysing solution and the standard protocol was followed [24].

CASP software was used for the quantitation of the DNA strand breaks of the stored images by which the percentage DNA in tail, tail length, tail moment, and olive tail moment [25]. The tail length of comet specifies the extent of damage as the smaller molecules move faster on the agarose gel. The longer tails of the comets

indicate that the strand breaks are more frequent. The tail moment normalizes the difference in the size of the nucleus studied, which is product of the percent DNA in the tail of the comet and tail length. Calculation of olive tail moment distance of centre of gravity of DNA is considered rather than usual tail length.

3.3 Metaphase preparation

Six groups of six animals each were used. At 22 h after irradiation all the animals were injected i.p. with 0.025 colchicine and sacrificed 2 h later by cervical dislocation. Bone marrow from the femur was aspirated, washed in saline, treated hypotonically (0.565% KCl), at 37°C for 30 min, fixed in 3:1::methanol:acetic acid, spread on clean slides and stained with 4% Giemsa [26].

The aberrations were scored with the help of a light microscope. Per animal 500 metaphases were scored. Chromatid breaks, chromosome breaks, fragments, rings and dicentrics as well as cells showing polyploidy and severely damaged cells (SDC), cells with 10 or more aberrations of any type, the different types of aberrations were scored. In 'chromosome type' aberration, breaks involved both the chromatids and in 'chromatid type' aberration involved only one chromatid. Fragments are those deleted portion having no apparent relation to any particular chromosome [27]. Data are mean ± (S.E).

3.4 Treatment of animals

Group I—double distilled water (DDW).
Group II—300 mg/kg body wt. of amifostine i.p. (30 min prior to irradiation).
Group III—20 mg/kg body wt. of glucan orally (5 min after irradiation).
Group IV—DDW + 4 Gy radiation (RT).
Group V—300 mg/kg body wt. of amifostine (30 min before irradiation) + RT 4 Gy.
Group VI—RT 4 Gy + 20 mg/kg body wt. glucan orally (5 min after irradiation).

4. Results and discussion

The compound isolated from *G. lucidum* answered anthrone and phenol sulphuric tests giving typical colour reactions indicating the presence of carbohydrates. From the IR spectrum, pyranoid form was suggested to be present due to the presence of three absorption bands at 1153.4, 1091.6 and 1029.9 cm^{-1}. In the HNMR spectrum H^{-1} signals were observed at less than 4.8 ppm (4.762, 4.683, 4.667, 4.658, 4.402 ppm), which suggest that component sugars have beta configuration. From gel filtration chromatography, the molecular weight of the compound was found to be 1.5×10^6 Daltons. From the acid hydrolysis treatment for the detection of monosaccharides, the sugars present in the compound were found to be glucose, mannose and rhamnose. The compound was identified to be beta-glucan.

4.1 DNA repair enhancement

The repair process in lymphocytes was found to be enhanced by the glucan at 50 μg/ml concentration. The percent DNA, tail length, tail moment and olive tail moment was reduced significantly. At 2 Gy 0 min, the comet parameters increased. Fifteen minutes after irradiation the comet parameters were reduced. The presence of glucan reduced the comet parameters further. After 2 h of irradiation the comet parameters were reduced by the glucan to the control level (**Figure 4** and **Table 1**).

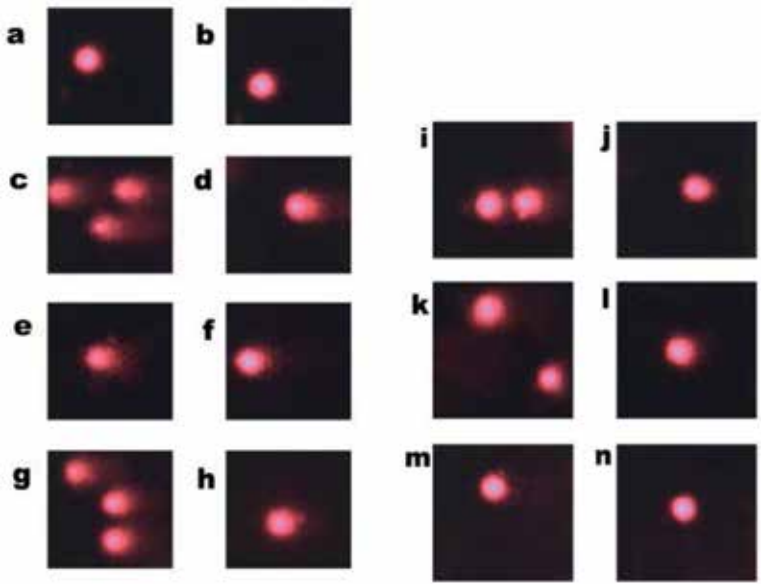

Figure 4.
DNA repair enhancement by glucan in human lymphocytes (comet assay). Untreated: (a) control; (c) 2 Gy 0 min; (e) 2 Gy 15 min; (g) 2 Gy 30 min; (i) 2 Gy 45 min; (k) 2 Gy 60 min; (m) 2 Gy 120 min. Treated with glucan: (b) control; (d) 2 Gy 0 min; (f) 2 Gy 15 min; (h) 2 Gy 30 min; (j)2 Gy 45 min; (l) 2 Gy 60 min; (n) 2 Gy 120 min.

Treatment (per 500 cells)	Fragments	Chromatid break	Chromosome break	Rings	Dicentrics
DDW (control)	6.3 ± 2.5	0.16 ± 2.5	0	0	0
Amifostine (alone)	8.0 ± 1.7	2.3 ± 0.3	0	0	0
Glucan (alone)	7.3 ± 0.8	1.0 ± 0.5	0	0	0
RT 4 Gy (alone)	384.1 ± 16.4[g]	13 ± 2.3[g]	8.5 ± 1.5[g]	3.8 ± 0.6[g]	11.3 ± 1.6[g]
RT 4 Gy + amifostine	31.5 ± 4.0[a,i]	9.5 ± 1.8[e]	2.3 ± 0.4[b,i]	0.8 ± 0.4[b]	2.1 ± 0.4[a,i]
RT 4 Gy + glucan	38.6 ± 4.6[a]	8.1 ± 0.7[e,k]	2.8 ± 0.4[a]	0.8 ± 0.3[b]	1.3 ± 0.4[a]

Datas are mean ± S.E. n = 6.
[a]P < 0.0001.
[b]P < 0.001.
[c]P < 0.01.
[d]P < 0.05.
[e]Marginally significant, compared to RT alone.
[f]P < 0.05 compared to RT + amifostine.
[g]P < 0.0001 compared to DDW.
[h]P < 0.001 compared to amifostine alone.
[i]P < 0.01 compared to amifostine alone.
[j]P < 0.05 compared to amifostine alone.
[k]P < 0.0001 compared to glucan alone.
[l]P < 0.001 compared to glucan alone.

Table 1.
*Effect of **G. lucidum** glucan and amifostine on the induction of different chromosomal aberrations in mouse bone marrow after whole body γ-irradiation (4 Gy).*

4.2 Chromosomal aberrations

Sham treated control showed 1% aberrant cells. Compared to control glucan or amifostine alone did not induce any significant changes. There was significant increase in the percentage of aberrant cells treated with radiation. Treatment with glucan after irradiation and amifostine before irradiation resulted in significant

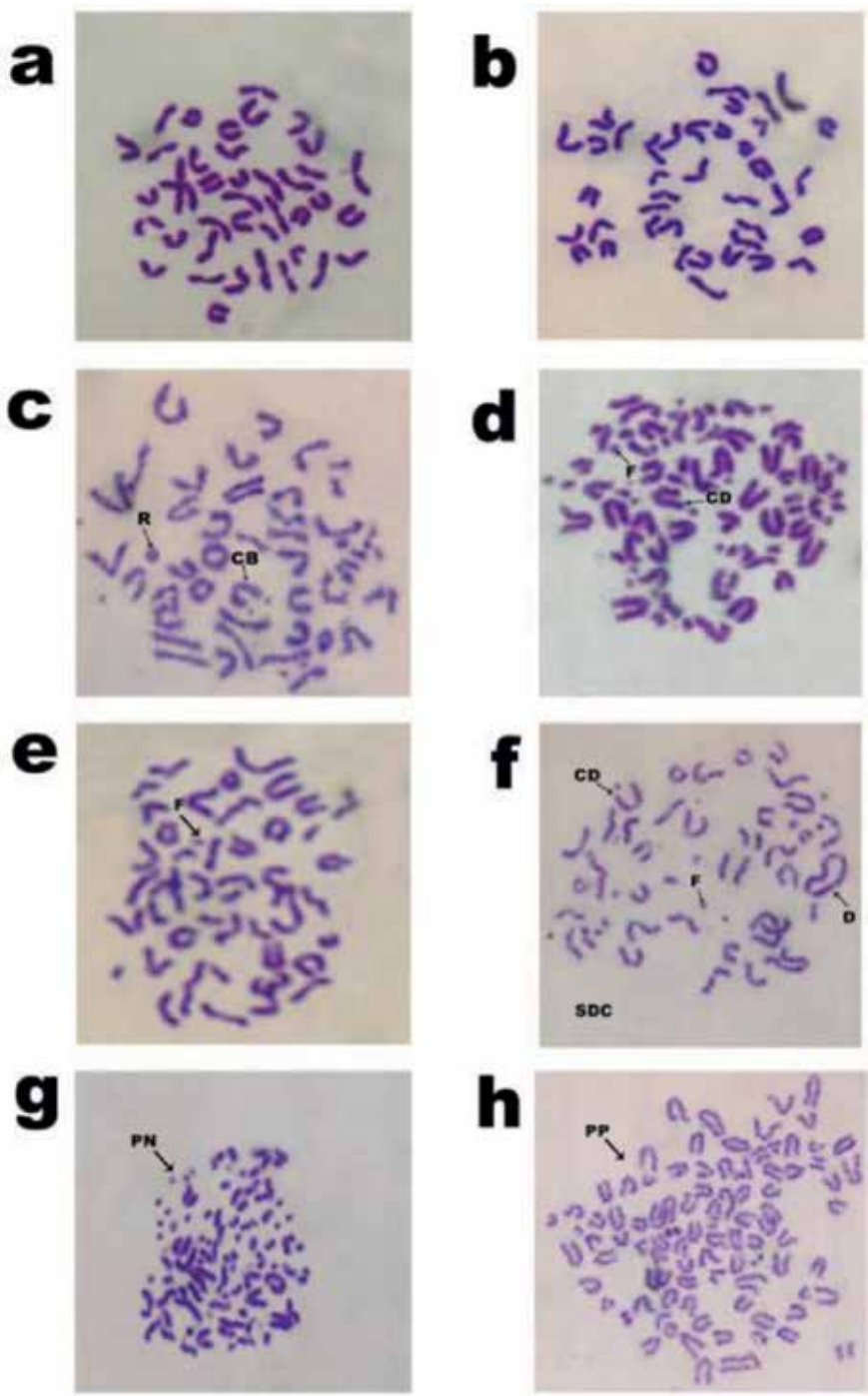

Figure 5.
Different types of chromosomal aberrations in mouse bone marrow. F, fragments; CD, chromatid break; CB, chromosome break; PN, pulverisation; SDC, severe damaged cell; PP, polyploidy; D, dicentrics; R, rings.

decrease in the percentage of aberrant cells and number of aberrations per cell compared to the group which received radiation alone. A decrease in all types of aberrations, as well as polyploidy and cells with pulverisation was observed. The number of severe damaged cells (SDC) significantly reduced to about 1.5 times after glucan treatment. The number of cells with multiple and complex damage was

Treatment	Polyploidy	SDC	Pulverised cells
DDW (control)	0	0	0
Amifostine (alone) (300 mg/kg body wt.)	0.6 ± 0.66	0	0
Glucan (alone) (20 mg/kg body wt.)	0	0	0
RT 4 Gy (alone)	4.8 ± 0.60[g]	14 ± 1.3[g]	10.6 ± 1.3[g]
RT 4 Gy + amifostine (300 mg/kg body wt.)	0.83 ± 0.40[a]	3.6 ± 0.71[a,i]	1.6 ± 0.33[a,j]
RT 4 Gy + glucan 20 mg/kg body wt.)	0.5 ± 0.22[a]	2.0 ± 0[a,f]	1.5 ± 0.22[a,l]

Datas are mean ± S.E. n = 6.
[a]P < 0.0001.
[b]P < 0.001.
[c]P < 0.01.
[d]P < 0.05.
[e]Marginally significant, compared to RT alone.
[f]P < 0.05 compared to RT + amifostine.
[g]P < 0.0001 compared to DDW.
[h]P < 0.001 compared to amifostine alone.
[i]P < 0.01 compared to amifostine alone.
[j]P < 0.05 compared to amifostine alone.
[k]P < 0.0001 compared to glucan alone.
[l]P < 0.001 compared to glucan alone.

Table 2.
Effect of G. lucidum polysaccharides and amifostine on the induction of polyploidy, SDC and pulverization in mouse bone marrow after whole body γ-irradiation (4 Gy).

Time	Olive tail moment without glucan	Olive tail moment with glucan
0 Gy 0 min	3.9444 ± 0.2582	3.677 ± 0.2362
2 Gy 0 min	26.1602 ± 0.5566	26.001 ± 0.3345
2 Gy 15 min	15.6947 ± 0.5193	15.0996 ± 0.7832
2 Gy 30 min	10.0415 ± 0.5287	7.9954 ± 0.57714
2 Gy 45 min	7.2821 ± 0.5541	6.1824 ± 0.5673
2 Gy 60 min	7.5109 ± 0.5966	4.4504 ± 0.3189
2 Gy 120 min	6.2424 ± 0.3847	3.6330 ± 0.3214

Table 3.
Effect of glucan on enhancement of DNA repair in human lymphocytes after 2 Gy gamma irradiation (comet assay).

significantly decreased by glucan post-treatment indicating that the former may help in the repair of the DNA breaks (**Figure 5**, **Tables 2** and **3**).

The lifespan of cells to radiation leading to a loss of cell viability can be greatly influenced by the ability of cells to repair injured DNA. The hazard in mammals exposed to ionizing radiation is to the haemopoetic system. Radiation induced damage to DNA can temporarily affect DNA replication allowing repair to happen involving a well-coordinated event of DNA repair enzymes such as DNA repair polymerase, DNA ligase and PARP [28]. The factors that influence the response of living cells to radiation are the DNA repair status, the physiological state of cells, the presence of oxygen and chemicals as well as pre and post-irradiation treatments [29].

By examining the comet parameters of human peripheral blood leucocytes the effect of polysaccharides on DNA repair was ascertained. Through the initial 30 min, most of the DNA repair processes were completed. The presence of polysaccharide boosted the process of DNA repair. The comet parameters were more at 30 min post-irradiation, in irradiated control and polysaccharide treated group which can be attributed to the commencement of excision repair process [30]. After 45 min

there was not much difference in the comet parameters, in control group. The comet parameters kept on reducing in the presence of polysaccharides and at 120 min the comet parameters were almost similar to the unirradiated control. Re-joining of DNA strand breaks by most cell types is known to be a rapid process within few seconds-minutes [31] and this kinetics are seen in comet assay too. In freshly isolated lymphocytes repair by Hydrogen peroxide induced breaks takes place very slowly which can be due to the additional DNA breakage as a result of quick exposure to atmospheric oxygen in the repair incubation period [32]. At the same time repair of endonuclease III- or FPG-sensitive sites (i.e., oxidized purine and pyrimidines) by base excision repair, is much slower process, taking few hours [33].

Background levels of DNA damage in normal cells, the variation in DNA repair capacity within human populations, and the regulation of DNA repair at the molecular level within the nucleus can be monitored by comet assay [34].

5. Conclusion

The integrity of DNA molecule at structural level has to be protected and preserved for the effectual transmission of the genetic information contained to progeny. Distinctions in the arrangement of nucleotides or changes in the configuration of bases or sugars, in the double helix of DNA can impede the replication or transcription of genome.

Multilation to DNA molecule is the crucial factor for cell death. Mechanisms of repair of damaged DNA molecules play a vital role in cell survival. No medicine has been invented that could successively be applied in DNA damage. Our study indicates that the polysaccharides from *G. lucidum* enhance the repair process.

5.1 Advances in area of DNA repair

Prevention is better than cure and cancer induction is greatly influenced by nutrition. The unaffordable discovery cost and failures at the completion of discovery pipeline makes medicines arbitrary to the developing countries. Newer technologies like reverse pharmacology, systems biology which are charming give innovation opportunities based on investigational wisdom and universal viewpoint of translation medicine. Chemotherapy and SSRI revolutionised longevity and quality of life in therapeutics. The Human Genome Project opened understanding towards personalised medicine. Glucan from *G. lucidum* possess immunomodulating activities and regulate a number of undiscovered cellular genes. New studies are needed to unravel these molecular targets giving insights into the interactions of the fungi like *G. lucidum* with our body system and provide strategies for the discovery of effective and safe approaches for drugs from natural sources.

Glucan was isolated from the mushroom *Ganoderma lucidum*, a basidiomycete white rot macro fungus that has been used extensively for therapeutic use in China and Japan for years. The compound was characterised by different chromatographic techniques, done by IR, NMR, and paper chromatography, gel filtration chromatography and spectroscopic techniques like infra-red spectrum and nuclear magnetic resonance spectrum.

The molecular weight of the isolated glucan was 1.6×10^6 Daltons. The rate of DNA repair in the presence and absence of the compound was determined. Comet assay was performed using the method of Singh in human lymphocytes. Chromosomal aberration was studied in mouse bone marrow. After radiation exposure, the comet parameters, percent DNA, tail length, tail moment and olive tail moment were changed in the presence of glucan. Chromosomal aberrations and

individual aberrations were also reduced by glucan. The result of present investigation reveals the potential application of glucan from *G. lucidum* in increasing the rate of DNA repair which makes it useful in medical scenario.

The path of science is always fascinating giving deep intuitions with new technologies. The term 'DNA repair' gained more significance in last decade. The beautiful discoveries in essential mechanisms of DNA repair extended Nobel prize in Chemistry in 2015 to T. Lindahl, P. Modrich and A. Sancar. Their discovery defined three pathways that essentially correct DNA damage, protecting the integrity of genetic code assuring perfect replication through generations allowing correct cell division. The mechanisms behind base excision repair, mismatch repair and nucleotide excision repair was explained. Since then the number of drugs and targeted pathways has increased remarkably. The DNA repair enzyme was declared as the molecule of the year in 1994. Though the studies from model organisms serve as a basis to elucidate of repair mechanism, the utilisation of cutting edge technology has channelled in a new era of DNA repair research. The DNA repair pathways have also become better understood. The accessibility of a wide-ranging spectrum of drugs with known molecular targets will provide the rationale to use those drugs in relation to various disease conditions and to combine DNA damaging agents with the appropriate DNA repairing agent. The journey of DNA repair continues. Our current research is carried out in this direction.

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This book offers a collection of chapters addressing different studies on DNA repair from a cellular and molecular point of view. The various contributions highlight the vastness of DNA repair process and the need for a deeper understanding. To this end, the recent considerations here presented can be a cue for scientists and students working on, or interested in, the subject of DNA repair in human cells. This book may suggest to readers new avenues of interplay between different kinds of DNA damage and cellular response for maintaining nuclear and mitochondrial genomic stability.

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