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Pancreatic Cancer

Molecular Mechanism and Targets

Edited by Sanjay K. Srivastava

PANCREATIC CANCER – MOLECULAR MECHANISM AND TARGETS

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http://dx.doi.org/10.5772/1271
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Notice

First published in Croatia, 2012 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Pancreatic Cancer - Molecular Mechanism and Targets
Edited by Sanjay K. Srivastava
p. cm.
ISBN 978-953-51-0410-0
eBook (PDF) ISBN 978-953-51-6935-2

Meet the editor

Dr Sanjay K. Srivastava is a Professor of Biomedical Sciences at Texas Tech University Health Sciences Center (TTUHSC), Amarillo, Texas, specializing in cancer biology, cell signaling and nutritional chemoprevention. Dr. Srivastava served as an Assistant Professor in the Department of Pharmacology, University of Pittsburgh School of Medicine, and did his post-doc from University of Texas Medical Branch at Galveston, Texas. He received a M.S. in Biochemistry from Lucknow University and a Ph.D. in Biochemical Toxicology from Industrial Toxicology Research Center, India. Dr. Srivastava is funded by grants from the National Cancer Institute, NIH. He has authored/co-authored more than 100 research papers and book chapters and is in the editorial board of several journals. Dr. Srivastava has been the recipient of several awards including TTUHSC "President's Excellence in Research Award". His research has been featured by news agencies including BBC, MSNBC, CBS, ABC, Science News etc.

Contents

Dedicated to my mother Vidya Srivastava and father Dr. Balramji Srivastava,
who provided me constant love and support.

Preface

Pancreatic cancer is one of the most fatal human malignancies with extremely poor prognosis making it the fourth leading cause of cancer-related deaths in the United States. The molecular mechanisms of pancreatic carcinogenesis are not well understood. The major focus of these two books is towards the understanding of the basic biology of pancreatic carcinogenesis, identification of newer molecular targets and the development of adjuvant and neoadjuvant therapies.

Book 1 on pancreatic cancer provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Apart from epigenetics, book summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil. The involvement of various survival pathways in chemo-drug resistance is discussed in depth. Major emphasis is given to the identification of newer therapeutic targets such as mesothalin, glycosylphosphatidylinositol, cell cycle regulatory proteins, glycans, galectins, p53, toll-like receptors, Grb7 and telomerase in pancreatic cancer for drug development.

Book 2 covers pancreatic cancer risk factors, treatment and clinical procedures. It provides an outline of pancreatic cancer genetic risk factors, signaling mechanisms, biomarkers and disorders and systems biology for the better understanding of disease. As pancreatic cancer suffers from lack of early diagnosis or prognosis markers, this book encompasses stem cell and genetic makers to identify the disease in early stages. The book uncovers the rationale and effectiveness of monotherapy and combination therapy in combating the devastating disease. As immunotherapy is emerging as an attractive approach to cease pancreatic cancer progression, the present book covers various aspects of immunotherapy including innate, adaptive, active, passive and

bacterial approaches. The book also focuses on the disease management and clinical procedures. Book explains the role of pre-existing conditions such as diabetes and smoking in pancreatic cancer. Management of anesthesia during surgery and pain after surgery has been discussed. Book also takes the reader through the role of endoscopy and fine needle guided biopsies in diagnosing and observing the disease progression. As pancreatic cancer is recognized as a major risk factor for vein thromboembolism, this book reviews the basics of coagulation disorders and implication of expandable metallic stents in the management of portal vein stenosis of recurrent and resected pancreatic cancer. Emphasis is given to neuronal invasion of pancreatic tumors along with management of pancreatic neuroendocrine tumors.

We hope that this book will be helpful to the researchers, scientists and patients providing invaluable information of the basic, translational and clinical aspects of pancreatic cancer.

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Risk Factors in Pancreatic Cancer

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1. Introduction

Pancreatic cancer is one of the most lethal malignant diseases with the worst prognosis. It is ranked as the fourth leading cause of cancer-related deaths in the United States. An unknown but important proportion of cancers develop in people who carry mutation in a cancer-predisposing gene. Identification of cancer-predisposing genetic mutations in susceptible individuals affords the opportunity to practise preventive medicine. Pancreatic cancer is an aetiologically complex disease whose development is contingent on the independent and joint effects of genes and environment. (Greer &Whitcomb, 2007). Recent analysis of human pancreas genomes showed that 12 common signaling pathways involved in cellular repair mechanisms, metabolism, cell-cycle regulation, genomic repair, and metastasis are affected in over two thirds of the pancreatic cancer genome, including mainly point mutations(Jones et al., 2008).

Many risk factors have been associated with PC such as genetic factors and premalignant lesions, predisposing diseases and exogen factors. Genetic susceptibility, observed in 10% of cases includes inherited pancreatic cancer syndromes and familial cancers. However, the rest of 90% of pancreatic cancer recognise as risk factors a mix between genetic factors and environmental factors, too, but the exact etiopathogenesis remains unknown.

2. Hereditary pancreatic cancer syndromes

2.1 Hereditary breast ovarian cancer syndrome

Hereditary breast ovarian cancer syndrome is associated with germ line mutation in the BRCA 2 and BRCA 1 gene and it is associated with a 7% lifetime risk in pancreatic cancer at 70 years old. BRCA1 and 2 are tumour suppressor genes that are inherited in an autosomal dominant fashion with incomplete penetrance. They controls cell growth and differentiation and their loss drives tumorigenesis by involving in transcriptional regulation of gene expression and reairing of damaged DNA. The 6174delT mutation of BRCA2, occur ten times more frequently in Ashkenazi Jewish population and it is responsible for breast and ovarian familial cancer. BRCA2 mutations are found in as many as 12 to 17 percent of

patients with familial pancreatic cancer. Single nucleotide polymorphism of BRCA 1 and 2 does not influence the risk for pancreatic cancer in sporadic pancreatic adenocarcinoma (McWilliams et al., 2009). For BRCA1 carriers, this relative risk is estimated to be 2-fold higher (Thomson et al., 2002) and for BRCA2 carriers, this relative risk is approximately 3-to 4-fold higher (The Breast Cancer Linkage Consortium, 1999). Within 24/219 BRCA1 and 17/156 BRCA2 families (representing 11% of overall individuals included in the study) there was at least 1 individual with pancreatic cancer. The onset of cancer was earlier than in general population : 59 in males and 69 in females in BRCA1families and 67 in males and 59 in females in BRCA2 families (Kim et al., 2009). Compared to SEER data which showed a 0.96:1 male:female ratio occurence of pancreatic cancer in general population, in BRCA1 families, showed a 2:1 male: female ratio, possible linked to the competing mortality for breast and ovarian cancer in their female relatives (Kim et al., 2009). For these reasons, males under 65 years old in families with a strong history of breast, ovarian, and pancreatic cancer be considered for BRCA1/2 testing along with their female relatives. Cigarette smoking and exposure to oestrogen influences pancreatic cancer risk, but in a direction opposite to that of breast cancer risk in BRCA1/2 mutation carriers (Greer & Whitcomb, 2007).

2.2 The Peutz-Jeghers syndrome

The Peutz-Jeghers syndrome is an autosomally dominant hereditary disease with characteristic of hamartoma polyps of the gastrointestinal tract, and mucocutaneous melanin pigmentation. Almost half of these patients are carriers of a germinal serine-treonine kinase 11*STK11/LKB1* gene mutation (Giardiello et al., 2000). Wild-type *STK11/LKB1* activates adenine monophosphate–activated protein kinase, which is a regulator of cellular energy metabolism. Activation of adenine monophosphate–activated protein kinase leads to inhibition of the mammalian target of rapamycin 1 (mTOR1), a serine/threonine kinase with a key position in the regulation of cell growth. The risk of PC is 132 times higher than for the general population (lifetime risk for cancer is 11-36%).

2.3 Familial atypical multiple mole melanoma syndrome (FAMMM)

Familial atypical multiple mole melanoma syndrome (FAMMM) is an autosomal dominant syndrome caused by a germline mutation in CDKN2A (or p16) gene on chromosome 9p21 or in a minority of cases in the CDK4 gene on chromosome 12 (Goldstein et al., 2000; Wheelan et al., 1995). This syndrome is characterized by multiple nevi, multiple atypical nevi, and an increased risk of melanoma. The relative risk of developing pancreatic cancer is 20 to 47 and the lifetime risk for pancreatic cancer is 16%(Vasen et al., 2000, De Snoo et al., 2008). Among cases who reported having a first-degree relative with pancreatic cancer or melanoma, the carrier proportions were 3.3 and 5.3%, respectively. Penetrance for mutation carriers by age 80 was calculated to be 58% for pancreatic cancer and the risk of pancreatic cancer in smokers was 25 compared to non-carriers (McWilliams et al., 2011). The onset of pancreatis cancer in a historical cohort of 36 patients from 26 families with FAMM was 65 years old. In a follow-up study group of 77 carriers of p16 mutation, 7 individuals developed a pancreatic cancer within 4 years and only 5 had curative resection, confirming rapidly growing tumor that could originate from small PanIN lesions in p16 mutation carriers(Vasen et al., 2010).

2.4 Lynch syndrome

Lynch syndrome is an autosomal dominant condition caused by defects in mismatch repair genes (MLH1, MSH2, MSH6 or PMS2). It has recently been shown that in addition to colorectal and endometrial cancers these individuals have a 9-fold increased risk of developing pancreatic cancer compared with general population(Kastrinos et al., 2009).

2.5 Hereditary pancreatitis

Hereditary pancreatitis is a rare autosomal dominant disorder, in more than two-thirds of cases caused by a mutation in the SPINK1 and PRSS1 genes, with a high risk of pancreatic cancer. For this population, the cumulative risks of pancreatic cancer at the age of 50 and 75 years are 11% and 49% for men and 8% and 55% for women, respectively(Rebours et al., 2008). The risk was higher for smokers and for those with diabetes mellitus.

2.6 Ataxia-teleangiectasia

Ataxia-teleangiectasia with mutation of ATM gene on chromosome 17p is associated with pancreatic cancer , but the relative risk is unknown yet.

3. Familial pancreatic cancer

It may be considered in families with at least two first-degree relatives suffering from the disease, thus suggesting an autosomal dominant penetrance (Greenhalf et al., 2009). Families with only one relative with pancreatic cancer or with multiple pancreatic cancers in more distant relatives are considered as sporadic PC. The lifetime risk increases with the number of relatives involved. Individuals with two first-degree relatives with pancreatic cancer have a 6-fold increased risk of developing pancreatic cancer, and individuals with three or more first-degree relatives with pancreatic cancer have a 14 to 32-fold increased risk (Klein et al., 2004) . The risk of pancreatic cancer was similar in familial PC kindred compared to sporadic pancreatic cancer kindred members. Analysing more than 9000 subjects, the presence of a young-onset pancreatic cancer patient, under 50 years old did not influence the risk of having pancreatic cancer inside familial PC kindred, but it added risk compared to sporadic pancreatic cancer (Brune et al., 2010). Smoking is a strong risk factor in familial pancreatic cancer kindred, particularly in males and people younger than 50 years of age, as it increases the risk of pancreatic cancer by 2 to 3.7 times over the inherited predisposition and lowers the age of onset by 10 years (Rulyak et al., 2003).

The genetic basis is not known, the BRCA2, palladin gene and PALB2 could play some role (Murphy et al., 2002; Couch et al., 2007; Pogue-Geile et al.,2006; Jones et al.,2009). The *PALB2* gene codes for a protein that binds to the BRCA2 protein and helps to localize BRCA2. (Tischkowitz et al.,2009, Jones et al.,2009). Palladin is a cytoskeleton-associated scaffold protein, with role in the formation of a desmoplastic tumor microenvironment (Giocoechea et al., 2010), but recent studies denied its involvement in carcinogenesis (Klein et al.,2009, Slater et al.,2007)

There has been developed and validated a risk prediction model PancPRO based on age, pancreatic cancer status, age of onset, and relationship for all biological relatives (Wang et al., 2007).

Even genetic testing may be of benefit to many families, more than 80% of the clustering of pancreatic cancer in families remains unknown or the known mutation are not found. Mutations in the *BRCA2*gene account about 11% of families, *PALB2* 1–3% and the remaining genes account for <1% of familial pancreatic cancer. Genetic susceptibility for developing pancreatic cancer has been recently atributed to a single nucleotide polymorphism of gene located on 13q22.1 chromosome, considered as specific for pancreatic cancer, or of a gene located on 1p32.1 chromosome, which interact with betacatenin pathway(Petersen et al., 2010).

3.1 Genetic predisposition: ABO blood group

Compared with blood group O, individuals with non-O blood group (type A, AB, or B) were significantly more likely to develop pancreatic cancer (adjusted hazard ratio for incident pancreatic cancer 1.32, 1.51. and 1.72, respectively)(Wolpin et al., 2009, Risch et al., 2010), probably based on genetic variants in ABO locus 9q34 (Amundadottir et al, 2009). Another extended study identified susceptibility loci on 3 chromosomes- 13q22.1, 1q32.1 and 5q15.33, the most specific being considered 13q22.1(Petersen et al., 2010). The incidence rates for pancreatic cancer (cases per 100,000 persons at risk) among White participants with blood types O, A, AB, and B were 28.9, 39.9, 41.8, and 44.5, respectively. In combination with smoking, overweight or diabetes, the non-O blood type was associated with ORs of 2.68, 1.66, and 2.29, respectively, compared to subjects who had O blood type and lacked the exposure(Wolpin et al., 2010). The mechanism of influence of blood group antigens on risk for pancreatic cancer might be the alteration of the systemic inflammatory state (Wolpin et al., 2010).

4. Premalignant lesions

There are three known precursor lesions to pancreatic cancer: intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasia (MCN) and pancreatic intra-epithelial neoplasia (PanIN). PanIN is by far the most common lesion and three grades of PanIN have been described as cellular atypia progresses from low grade dysplasia (PanIN 1) to high grade dysplasia (PanIN3), similar to colorectal cancer carcinogenesis. The 5-year-risk of PC is about 50% for MCN, 50% for main ductal IPMN while only 15% for branch IPMN.

5. Predisposing diseases

5.1 Chronic pancreatitis

The risk of developing pancreatic cancer is about 5%(Raimondi et al., 2010), probably due to PanIN lesions or chronic inflammation. In a large multicentric study, the total risk reached 1.8 percent at 10 years and 4 percent at 20 years, independently of the type of pancreatitis(Lowenfels et al., 1993; Howes et al., 2004). There is no need for systematic screening in patients with chronic pancreatitis, but acute onset of pain after long free-pain interval, a non-equilibrated diabetes without explanation, the onset of jaundice or weight loss require looking for pancreatic cancer. The risk is higher for non-alcoholic pancreatitis, as hereditary pancreatitis linked to PRSS1 mutations (40% at 70 years old) or tropical pancreatitis, form of hereditary pancreatitis linked to SPINK1 mutation (a 100 times higher risk than for the general population)(Lowenfels et al., 1993).

5.2 Diabetes mellitus

Diabetes is associated with pancreatic cancer in about 40 to 60% of patients at the onset of symptoms, being a consequence or the cause of the disease. A meta-analysis of 20 studies (predominantly of patients with type 2 diabetes) estimated that the pooled relative risk for pancreatic compared to patients without diabetes was 2.1, especially among patients with long-standing diabetes(Everhart&Wright, 1995; Huxley et al., 2005).Diabetes associated with pancreatic cancer is often new-onset (<2-year duration), it resolves following cancer resection and appears to be associated with conventional risk factors for diabetes such as age, obesity and familial history (Pannala et al., 2008; Gupta et al., 2006). Even in the absence of frank diabetes mellitus, abnormal glucose metabolism and insulin resistance have been associated with pancreatic cancer(Stolzenberg-Solomon et al., 2005; Gapstur et al.,2000), and the insulin-growth factor(IGF) involvement might be the pathway in the pathogenesis. Although not all studies found an association between the risk of pancreatic cancer and the level of IGF, it seems that the polymorphism of IGF is associated with lower susceptibility to pancreatic cancer(Lin et al., 2004; Wolpin et al., 2007; Suzuki et al., 2008).The risk is higher in insulin ever users compared with nonusers (OR = 2.2, 95% CI = 1.6-3.7) and was restricted to insulin use of ≤3 years (OR = 2.4), but decreases after ten years of insulin use(Li et al., 2011). The explanation might be that the two diseases could share genetic risk factors in common. The CT screening is recommended for older patients with new-onset diabetes, especially those with family history or symptoms, as shown in a recent description of French families.

5.3 Postgastrectomy or postcolecystectomy status

Postgastrectomy or postcolecystectomy status were associated with an increased risk of pancreatic cancer, probably due to high level of circulating colecystokinin(Smith et al., 1990).

5.4 Helicobacter pylori and hepatitis B

Helicobacter pylori and hepatitis B have been found as associated factors to pancreatic cancer. The pathway may be represented by the polymorphism of genes involved in the inflammatory response, but further studies are needed for confirmation.

6. Environmental factors

6.1 Smoking

The risk for pancreatic cancer is 1.5-2.5, higher with the numbers of cigarettes and in glutathione-S-transferase deficient persons and decreases 10 years after the smoking cessation. (Iodice et al, 2008). It increases the risk in hereditary chronic pancreatitis. Mutations in carcinogen-metabolizing genes, such as glutathione-S-transferase, N-acetyl-transferase, cytochrome P450 and DNA-repair genes in oxidative metabolism(XRCC1, OGG1) with multiple sequence variants may be genetic modifiers for smoking-related pancreatic cancer (Duell et al., 2002; Li et al., 2006). In a recent case-control publication, the risk more than 15 years after smoking cessation was similar to that for never smokers. Also, there was a more significant risk for total exposure delivered at lower intensity for longer duration than for higher intensityfor shorter duration. These findings and the decline in risk after smoking cessation suggested that smoking has a latestage role in carcinogenesis. (Lynch et al., 2009). There is a synergistic interaction with diabetes mellitus and family

history of pancreatic cancer (Hassan et al.,2007). Smoking can be reponsible for familial agregation of pancreatic cancer individuals with lung and larynx cancer (Hiripi et al., 2009).

6.2 Obesity

A body mass index of at least 30 kg/m2 was associated with a significantly increased risk of pancreatic cancer compared with a BMI of less than 23 kg/m2 (relative risk 1.72), but an inverse relationship was observed for moderate physical activity when comparing the highest versus the lowest categories (relative risk 0.45) (Michaud et al., 2001). Centralized fat distribution may increase pancreatic cancer risk,especially in women, (Arslan et al., 2010).

There have recently been discovered genetic factors which can reduce the risk of PC (PPARγ P12A GG genotype, NR5A2 variants) or which can enhance th risk in overweight patients (FTO, ADIPOQ) (Tang et al., 2011). Others have suggested that overweight and obese individuals develop pancreatic cancer at a younger age than do patients with a normal weight, and that they also have lower rates and duration of survival once pancreatic cancer is diagnosed (Li et al., 2009). Obesity in early adulthood was a risk factor for pancreatic cancer (Genkinger et al., 2010).

6.3 The diet

The diet based on fat and meat has been linked to the development of pancreatic cancer in many (Nothlings et al., 2005; Thiebaut et al., 2009), but not all studies (Michaud et al,2003, 2005). The consumption of fresh fruits and vegetables were not associated with pancreatic cancer risk (Coughlin et al.,2000). Lower serum levels of lycopene and selenium have been found in subjects who subsequently developed pancreatic cancer (Burney et al.,1989). Although the majority of prospective cohort studies found no significant increase in the risk of pancreatic cancer with moderate to high levels of alcohol intake in a general population., a recent study has shown that a certain polymorphism of genes involved in the production and/or oxidation of acetaldehyde is associated with an increasing risk in developping pancreatic cancer (Michaud, 2004;Kanda et al., 2008). Folate deficiency, involved in DNA mutations and DNA methylation, may increase the risk of cancer. Although at least two variants of genes involved in folate metabolism were found to be associated to pancreatic cancer and smoking, these findings were not confirmed in all studies. Because the sample size was considered to be insufficient and the criteria for control selection of patients were different,these evidence were considered inadequately powered for drawing a conclusion. (Wang et al., 2005; Matsubayashi et al., 2005; Suzuki et al., 2008; Ohnami et al., 2008). No epidemiologic study has provided evidence to support the hypothesis that high glycemic index or glycemic load increases the risk of pancreatic cancer (Jiao L et al., 2009).

Also, the role of TGF-beta pathway, proved to be linked to pancreatic cancer, and its genetic variants, but it still remains unclear.

6.4 Exposure to sunlight

Exposure to sunlight with increase of *vitamin D* synthesis might decrease the cancer risk and polymorphic variants in genes encoding the for synthesis enzyme is an important task for future research, as the role of melatonin receptor and genetic variants in clock genes. Based on different sun exposure in different geographic latitude, several studies sustained the

protective role of vitamin D against pancreatic cancer, in association with other factors as age and obesity (Grant, 2002, Guyton et al., 2003). The quantification of Vitamin D concentration must consider also the race (Afro-Americans has a higher risk for PC), the season of blood drawn and presence of supplemental in diet (Stolzenberg-Solomon, 2009).

6.5 Alcohol consumption

A recent study showed a moderate risk to heavy alcohol drinkers (about 40 g alcohol daily) and liquor users (relative risk 1.45-1.62) , probably due to their nitrosamine content (Jiao et al., 2009), sustained by other studies only in men (Hassan et al., 2007).

6.6 Demographic factors

Advanced age, between 60 and 80 is associated with 80% of pancreatic cancers. Other demographic factors that are associated with a modest (about 2-fold) increased risk include male gender, Jewish descent and black ethnicity(Lillemoe et al., 2000).

Gene function	Gene symbol	Gene full name	Gene location	Concentration tumor vs normal
Transcription	ZNF	zinc finger protein	19q13.31	3.38
	MIXL1	Mix1 homeobox-like 1	1q42.12	6.24
	SEPT1	Septin 1	16p11.1	3.42
Intracellular signaling	FLJ 42953	breakpoint cluster region pseudogene 2	22q11.21	3.02
	AGRP	agouti related protein homolog	16q22	6.51
Intracellular transport	CCDC 88	coiled-coil domain containing 88B	11q12.3	4.61
	UTP14 A	U3 small nucleolar ribonucleoprotein	Xq26.1	3.44
	VPS11	vacuolar protein sorting 11 homolog	17p11.2	3.33
	LLRC 21	leucine-rich repeat, immunoglobulin-like and transmembrane domains	10q23	3.33
	CHRM3	cholinergic receptor, muscarinic 3	1q43	3.01

Table 1. Genes with significant different expression (overexpressed or underexpressed) in pancreatic cancer compared to normal pancreatic tissue.

Our research on 16 tissue samples of T3 pancreatic cancer comparing to normal tissue in the same patients analysed by microarray showed that there were 41 overexpressed genes and 402 underexpressed genes. From those with tumor concentration three times modified compared to normal tissue we noticed genes involved in transcription, intracellular signaling and intracellular transport (Table I), which need further validation on larger sample groups (data unpublished). This showed that genomic tissue microarray analysis represents a powerful strategy for identification of potential biomarkers in pancreatic cancer.

7. Conclusions

Pancreatic cancer is a pathological status with clear inheritance in only 10% of cases, the others seems to be linked to premalignant situations, other diseases or environmental factors in which genetic implications need further investigations. The gene-gene and gene-environment interactions have to be more extensively studied, especially because there are not only single-nuclear polymorphisms, but also DNA copy number variations and variable-number tandem repeats which can be linked to the risk of pancreatic cancer.

8. Acknowledgments

We thank Ovidiu Balacescu MD, PhD, and his team from Institute of Oncology, Cluj-Napoca, Romania, for his work in tissue microarray analysis in pancreatic cancer.

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Epigenetics and Pancreatic Cancer: The Role of Nutrigenomics

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1. Introduction

Pancreatic ductal adenocarcinoma cancer is the 10th most commonly diagnosed cancer but is the 4th leading cause of cancer death in the United States. A number of risk factors have been proposed to play a role in the etiology of pancreatic cancer (1,2). Life-style factors such as smoking accounts for 20-30% of pancreatic cancer death, with approximately 10% having germline or somatic mutations association (3). Other risk factors include age, race, gender, chronic pancreatitis and diabetes; however, the role of dietary intake and specific nutrients remain an unexplored area of research, although diet is a risk factor (4,5). Epidemiological studies have long suggested the possibility that what we eat influence the state of our health. It is believed that dietary habits are important modifiable factors that can influence cancer risk and tumor behavior (6,7). *In vivo, in vitro* and epidemiological studies have shown that an individual's diet may contribute to their susceptibility to develop cancer (8-11).

Pancreatic cancer remains a very complex and challenging disease. This cancer carries one of the worst prognosis of any major malignancy, mainly due to its lack of early detection and lack of effective therapeutic agents. The American Cancer Society projected 43,140 new cases of the disease in 2010, and over 36,800 deaths (12). Improvements in imaging technology has aided in diagnosis and identification of patients with the disease; however, these new technologies have not greatly improved the mortality rate of pancreatic cancer. Clinical, pathological and genetics studies have identified three important different preneoplastic lesions of the pancreatic ductal adenocarinoma, the pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCM) which could be studied to identify early changes in pancreatic cancer (13,14). Understanding molecular changes within these preneoplastic lesion, whether genetic or epigenetic, will greatly improve detection of pancreatic cancer at its earliest stages. Furthermore, the examining of these lesions with emerging "omics' technologies and the emerging new science "nutriogenomics" will greatly contribute to our knowledge of this deadly cancer.

2. Nutrigenomics

Nutrigenomics is an emerging new field of science in which attempts are being made to study the effects of nutrition on the whole genome (15). Nutrigenomics is the study of

specific genes or the affect of functional single nucleotide polymorphisms and bioactive food components interactions. Although great emphasis has been placed on understanding the role of nutrigenomics on regulation of gene expression in regards to polymorphisms, very little data are available on the role of nutrigenomics and its role in epigenetic regulation. We must also include in this new area of science, high energy or caloric intake because of its contribution to obesity. Nutrients are thought to be dietary signals that can be detected by various cellular systems involved in regulating gene and protein expressions, as well as affecting the production of metabolites (16,17). Therefore, each individual can establish dietary signatures in specific cells, tissues or organs according to their daily diets, which could utlimately influence homeostasis and their susceptibility to diseases, such as cancer. Studying the effects of nutrients at the genomic level can be through genetic or epigenetic mechanisms. This chapter focuses on the role of epigenetic mechanisms in pancreatic cancer and their modulation through dietary agents found in daily food intake. The influence of bioactive components in foods on various biological and physiological functions at the genomic level is a vastly unexplored area of research in cancer research. Dietary components are beginning to be observed as major determinants of cancer risk in humans (18-22). Nutrition can potentially modify, through epigenetic mechanisms molecular changes associated with carcinogenesis. Furthermore, employing this new science in understanding how bioactive components can affect the constant insults from external and internal factors to DNA, which results in chromatin changes, alteration in DNA repair, apoptosis and inflammation epigenetically will enhance our knowledge on pancreatic cancer. This new field of science can begin to investigate the role of various nutrients on mechanisms that may influence the etiology or progression of pancreatic cancer.

3. Epigenetic mechanisms

Epigenetic modifications can be altered by external or internal environmental factors, such as diets, and has the potential to also be reversed (23,24). Epigenetic mechanisms include DNA methylation, histone modifications, and changes in microRNAs (25-28). These mechanisms can lead to changes in gene expression and have been the focus of a number of diseases including cancer, type 2 diseases, obesity, cardiovascular diseases, neurodegenerative diseases and immune diseases (29-33). Tumors can exhibit widespread global DNA hypomethylation, region-specific hypermethylation and increased activities of the DNA methyltransferases. DNA methylation modification is established and maintained by a family of DNA methyltransferases (DNMTs), DNMT1, DNMT3a and DNMT3b (34,35). These enzymes catalyze the transfer of methyl groups from S-adenosylmethionine (SAM) to cytosine residues in the DNA. These critical enzymes have been shown to be highly expressed in pancreatic cancer and play critical roles in silencing important genes, such as p16, RASSFIA, cyclin D2, APC and others through promoter hypermethylation in various cellular pathways (36-38). Approximately 60% of human genes are associated with CpG islands that are subject to methylation in tissue specific patterns; however, these islands have been shown to increase their methylation status during aging and the development of certain diseases such as cancer (39,40). Several of the classic tumor suppressor genes, such as p16/CDKN1A, p53, SMAD4 and STKll, have been genetically inactivated through DNA methylation in pancreatic cancer. hMLH1, which is associated with microsatellite instability, has been also shown to undergo methylation in pancreatic cancer (41,42). Several other genes with tumor suppressor properties have also been associated with pancreatic cancer (43).

Although much of the focus of cancer epigenetics is on inactivation of tumor suppressor genes by promoter methylation, the earliest observation of altered methylation patterns identified DNA hypomethylation as an important event in the etiology of cancer (44-46). Global DNA hypomethylation was first associated with the lack of critical nutrients such as methonine, folate, and vitamin B12 (47-49). These observations raised the importance of nutritional causes of methyl group deficiency and its association with the tumorigenesis. DNA hypomethylation is often associated with gene overexpression or gene activation. Nutrients deficiency can, therefore, influence the methylation status of an individual and increase their susceptibility to diseases such as pancreatic cancer. Given the role of the pancreas in digestion and absorption, diet may play a larger role in pancreatic disease and prevention.

In addition to DNA methylation, histone modification has also been implicated in pancreatic cancer, particularly genes of the mucin family (50-52). These genes have been found to undergo histone modifications in pancreatic cancers (53,54). Mucin gene products are high molecular weight glycoproteins that are produced by pancreatic cancers. MUC1, MUC2 and MUC3 histone modifications have been investigated and their role in pancreatic cancer is described in relation to nutrigenomics (55,56). MUC1 in normal pancreas is the main membrane-bound mucin expressed. MUC1 has been used as a marker of pancreatic ductal cells. MUCs are known to play important roles in protection and epithelial repair in the intestinal mucosal (57). MUC2 is absent or weakly expressed in ductal and acinar cells in normal pancreas. MUC2 has been shown to demonstrate tumor suppressor properties (58). However, in pancreatic cancer there is an altered expression pattern of mucins at different stages of pancreatic tumor progression (59). MUC1 gene expression is regulated by a combination of DNA methylation and histone H3-K9 modification (60).

4. Nutrigenomics and epigenetic regulation of signaling pathways

The past decades have focused mainly on research involving genetic alterations or genetic susceptibility due to germline mutations (61-64). Mutated KRAS has high mutation prevalence in pancreatic cancer, reaching as much as 100% in advanced stages of the disease (65,66). However, dietary agents such as high fat diets have been shown to increase KRAS expression (67-69), while other studies have shown decreased expression with caloric restriction (70,71) and intake of bioactive components found in some vegetables and fruits (72-75). Using global genomic screening, 12 altered core signaling pathways due to mutations have been found in pancreatic cancer (76). In addition to widespread genetic alterations, it is now apparent that epigenetic factors also play an important role in modulating a number of these signaling pathways in pancreatic cancer (77). Regulation of specific genes in a subset of regulatory pathways has been identified to be disrupted in pancreatic cancer and modulated by dietary agents (78). These pathways involve apoptosis, DNA damage control, K-ras signaling, JNK signalings, invasion, Hedgehog signaling, Wnt-Notch signaling, TGF-ß, and regulation of the G1/S phase transition (79-81). The dietary agent curumin, a yellow spice found in both turmeric and curry powder, inhibits JNK, COX2, NF-kappaB, STAT3 and AP-1 activation (82) through epigenetic mechanisms. The Wnt-Notch signaling pathway, which is altered in pancreatic cancer, control key biological processes that impact tumor progression and patient survival. Epigenetic inactivation of key components, such as the secreted frizzed-repeated protein (SERP1), in this pathway can lead to constitutively

activation of this pathway (83). EGCG, a component found in green tea extract, induces apoptosis and inhibits JNK signal pathway in pancreatic cancer (84,85). Inactivation of the human Runt-related transcription factor 3 (RUNX3), which play a role in TGF-ß signaling, decreases TGF-ß expression in pancreatic cancer (86). TGF-ß has been shown to be a potent inhibitor of pancreatic cancer cells *in vitro* (87). Recent data revealed the inactivation of the Hh-interaction protein (HHIP) through promoter hypermethylation in pancreatic cancer cells *in vitro*. HHIP is a negative regulator of the Hedgehog signaling pathway which is up-regulated in pancreatic cancer (88). The Hedgehog signaling pathway has been highly conserved through evolution and plays a crucial role during embryonic development (89). Dietary agents have been shown to modulate homologus of this pathway (90). In humans, there are three different homologues of the pathway, Sonic Hedgehog (Shh), Indian Hedgehog (IH) and the Desert Hedgehog (Dhh). Epigenetic mechanisms involve altered gene expression without changes in genomic sequences, thus these mechanisms can alter the above pathways through many factors, such as diet and life-style factors (e.g., smoking).

5. Dietary nutrients, obesity and caloric restriction

In the nutritional field, epigenetics is important because nutrients and bioactive food components can modify the expression of genes at the transcriptional level (91-93). There is a critical lack of research examining the role of critical nutrients on the etiology of cancers such as pancreatic cancer, although animals studies have indicated its role in cancer development for a number of years (94,95). However, to critically examine an individual's nutrients intake will require improvement over the current 24-hour recall survey often used in dietary studies.

Deficiency in proper nutrients, critical micronutrients and increase in high fat-diets or high caloric intake have been implicated in a number of diseases, including cancers, such as pancreatic cancer (96,97). The relationship between food, nutrition science and diseases such as cancer through epidemological studies have been analyzed for a number of years. However, the genomic variation among individuals and populations remains an unexplored area of research, which can enhance our knowledge in understanding complex diseases such as pancreatic cancer and its impact on the etiology and progression of this disease. The genomic era has ushered in a new science called "nutriogenomic" to began to understand the importance of nutrition on complex diseases such as pancreatic cancer, in which the disease presents little or no early symptoms for early detection or diagnosis. Obesity is a risk factor for pancreatic cancer in certain populations (98). Understanding these interactions will provide critical information for understanding how the health consequences of eating behaviors may vary across individuals or different ethnic groups. Although the survival rate of pancreatic cancer has slightly improved, African Americans continue to have the highest incidence rate of pancreatic cancer than any other ethnic groups (99). Eating behaviors and types of diets in this group as it relates to its effects on changes in the genome related to diseases such as cancer, remains an unexplored area of research. Bioactive components in foods can act on the human genome directly or indirectly to affect gene expression or their gene products. This new research area "nutrigenomics", in relation to pancreatic cancer, can ultimately identify molecular targets for nutritional intervention.

Numerous dietary components are known to alter epigenetic events, and thus can influence the health of individuals. Folic acid and vitamin B12 play an important role in DNA

metabolism and are required for the Synthesis of Methionine and S-adenosylmethionine (SAM), the common methyl donor required for the maintenance of DNA methylation patterns (100). Essential and non-essential nutrients or bioactive components have been shown to modulated and number of cellular processes through epigenetic mechanisms involved in carcinogen metabolism, cell signaling, cell cycle control, apoptosis, hormonal balance and angiogenesis (101).

Epidemiological evidence and the relation of nutrition and pancreatic cancer has been extensively reviewed (102). However, a number of these studies have included descriptive, case-control and often cohort studies, all showing a consistent pattern of positive association with nutrition and recently, research data showing correlation with increase pancreatic cancer and obesity (103). Some current studies have confirmed our early studies showing decreased rates of pancreatic cancer with caloric restriction (104). We reported this finding in the mid-90s and demonstrated that it occurred through DNA methylation, an epigenetic mechanism. Case-control studies have shown a correlation between caloric intake and higher risk of pancreatic cancer in African American and identified obesity as a risk factor for pancreatic cancer (105). Obesity during pregnancy and high-fat maternal diets have been shown to be associated with obesity in offsprings suggesting early imprinting (106). Studies are needed to address the specific nutrients or fats that may modulate gene expression through epigenetic mechanisms. Epigenetic biomarkers of obesity that have been identified include epigenetic regulation of genes involved in adipogenesis (SOCS1/SOCS3), methylation patterns of obesity-related genes (FGF2, PTEN, CDKN1A and ESR1), inflammation genes as well as genes involved in intermediary Metabolism and insulin signaling (107).

The degree of methylation can be determined by the availability of methyl donors, methyl transferase activity, and also demethylation activity. Studies have shown that chronic administration of methionine- and choline-deficients diets results in global hypomethylation of hepatic DNA and development of spontaneous tumor formation (108). In those studies when the pancreas was examined in the methionine- and choline-deficients diets, a transdifferentiated (hepatocyte-like) phenotype was observed (109). The progentic of these cells have now been identified as pancreatic stem cells (PSCs) that are capable of producing cells with multiple markers of other non-pancreatic organs (110). The fact that pancreatic cancer contains tumorigenic cancer stem cells and are highly resistant to chemotherapy and can be induced by a lack of micronutrients strongly suggest this area of research greatly needs exploring. Research using nutrigenomics can address the importance of tumorigenic cancer stem cells in pancreatic cancer.

6. DNA methylation and nutrigenomics

Bioactive food components have been shown to have benefical effects on the genome through epigenetic mechanisms. Certain bioactive components, such as tea polyphenols, genistein from soybeans, and isothiocyanates from plant food, may have inhibitory effect on certain cancer, including pancreatic cancer. Dietary polyphenols is thought to have a direct inhibition by interaction with the catalytic site of the DMNT1 or it could have an influence on the methylation status indirectly. A number of cultured cells, animal models and human clinical trials have shown the protective role of dietary polyphenols against a number of cancers, including pancreatic cancer (111). However, understanding the timing of

intervention is critical in cancer prevention, particularly for an aggressive cancer such as pancreatic cancer which lacks early biomarkers of detection. Epigenetic mechanisms are thought to play an early role in pancreatic cancer, such as inactivation of tumor suppression genes through hypermethylation of CpG islands in promoter regions of genes. Reversal of gene hypermethylation has been achieved by inhibiting DNMT activity in cancer cells. A number of studies are showing inhibition of DNMT activity with dietary components. We have shown reactivation of p16 in pancreatic cancer cells through DNA hypomethylation with the dietary agent indole-3-carbinol. Recently our laboratory has also shown that indole-3-carbinol can greatly enhance the efficacy of gemcitabine, which is the first line treatment for pancreatic cancer (112).

Epigallocatechin-3-gallate (EGCG) one the major components of green tea has been shown to be an effective DNMT1 inhibitor directly. Thus, activation of tumor suppression genes p16, RAR, MGMT and MLH1 have been demonstrated by EGCG. In addition, the protected effects associated with consumption of fruits and vegetables and various chemical components in pancreatic cancer have demonstrated various effects on pancreatic cancer cells, such as induction of apoptosis, inhibition of proliferation, inhibition of transcription factors, activation of suppressor genes and inhibiting K-ras signaling through epigenetic mechanisms (113). Modulation of these critical events by dietary factors through epigenetic changes is an important area of research that is needed in clinical trials with or without association with current chemotherapeutic agents. Table 1 shows a list of dietary factors know to regulate DNA methylation.

Bioactive Component
Coumestrol
Methionine
Genistein
Vitamin B12
EGCG
Indole-3-Carbinol
Vitamin B6
Folate
Equol
Choline
Curcumin

Table 1. Bioactive Components of Food that Influence DNA Methylation in Pancreatic Cancer

7. Histone modifications and nutrigenomics

Another epigenetic mechanisms that has been shown to be modulated by bioactive components in foods are histone modifications. Histones, which are the structural component of chromatin, are modified by methylation, acetylation, phosphorylation, biotinylation, ubiquitination, sumoylation, and ADP-ribosylation (114). Diverse histone modification is known to play an important role in gene regulation and tumorigenesis. The

modification involving epigenetic mechanisms occurs at the histone tails, that usually consist of about 15-38 amino acids. Majority of the modifications takes place at lysines, arginine and serine residues. These modifications can lead to either activation or repression depending on which resides are modified. Lysines residues in the tails can be either methylated or acetylated. Usually histone modification status is often balanced by a group of enzymes called histone acetyltransferases (HATs) and histone methyltransferases (HATs) which add acetyl and methyl groups; and histone deacetylases (HDACs) and histone demethylases (HDMs) which remove acetyl and methyl groups from histone protiens. Histone methylation is maintained by histone methyltransferases and histone demethylases. Histone acetylation results in an "open" chromatin structure thus allowing access to DNA and gene transcription. Acetylation of N-terminal lysine residues at positions 9,14,18, and 23 of H3 and 5, 8,12, of H4 mediates the decondensation of the chromatin for accessibility to transcription factors. Histone acetylation is one the most extensively studied histone modification. Deacetylation is often associated with silencing of gene expression. Dietary agents have been identified that have structural features similar to the HDAC inhibitors (115,116). HDAC inhibitors are known to reactivate epigenetically silenced genes.

Bioactive components have been found to act as HDAC inhibitors, such as butyrate, sulforophane, curcumin, resveratrol and diallyl disulphide. Butyrate, a short-chain fatty acid formed from the fermentation of fibre when consumed has been shown to downregulate transcription factors such as Sp1 and Sp2, which have been reported to be acetylated targets for HDAC1 and HDAC2 (117). This effect has been shown to increased p21 expression which will ultimately cause cell cycle arrest and an increase in Bax expression thus causing apoptosis. In pancreatic cancer cells sodium butyrate has been shown to sensitize these cells to Fas-mediated apoptosis as well as down regulation of Bcl-xL expression and apoptosis. Further research is needed to understand the role of dietary agents on histone modifications in pancreatic cancer. A number of studies have shown dietary agents such as curcumin, anacardic acid, garcinol, polyphenols, isothiocyanates, isoflavone and resveratrol to affect histone modifications. Resveratrol, a bioactive component of grape skins, exert its anti-inflammatory effect through repression of NF-κB induced by histone deacetylation (118).

Bioactive Component
Butyrate
Sulforophane
Curcumin
Resveratrol
Diallyl disulphide
Anarcardic acid
Garcinol
Polyphenols

Table 2. Bioactive Components of Food that Influence Histone Modification in Pancreatic Cancer

In addition to bioactive nutrients modulating histone modifications, studies have also shown that caloric restriction, another unexplored area of research on epigenetics, reduces the expression of inflammatory genes such as NF-κB, AP1, COX-2, and iNOS (119).

Reduction in total caloric intake has numerous health benefits, including reducing risk to certain cancers such as pancreatic cancer ( ).NF-κB is known to be activated by histone aceylation. Activation of NF-κB occurs through p300 HAT acetylation of the p50 subunit of NF-κB. This increases NF-κB binding and transactivation. Caloric restriction modulation of these pathways through epigenetics mechanisms allows numerous opportunities for prevention of diseases such as cancer.

8. microRNAs and nutrigenomics

In addition to DNA methylation and histone modification, another epigenetic mechanism, microRNAs is emerging as a key mediator in gene regulation which may be affected by bioactive dietary components. These small single-stranded RNAs, ~19-24 nucleotides in length, regulate gene expression through post-transcriptional silencing of targeted genes. MicroRNAs can play important roles in controlling both DNA methylation and histone modifications. This regulation creates a highly controlled feedback mechanism. In contrast, promoter methylation or histone acetylation can also modulate microRNA expression (120). Usually microRNAs can control a wide spectrum of biological function that may be relevant in cancer, such as cell proliferation, apoptosis, and differentiation. Aberrant expression of these small nucleotides have been associated with cancer. Several microRNAs have been identified that are regulated by DNA methylation in pancreatic cancers (121). Noncoding RNA and miRNAs are known to be involved in post-transcriptional gene silencing. Methyl-deficient diets and folate deficiency induce global increase in microRNA expression in some cancers.The relevance of microRNA and nutrigenomics is a greatly unexplored area of research as it relates to pancreatic cancer. However, curcumin has been linked to changes in microRNA expression in pancreatic cancer cell lines. Curcumin represses human pancreatic cancer cells by upregulating miR-22 and downregulating miR-199a. MicroRNA-10a expression, which has been identified as a mediator of metastatic in pancreatic cancer, is repressed by retinoic acid receptor antagonists (122,123).

Bioactive Component
Curumin
Polyphenols
Resveratrol

Table 3. Bioactive Components of Food that Influence microRNAs in Pancreatic Cancer

9. Conclusion

Finally, understanding the role of nutrigenomics on pancreatic cancer etiology through epigenetic mechanisms could have a tremendous impact on decreasing the mortality of this disease. The beneficial aspects of various nutritional bioactive components and their effects on inhibiting or decreasing pancreatic cancer could also enhance the efficacy of current therapeutics used in treating pancreatic cancer. Understanding the role of nutrigenomics and its impact on modulating epigenetic mechanisms such DNA methylation, histone modification and microRNAs in pancreatic cancer will greatly enhance intervention or prevention stagergy for this disease. Our knowledge in the field of this emerging science is currently very limited, but the potential is vast in understanding the role of various

nutrients on the genome and its ability to contribute to healthy life-style, thus decreasing individuals risk to diseases such as cancer. Although intake of some dietary components may not improve health, research in this field will identify the interaction of these components with various macromolecules in the cell that are not Benefical. The study of nutrigenomics could identify molecular targets for nutritional preemption and information obtained from these studies are key to personalized nutrition.

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3

Characterization of the Molecular Genetic Mechanisms that Contribute to Pancreatic Cancer Carcinogenesis

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1. Introduction

Molecular genetic analyses have provided evidence that has helped characterize the carcinogenesis of pancreatic adenocarcinoma. Pancreatic carcinogenesis is a multistep process during which oncogenes are activated, and the function of tumor suppressor genes is lost. *K-ras* mutations, telomere shortening, loss of p16, loss of p53 and loss of smad4 are thought to contribute to pancreatic carcinogenesis. Recent studies have shown that some new signaling pathway contribute to pancreatic cancer development. Because the model of pancreatic cancer development suggests that several genetic alterations accumulate progressively, the molecular mechanisms underlying this disease should be investigated thoroughly. In addition, we have considered of the appearance of epigenetic and microRNA abnormalities in creating a profile of the molecular genetic mechanisms at work in pancreatic cancer carcinogenesis.

This chapter provides an overview of the most relevant molecular genetic alterations that have been implicated in pancreatic cancer development and includes the characterization of the development of precancerous lesions and invasive carcinoma.

2. Molecular genetics understanding of pathway in pancreatic cancer

2.1 Alterations in oncogenes

Many gene mutations have been implicated in the molecular mechanisms of pancreatic cancer formation. In this section, we focus on the oncogenic gene mutations that have been linked to pancreatic cancer.

2.1.1 K-ras

The most frequent genetic abnormality in invasive pancreatic cancer is mutation of the activating K-ras oncogene, which occurs in 75-90% of pancreatic cancers (Ji et al., 2009). K-

ras is a member of the Ras gene family, which is located on chromosome 12p and encodes a 21-kDa membrane-bound GTP-binding protein. This GTP-binding protein mediates various cellular functions, such as proliferation, cellular survival, motility, and cytoskeletal remodeling. The K-ras activating mutations abolish the regulated GTPase activity of the K-ras protein, which converts the Ras protein to the 'on' state and permanently activates downstream signaling events that may contribute to carcinogenesis. K-ras is activated by point mutations, most often in codon 12 but also in codons 13 and 61 (Jones et al., 2008). The role of H-ras, another member of the Ras family, in carcinogenesis is not as well characterized, but it has been reported that H-ras is responsible for mediating the growth-promoting effects in pancreatic cancer cells that possess K-ras mutations (Seufferlein et al., 1999).

The critical role of Ras signaling in pancreatic cancer has been confirmed by many experimental studies. The mutations in the K-ras gene are observed in the earliest form of pancreatic intraepithelial neoplasia (PanIN) lesions and are considered to be one of the earliest genetic events to take place during pancreatic tumorigenesis (Jones et al., 2008; Tada et al., 1996). However, the hyperactivation of the Ras signaling cascade alone is neither sufficient for the malignant transformation nor restricted to malignant pancreatic cells. Instead, Ras hyperactivation may be combined with many genetic abnormalities and signaling pathways to promote pancreatic cancer development. Moreover, K-ras mutations were also detected in nearly 25% of chronic pancreatitis patients and even in healthy elderly subjects (Guerra et al., 2007).

Until now, several studies have focused on K-ras as a therapeutic target and have worked to develop treatments, such as antisense therapy and RNA interference. In a phase II trial of patients with locally advanced and metastatic pancreatic cancers, the Ras family antisense inhibitor showed a response rate of 10.4% and a median survival of 6.6 months when the therapy was combined with gemcitabine treatment (Alberts et al., 2004). RNA interference technology is highly specific, but it has not yet entered the clinical trial stage. However, *in vitro* and *in vivo* studies have provided promising results for the use of RNAi as a pancreatic cancer therapy (Rejiba et al., 2007).

2.1.2 The PI3K/AKT pathway

The PI3K-AKT pathway is one of several signaling pathways that function downstream of K-ras, and it is also activated by mutations during carcinogenesis. AKT proteins are activated through PI3K in response to mitogenic stimulation, such as the activation of EGFR. Several downstream targets, including the mammalian target of rapamycin (mTOR) and the transcription factor NFκB, have a variety of roles in cell proliferation, survival, resistance to apoptosis, angiogenesis and invasion (Schneider & Wolf, 2009).

AKT is amplified and the PI3K–AKT pathway is activated in 20% and 59% of pancreatic cancers, respectively (Schlieman et al., 2003). The amplification of AKT2 genes are also observed in 10% to 20% of pancreatic cancers, and its suppression by antisense RNA results in the reduced growth and tumorigenicity of pancreatic cancer cell lines (Cheng et al., 1996).

Inhibition of this pathway through aberrant expression of PTEN (phosphatase and tensin homolog), which is a natural antagonist of PI3K, is frequently observed in pancreatic cancers (Asano et al., 2004). Furthermore, an architectural transcription factor, HMGA1, activates

PI3K–AKT signaling and appears to mediate resistance to gemcitabine. Together, these observations suggest that this gene is another potential target for inhibition therapy(Kim & Gallick, 2008; Liau & Whang, 2008). Other agents, including everolimus and sirolimus, are currently in phase II clinical trials (Azzariti et al., 2008). Furthermore, PTEN has also been described as a target for treating human pancreatic cancer.

2.1.3 EGF receptor

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein receptor with an intracellular tyrosine kinase domain. Once bound to its ligands, the protein forms homodimers or heterodimers with other members of the ErbB family, which leads to the phosphorylation of tyrosine residues in its intracellular domain. Intracellular proteins were subsequently activated, which induce downstream signaling events through the PI3K–AKT family, STAT family, and, most notably, the MAPK signaling pathway. STAT proteins have roles in cell proliferation, survival, motility, invasion and adhesion. The mechanisms that lead to inappropriate activation of EGFR include receptor overexpression, activating mutations, overexpression of receptor ligands, and/or the loss of negative regulatory pathways. The overexpression of EGFR and its ligands (EGF and others) and/or the loss of the mechanisms that down-regulate the activity are frequently observed in pancreatic cancer (Bloomston et al., 2006; Preis & Korc, 2010).

A phase III trial that combines gemcitabine and erlotinib, an orally active small molecule that binds to the ATP-binding site of EGFR, has revealed a small but statistically significant increase in the survival of patients with advanced pancreatic cancer compared with gemcitabine treatment alone (Moore et al., 2007).

2.1.4 IGF

The insulin-like growth factor receptor (IGF-R) is structurally similar to the insulin receptor. Insulin-like growth factor-1 (IGF) exhibits structural homology to proinsulin and binds to IGF-R with high affinity and to the insulin receptor with a much lower affinity. Therefore, the insulin-receptor substrate is able to interact with many signaling molecules. These interactions facilitate the activation of multiple downstream signaling pathways, including the PI3K/AKT, MAPK, and JAK/STAT3 pathways, and result in anti-apoptosis and growth-stimulating effects. IGF and its receptors have been extensively studied in various cancers, such as colon, breast and prostate cancer (Moschos & Mantzoros, 2002).

A large portion of the exocrine pancreas is exposed to high levels of insulin, which may act on the exocrine cells via a proxicrine mechanism to provide the pancreatic cancer cells a growth advantage. These high insulin levels can activate both the insulin and IGF receptors. IGF-R is overexpressed in 64% of pancreatic cancers (Moschos & Mantzoros, 2002).

Together, these alterations may work in combination to further enhance cancer growth, indicating that IGF-R may be an important therapeutic target in pancreatic cancer. There are several IGF-1R-targeting agents that are currently being tested in clinical trials. The anti-IGF-R monoclonal antibodies, AMG-479 and IMC-A12, are in Phase I/II studies, which are currently enrolling patients. Moreover, small molecule inhibitors of IGF-R, such as BMS-754807, may provide an alternate approach for targeting this important pathway in pancreatic cancer treatment (Ma & Adjei, 2009).

2.1.5 VEGF

Tumor angiogenesis is essential for tumor growth and is largely mediated by the vascular endothelial growth factor (VEGF) family of proteins and receptors. VEGF is a glycoprotein that promotes endothelial cell survival, mitogenesis, migration, differentiation and vascular permeability. The upregulation of VEGF expression is stimulated by hypoxia and oncogenic proteins, such as Ras. In addition, growth factors, such as EGF, TGF-α, TGF-β, PDGF, and HIF, and cytokines, such as IL-1α and IL-6, can also upregulate the expression of VEGF. VEGF and its receptors are overexpressed in more than 90% of pancreatic cancers and are associated with increased microvessel density, tumor progression and poor prognosis (Seo et al., 2000).

The importance of VEGF and its receptor pathway for the growth of pancreatic tumors was demonstrated in several studies with animal models. These studies showed that VEGF and its receptors are the targets of numerous ongoing clinical trails that are evaluating the efficacy of these treatments in pancreatic cancer (Seo et al., 2000). Several other trials are being conducted to examine bevacizumab in combination with other agents or treatment modalities for pancreatic cancer; however, this agent seems unlikely to confer sufficient benefit to justify licensing for this condition. It has been suggested that angiogenic inhibitors that target other non-VEGF pathways may be better able to gain access to the tumor environment than an antibody (Whipple & Korc, 2008).

2.2 Tumor-suppressor genes and pathways

Tumor suppressor genes inhibit cell proliferation and signaling pathways and induce apoptosis and support DNA repair systems, which are thought to be key events that suppress transformation during tumor carcinogenesis. However, these genes are subjected genetic alterations that reduce or eliminate their normal function.

In pancreatic cancer, the frequently affected tumor suppressors include *p53*, *APC*, SMAD4/*DPC4*, *p16INK4A* and some additional candidate genes. The loss of these tumor-suppressor genes may participate and dominate the signaling pathways in pancreatic tissue carcinogenesis. A summary of these and other tumor-suppressor genes that are altered in pancreatic cancer are discussed below.

2.2.1 p16INK4A/retinoblastoma

The loss of function of the p16 gene, due to mutation, deletion or promoter hypermethylation, occurs in 80-95% of sporadic pancreatic cancers, which is a higher rate than that reported in any other tumor type (Caldas et al., 1994; Rozenblum et al., 1997). The p16 locus is located on chromosome 9q21, and it regulates cell cycle progression by limiting Rb phosphorylation through inhibition of the cyclin D/CDK4/6 complexes (Serrano et al., 1996). The inactivation of the pRb/p16 tumor-suppressor pathway may alter the activity of pRb, CDK4, and cyclin D to promote tumor development (Freeman et al., 2004).

The loss of p16 alone or in combination with the activity of other oncogenes has a significant role in the formation of pancreatic precursor lesions and the development of pancreatic cancer. Immunohistochemical analyses revealed that the loss of p16 protein expression occurred in approximately 30% of PanIN-1A lesions, 55% of PanIN-1B lesions and PanIN-2

lesions, 71% of PanIN-3 lesions and 100% of PDAC (Real et al., 2008). Recently, Aguirre et al. found that p16 limits the malignant conversion of these PanIN lesions to ductal adenocarcinoma in activated *KRAS*-initiated PanIN formation, which suggested that p16 is not the earliest event but is an important event in the progression of pancreatic carcinogenesis (Aguirre et al., 2003).

Clinical research has focused on the contribution of p16 in pancreatic cancer. It appears that p16 plays a significant role in pancreatic carcinogenesis and is an important diagnostic or therapeutic target. Rosty et al. proposed that the loss of the expression of the suppressor gene p16 was a major risk factor for the development of pancreatic cancer in patients with chronic pancreatitis (Rosty et al., 2003). DNA hypermethylation of p16 in pancreatic juice was demonstrated to be a valuable diagnostic marker to predict pancreatic cancer progression. However, further studies are needed to provide evidence for the clinical applications that target the p16 gene (Matsubayashi et al., 2006; Yan et al., 2005).

2.2.2 p53

The p53 locus, which is on the 17p13 chromosome, regulates the cell cycle by integrating numerous signals to control cell death (Rozenblum et al., 1997). The abrogation of p53 activity through mutation occurs in more than 50% of sporadic pancreatic cancers. Wild-type p53 maintains a G2-M arrest and regulates the G1-S checkpoint to facilitate normal cell cycle progression (Vogelstein & Kinzler, 2004). The inactivation of p53 affects PTEN, which inhibits the AKT signaling pathway and induces apoptosis in pancreatic cancers. p53 is short-lived and expressed at very low levels in normal cells, but p53 becomes stable and accumulates if the cell has DNA damage. Pinho AV et al. found that p53 controls both growth and epithelial cell differentiation in the pancreas, which indicates that p53 inactivation in tumors is associated with aggressive biological behavior (Pinho et al., 2011).

Because p53 mutations accumulate relatively late in carcinogenesis, clinical research has focused on the therapeutic contribution of p53 in pancreatic cancer. Patients with pancreatic cancer that carry a p53 mutation have shorter survival rates than patients with wild-type p53. Moreover, tumors that contain a mutated p53 are typically radioresistant and/or chemoresistant, indicating that p53 may serve as treatment indicator in pancreatic cancer (Dergham et al., 1998). In addition, p53 gene therapy strategies can induce tumor regression in patients with advanced NSCLC and with recurrent head and neck cancer (Roth et al., 1999).

2.2.3 SMAD4/DPC4

The SMAD4/DPC4 locus on 18q21 is the critical component of the TGFβ signaling pathway and negatively regulates the growth of epithelial cells (Massague et al., 2000). SMAD4 (DPC) is another commonly mutated gene in PDAC, and it is activated in approximately 50% of pancreatic cancers as a result of homozygous deletion mutations. Wilentz et al. revealed that expression of the SMAD4 protein is associated with the histopathological grades of pancreatic cancer (Hahn et al., 1996). In addition, immunohistochemical assays revealed that the smad4 protein was not expressed in 31% (9/29) of the high-grade lesions (PanIN-3).

Conversely, the loss of SMAD4 expression did not occur in PanIN-1 and -2, indicating that the loss of SMAD4 typically occurs late in PanIN progression to PDAC, similarly to p53 (Miyaki & Kuroki, 2003; Wilentz et al., 2000).

SMAD4 is an integral member of the TGF-β signaling cascade, which plays an integral role in tumor initiation and progression (Bierie & Moses, 2006; Massague, 2008). There are three TGF-β ligands (TGF-β1, TGF-β2 and TGF-β3), which bind to TbRII, TbRI and phosphorylate the downstream mediators SMAD2 and SMAD3. The phosphorylated SMAD2 and SMAD3 for a complex with SMAD4 and enter the nucleus to modulate gene transcription (Derynck & Zhang, 2003).

Clinical research has focused on the therapeutic contribution of smad4 in pancreatic cancer. Melisi D et al. found that the TGF-β/Smad-independent pathway can increase apoptosis inhibitors to produce pancreatic cancer cells that are resistant to the pro-apoptotic effects of gemcitabine (Melisi et al., 2011). Some ongoing clinical trials are employing different TGF-β inhibitors to inhibit the TGF-β signaling pathway in advanced pancreatic carcinoma (Korpal & Kang, 2010; Nagaraj & Datta, 2010). The loss of SMAD4 plays a crucial role in abrogating the TGFβ-mediated cancer cell growth and metastasis. However, further studies are needed to investigate and improve the effectiveness of combined TGFβ inhibitor treatment and SMAD4 gene therapy.

2.2.4 Candidate tumor suppressor genes

2.2.4.1 ARHI gene

The maternally imprinted gene Aplesia Ras homolog member I (ARHI, *DIRAS3*) is a member of the Ras superfamily locus on chromosome 1q. It is a small 26-kDa GTPase that inhibits anchorage-dependent and independent growth, motility, invasion and angiogenesis, despite sharing 54-62% amino acid homology with *Ras* and *Rap* (Yu et al., 1999). Artificially induced expression of ARHI in mice leads to small body size, infertility and decreased lactation (Xu et al., 2000) . Ectopic overexpression of ARHI in cancer cells that express low levels of ARHI triggers apoptosis through a caspase-independent, calpain-dependent mechanism (Bao et al., 2002). Recent studies suggest that the return of ARHI to normal physiological expression levels also induces a G2/M cell cycle arrest, autophagy and tumor dormancy in ovarian cancer (Lu et al., 2008) . The expression and function of ARHI in pancreatic cancer has received relatively little attention. Because ARHI appears to oppose Ras function, and *K-ras* is frequently activated in pancreatic cancers, it is possible that the loss of ARHI contributes to pancreatic carcinogenesis. In the present study, we measured the expression of ARHI in normal and cancerous pancreatic tissue. Yang et al. found that ARHI is widely expressed in the ductal and acinar cells of normal pancreatic tissue but is down-regulated or lost in approximately 50% of pancreatic cancers (Yang et al., 2010). This study also examined the methylation status of ARHI in pancreatic cancer cell lines with low ARHI expression and found that hypermethylation was the main mechanism for the loss of function of ARHI. Stable transfections of ARHI can inhibit cell cycle progression and induce cell apoptosis in pancreatic cancer cells through the inhibition of PI3K/AKT signaling (Lu et al., 2009). The role of ARHI in regulating growth and its loss in half of pancreatic cancers suggest that the loss of ARHI could be an important event in the pathogenesis of pancreatic cancer. However, the identification of clinical applications of ARHI requires further studies.

2.2.4.2 KLF4 gene

The KLF4 gene, which locus on chromosome 9q31.1-3, negatively regulates G protein-coupled mitogenic signal transduction, cell proliferation, transformation, and oncogenesis. Zammarchi F et al. used immunohistochemical analysis to show that the KLF4 protein is expressed in 86.8% cases of DPC (33/38). The overexpression of KLF4 in a human pancreatic carcinoma cell line induced the up-regulation of p21 and the down-regulation of cyclin D1. It appears that the KLF4 gene may be a key suppressor in pancreatic tumorigenesis (Zammarchi et al., 2011).

2.3 Telomere length abnormalities

2.3.1 The definition and function of telomeres

A telomere is a region of repetitive DNA sequences at the end of a chromosome. This region protects the end of the chromosome from deterioration and from fusion with neighboring chromosomes. Human telomeres are nucleoprotein complexes consisting of 8–15 kb of hexameric DNA repeat sequences (TTAGGG) and specifically bound proteins at chromosomes ends (Blackburn, 1991). These structures prevent the chromosome termini from being recognized as double-stranded DNA breaks and are essential for genomic stability (Artandi et al., 2000). During DNA replication, the DNA polymerase protein complex cannot replicate the sequences that are present at the ends. In somatic cells, telomeres become progressively shorter during each round of cell division through replication-dependent loss of the DNA termini (Harley et al., 1990). Over time, due to each cell division, the telomere ends become shorter. This is the reason why telomeres are so important in context of successful cell division; they "cap" the end sequences and are lost in the process of DNA replication. The cell has an enzyme termed telomerase, which carries out the task of adding repetitive nucleotide sequences to the ends of the DNA. Telomerase is the natural enzyme that promotes telomere repair. Its expression is low or absent in somatic cells, but it is active in stem cells, germ cells, hair follicles, and 90 percent of cancer cells (Blackburn, 1991).

The consecutive shortening of telomeres ultimately leads to excessive telomere erosion, loss of telomere capping function, and eventually genetic instability and cellular senescence when telomeres become critically short (Counter et al., 1992). Consequently, epithelial cells with excessive telomere shortening are largely eliminated by protective mechanisms (Artandi et al., 2000). Therefore, telomere shortening has been suggested to be an important biological factor in aging and cellular senescence, which could limit the over-growth of cells and prevent them from transforming into cancer cells.

2.3.2 The relationship between telomeres and human cancer

It is clear that telomeres could function as protectors of chromosome stability and prevent uncontrolled cellular growth. In cancer progression, telomeres help to maintain genomic integrity, similar to the role played by caretaker genes. It is assumed that the loss of telomere function might permit subsequent accumulation of additional genomic changes at the chromosomal level, which may facilitate the progression toward a fully malignant phenotype (Hackett & Greider, 2002).

Telomeres can be maintained through recombination or by telomerase activation. Telomerase is an RNA-dependent DNA polymerase that is generally inactivated in normal human somatic cells. Introduction of telomerase into normal human somatic cells may facilitate unlimited cellular growth and extend the cellular lifespan (Bodnar et al., 1998).

In most human cancers, telomerase was activated through the accumulation of multiple genomic and epigenetic aberrations, and these changes help the cells restore the minimal length of telomeres required to maintain cell function and escape from cellular senescence (O'Hagan et al., 2002). Therefore, the reactivation of telomerase has become an additional hallmark of some human cancers, including pancreatic cancer (Hiyama et al., 1997).

Telomeric fusion is mechanism of telomere dysfunction and leads to uncontrolled mitosis of cancer cells. Telomeric fusions between chromosomal arms may occur in the presence of critically shortened telomere repeat sequences; these fusions lead to ring and dicentric chromosomes that form anaphase bridges during mitosis (Gisselsson et al., 2001).

Highly recombinogenic free DNA ends are generated when anaphase bridges are broken, and fusion of the broken ends results in novel chromosomal rearrangements. Some of these abnormal chromosomes may then form bridges during the next cell division, setting in motion a self-perpetuating breakage-fusion-bridge cycle. The presence of unbalanced chromosomal rearrangements is an essential feature of most human epithelial cancers (Gisselsson et al., 2001) .

2.3.3 The relationship between telomeres and pancreatic cancer

Pancreatic adenocarcinomas, which are remarkable for their highly complex karyotypes, numerous chromosomal abnormalities, and multiple deletions, often possess chromosome ends that lack telomeric repeat sequences (Griffin et al., 1995). The evidence for up-regulated human telomerase reverse transcriptase expression has been demonstrated in invasive pancreatic cancer (Hiyama et al., 1997) and in the intraductal papillary mucinous neoplasms (IPMN) of the pancreas (Hashimoto et al., 2008). Telomere dysfunction was also found to play a role in the multistep progression model for the development of pancreatic cancer. In this multistep model of pancreatic cancer development, noninvasive precursor lesions in the pancreatic ductules accumulate genetic alterations in cancer-associated genes that ultimately lead to the development of an invasive cancer. In the pancreas, the noninvasive precursor lesions are called pancreatic intraepithelial neoplasia or PanIN. PanINs are believed to progress from a flat and papillary appearance without dysplasia to a papillary appearance with dysplasia to carcinoma in situ (van et al., 2002). Telomere fluorescence in situ hybridization and immunostaining was used to assess the telomere length in tissue microarrays containing a variety of noninvasive pancreatic ductal lesions (van et al., 2002) found that the telomere signals were strikingly reduced in 79 of 82 (96%) of PanINs compared with adjacent normal structures. The 82 PanIN lesions that were examined included all histological grades (PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3). Thus, this study reveals that telomere shortening is the most common early genetic abnormality in the progression of pancreatic adenocarcinomas. Telomeres may be an essential gatekeeper for maintaining chromosomal integrity and normal cellular physiology in pancreatic ductal epithelium. A critical shortening of telomere length in PanINs may predispose these noninvasive ductal lesions to accumulate progressive chromosomal abnormalities and to

progress toward the stage of invasive carcinoma. Another research group also found that the telomeres were significantly shortened (97.3%) in 37 intraductal papillary mucinous neoplasm (IPMN) loci of the pancreas, which has been increasingly identified as a precursor to infiltrating ductal adenocarcinoma (Hashimoto et al., 2008) .

Therefore, telomere abnormalities may function as a cancer marker in invasive pancreatic cancer and may also function as the earliest known event in the cascade of pancreatic cancer development.

Telomere shortening has been suggested to be an important biological factor in aging, cellular senescence, cell immortality, and transformation to cancer. Cellular immortality and transformation are associated with the reactivation of telomerase and with telomere dysfunction in cells with critically shortened telomeres and may play an important role in the development of pancreatic cancers.

2.4 Epigenetic abnormalities

Both epigenetic abnormalities and genetic alterations contribute greatly to cancer development at all stages and may drive the initial steps of cancer progression. DNA methylation and chromatin configurations underlie the abnormal patterns in cancer, and cumulative epigenetic abnormalities of the host genes without accompanying changes in the DNA sequences are critical contributors to oncogenesis. Interestingly, cancer-specific epigenetic alterations can be reversed by pharmacological targeting, and increasing attention has been given to this field as a means to treat cancer.

In the United States, it is estimated that 44,030 new cases of pancreatic cancer were diagnosed and 37,660 deaths occurred in 2011(Siegel et al., 2011), which indicates that pancreatic ductal adenocarcinoma is an extremely aggressive and devastating neoplasm. Therefore, a better understanding of pancreatic cancer molecular genetics is important and can provide the basis for the development of valuable biomarkers and targets for therapeutic intervention.

Over the past two decades, extensive interest has revealed many advances in the understanding of genetic alterations that are important in pancreatic cancer. The mutations and deletions of oncogenes and tumor suppressor genes, such as k-ras,p53 CDKN1A/p16, SMAD4/DPC4, etc., appear to play an important role in pancreatic carcinogenesis. In addition, by understanding of the progression of pancreatic cancer, a model of pancreatic carcinogenesis, from precursor lesions to invasive cancers with genetic alterations, was proposed.

Recently, the epigenetic abnormalities found in pancreatic cancers were also of considerable interest among researchers and clinicians. This interest was especially piqued after demethylating drugs, 5-azacytidine (5-aza-CR) and 5-aza-29-deoxycytidine (5-aza-dC), were shown to be effective in treating myelodysplastic syndrome and were approved by the Food and Drug Administration (FDA) (Venturelli et al., 2011). The key epigenetic mechanisms that may affect gene expression include DNA methylation, histone modification, and microRNA expression (Hong et al., 2011). Epigenetic abnormalities may be functionally involved in precursor lesions, tumor growth, invasion and metastasis in pancreatic cancer. In the following section, we will review recent advances in our understanding of the

epigenetic features associated with pancreatic neoplastic progression, specifically focusing on their role in precursor lesions and their potential clinical benefits.

2.4.1 DNA methylation

DNA methylation is a biochemical process where a methyl group is added to the fifth positon of the cytosine pyrimidine ring or the sixth nitrogen of the adenine purine ring. DNA methylation stably alters the gene expression pattern to provide cellular memory or decrease gene expression. DNA methylation also plays a crucial role in the development of nearly all types of cancer. Both hypermethylation and hypomethylation distinguish normal tissue from tissue associated with pancreatic cancer (Jaenisch & Bird, 2003). Hypermethylation is one of the major epigenetic modifications that repress transcription via the promoter region of tumor suppressor genes. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with gene inactivation. Global hypomethylation has also been implicated in the development and progression of cancer through alternative mechanisms (Jeffrey & Nicholas, 2011).

2.4.2 DNA methylation and precursor lesions

It has been shown that PDAC develops through a stepwise progression from preinvasive lesions, including PanINs, IPMNs, and MCNs, to invasive neoplasms (Haugk, 2010). The discovery of abnormal methylation in pancreatic cancer has been followed by the investigation of methylation in precursor lesions. Many genes that are epigenetically silenced in pancreatic cancers also are silenced or have reduced expression in precursor lesions of pancreatic cancer. The molecular genesis of precursor lesions may lay the foundation for our understanding of pancreatic carcinogenesis and the identification of valuable tumor markers and therapeutic targets.

Many genes showed epigenetic abnormalities in precursor lesions of pancreatic cancer, including Reprimo, SPARC, SAPR2, NPTX2, LHX1, CLDN5, CDH3, and ST14 for PanIN and 119 CDKN1C/p57KIP2 and CyclinD2 for IPMN (Fukushima et al., 2002, 2003; Gerdes et al., 2003; Matsubayashi et al., 2003; Sato et al., 2008). Using methylation-specific PCR analysis (Singh & Maitra, 2007), eight genes (Reprimo, SPARC, SAPR2, NPTX2, LHX1, CLDN5, CDH3, and ST14) were tested in 65 PanIN lesions. The results revealed that these eight genes may be detected in more than 70% of the earliest lesions (PanIN-1A). In addition, aberrant DNA methylation can be detected in PanIN-2 and PanIN-3 lesions, which suggests that DNA methylation alterations may begin in the early stages of precursor lesions, such as in PanINs, IPMNs, and MCNs. Moreover, their prevalence was shown to progressively increase during pancreatic carcinogenesis. Because DNA methylation of particular genes can occur in the precursor lesions, the methylation targets may be valuable tumor markers and treatment strategies.

2.4.3 DNA methylation and pancreatic cancer

Changes in the DNA methylation program are closely associated with pancreatic carcinogenesis, including CpG island hypermethylation and hypomethylation (Sato & Goggins, 2006). Recently, high-throughput screening technologies and single gene methylation technologies have identified several genes that are affected by aberrant DNA

methylation in pancreatic cancer. Tan AC et al. detected 1505 CpG sites across 807 genes to identify DNA methylation patterns in the pancreatic cancer genome and found that 289 CpG sites show different patterns in the normal pancreas, pancreatic tumors and cancer cell lines (Tan et al., 2009). The promoter and CpG island array was used to compare the Panc-1 cell lines with a non-neoplastic pancreatic duct line, and 1,010 of 87,922 probes on the 88 K promoter array (606 genes) had higher signals (log2 > 2) in the pancreatic cancer line.

The aberrant hypermethylation of CpG islands is an important cause of altered tumor suppressor gene function in pancreatic cancers. Several of the classic tumor suppressor genes, such as p16, p53, and SMAD4/DPC4, showed DNA hypermethylation, which suggests that DNA hypermethylation is an important mechanism in pancreatic carcinogenesis. DNA hypermethylation has also been observed in many other genes that are implicated in pancreatic carcinogenesis, including TNFRSF10C, NPTX2, SPARC, FOXA1/2, RUNX3, GATA-4, GATA-5, ppENK, CDKN1C/p57KIP2, HHIP, DUSP6, CXCR4, TFPI-2, HIN-1, SOCS-1, WWOX, RASSF1A, CACNA1G, TIMP-3, E-cad, THBS1, hMLH1, DAP kinase, and ARHI (Cai et al., 2011; Dammann et al., 2003; Fendrich et al., 2005; Fu et al., 2007; Gao et al., 2010; Komazaki et al., 2004; Krop et al., 2004; Kuroki et al., 2004; Martin et al., 2005; Nakayama et al., 2009; Nomoto et al., 2008; Ohtsubo et al., 2006; Park et al., 2007, 2011; Sato et al., 2003, 2005, 2005, 2005, 2005; Song et al., 2010; Ueki et al., 2000; Xu et al., 2005).

DNA hypomethylation an additional type of epigenetic alteration that is found in pancreatic cancer (Ehrlich, 2002). Global DNA hypomethylation and hypomethylation of specific genes have been observed. Global DNA hypomethylation is associated with folate metabolism, indicating that essential nutrients are helpful for preventing cancer progression (Gaudet et al., 2003; Kim, 2004). DNA hypomethylation of many oncogenes, such as claudin4, lipocalin2, 14-3-3 sigma, trefoil factor 2, S100A4, mesothelin, PSA, has also been shown to be important for facilitating their over-expression during pancreatic carcinogenesis.

2.4.4 DNA methylation and clinical applications

Does targeting DNA methylation in pancreatic cancer show a clinical benefit as an early detection method or an effective treatment strategy? Initially, the serum level of the hypermethylation of specific genes appeared to hold potential diagnostic value. Gotoh M found that the methylation status of twelve bacterial artificial chromosome (BAC) clones could predict pancreatic tumors with 100% sensitivity and specificity and could also identify patients that would show early relapse with 100% specificity (Gotoh et al., 2011). Park JK found that the level of serum NPTX2 hypermethylation was a valuable diagnostic marker for identifying pancreatic cancers with 80% sensitivity and 76% specificity (Park et al., 2011). Gerdes B et al. found that p16(INK4a) alterations can be observed in a significant number of PanIN1 in chronic pancreatitis tissues, and methylation of the p16(INK4a) promoter may indicate a high-risk for progression from chronic pancreatitis to cancer (Gerdes et al., 2001). In addition, DNA methylation of p16, ppENK, SARP2 and some additional genes was demonstrated to be a valuable diagnostic tool to predict pancreatic cancer (Yan et al., 2005). Overall, the detection of DNA methylation, either alone or in combination with other tumor markers, will be helpful for screening and diagnosing pancreatic cancer.

Importantly, DNA methylation, unlike genetic changes, are considered to be reversible biological alterations, so pharmacological agents that target this change are attractive potential

strategies for treating cancer. Drugs that target the DNA methyltransferase are promising chemotherapeutic agents because this enzyme is a limiting factor for DNA methylation.

Yang et al (Yang et al., 2010). demonstrated that the inhibitor decitabine (5-aza-dC, 2'-deoxy-5-azacytidine DNMT inhibitor) could inhibit pancreatic cancer cell growth, induce apoptosis, induce ARHI gene demethylation and induce ARHI re-expression. Many studies have demonstrated that tumor-suppressor gene expression can be restored by DNMT inhibitors to induce pancreatic cancer apoptosis, including NPTX2, BNIP3, SOCS-1, WWOX, and cyclin D2. Although demethylating drugs have been approved by the FDA to treat MDS, these demethylating drugs must be further investigated to understand the mechanism that prevents pancreatic cancer progression and to predict potential side effects (Matsubayashi et al., 2006; Sato & Goggins, 2006).

2.4.5 Histone modifications and pancreatic cancer

Histone proteins influence chromatin accessibility and gene activity through post-translational modifications (Bernstein et al., 2007; Gaudet et al., 2003; Ting et al., 2006). Histone acetylases/deacetylases, the polycomb group proteins, and HP1 are the key histone protein complexes that influence chromatin accessibility and gene activity. Histone modifications have been linked to the altered expression of several critical genes in pancreatic cancer, including the IL-13 receptor, MUC17, MUC4, MUC1 and MUC2 (Esteller, 2007; Fujisawa et al., 2011; Kitamoto et al., 2011; Vincent et al., 2008; Yamada et al., 2008).

The importance of histone modifications lies in their potential use as a diagnostic and therapeutic intervention. For instance, it has been shown that histone deacetylase inhibitors induce apoptosis of human pancreatic cancer cells. Donadelli M found that histone deacetylase inhibitors, in combination with conventional chemotherapeutic drugs, such as gemcitabine, leads to a synergistic inhibition of pancreatic adenocarcinoma cell growth. In addition, targeting the Polycomb members and HP1 has also been shown to be effective in inhibiting pancreatic cancer cells. Furthermore, Manuyakorn A et al. showed that the pattern of H3K4ME2, H3K9me2 and H3K18ac can predict the prognosis and treatment response of patients (Donadelli et al., 2007; Garcia-Morales et al., 2005; Haefner et al., 2008; Manuyakorn et al., 2010; Yamada et al., 2006).

Recently, many studies have focused only on somatic genetics; however, these areas represent only a small portion of mechanisms that contribute to gene alteration in pancreatic cancer. Epigenetic changes, including CpG island hypermethylation, hypomethylation, and histone modifications, comprise a new arena for pancreatic cancer research, which may provide new diagnostic and therapeutic tools to combat pancreatic cancer. However, many fundamental questions about the biological and clinical significance of epigenetic changes have yet to be answered, and further studies are needed to do to create effective clinical applications for pancreatic cancer.

2.5 Aberrant microRNA expression in pancreatic cancer

2.5.1 Introduction to microRNA

MicroRNAs(miRNAs) are non-protein-coding RNA molecules that are approximately 22 nucleotides and regulate gene function in various silencing pathways. These molecules are

also encoded by genes and are transcribed by RNA polymerase II. miRNAs are phylogenetically conserved and play an important role in cell survival, proliferation, differentiation, apoptosis and angiogenesis (Ambros, 2004; Farh et al., 2005). miRNAs expression patterns differ, depending upon the cell, tissue, and disease type.

miRNAs regulate their targets by direct mRNA cleavage or translational inhibition and each miRNA can regulate multiple target genes.In the most recent database (miRBase release 15), over 21,643 mature miRNAs have been identified in 168 species (Kozomara & Griffiths-Jones, 2011).

2.5.2 miRNAs and pancreatic cancer

The overexpression and deregulation of several miRNAs has been observed in human cancers (Lu et al., 2005; Metzler et al., 2004; Takamizawa et al., 2004). These studies have also shown that miRNA expression signatures correlate well with specific cancer clinical characteristics and could be used to differentiate normal and cancerous tissues, as well as subtypes of malignancy (Calin & Croce, 2006; Cummins & Velculescu, 2006; Dalmay & Edwards, 2006).Deregulation of miRNAs in cancer may be caused by several changes: (1) chromosomal regional gain, loss or translocation, (2) aberrant expression and activation of transcriptional factors, (3) epigenetic alterations, or (4) changes in miRNA processing (Deng et al., 2008).

The miRNA expression profiles in pancreatic tumor tissues are different from those observed in the normal pancreas or in patients with chronic pancreatitis. Most miRNA expression profile analyses show that miRNAs are deregulated in tumor tissues compared with normal pancreatic tissue, and the expression pattern is tissue specific.

Szafranska *et al*. (Szafranska et al., 2007) demonstrated that two miRNAs, miR-216 and miR-217, are pancreas specific, which was in agreement with two previous studies (Sood et al., 2006). Furthermore, both miR-216 and miR-217 are absent or only minimally expressed in pancreatic carcinoma tissues and cell lines. Therefore, miR-216 and miR-217 are potential biomarkers. Based on clustering analysis, the three pancreatic tissue types (normal pancreas, chronic pancreatitis and pancreatic cancer) can be classified according to their respective miRNA expression profiles. Among 26 miRNAs that have been identified as most prominently deregulated in PDAC, only miR-217 and miR-196a have been found to discriminate between normal pancreas, chronic pancreatitis and tumor tissues. These miRNAs are also potential biomarkers.

Zhang *et al.* (Zhang et al., 2009) evaluated 95 miRNAs, which were selected from pancreatic cancer profiling, and correlated them with their potential biological functions, such as cancer biology, cell development, and apoptosis. Among them, eight miRNAs (miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b and miR-95) are differentially expressed in most pancreatic cancer tissues and cell lines. These eight genes are all significantly up-regulated, from 3- to 2018-fold, in pancreatic tumors compared with normal control samples.

miRNAs are functionally classified as oncogenes or tumor suppressors based on whether their targets are oncogenes or tumor suppressor genes. Therefore, oncogenic miRNAs are upregulated in tumors, whereas tumor suppressor miRNAs are downregulated. Torrisani *et*

al. (Torrisani et al., 2009) have reported that the tumor suppressor let-7 miRNA is expressed in normal acinar pancreatic cells but is extensively downregulated in PDAC samples compared with adjacent unaffected tissues.

2.5.3 miRNAs and clinical applications

2.5.3.1 miRNAs as biomarkers for pancreatic cancer diagnosis

Recent studies indicate that aberrant miRNA expression occurs early in the precursor lesions during the multiple stages of pancreatic cancer development. In addition, miRNA profiles may be assessed in more clinically accessible samples, such as pancreatic juice, and may be used as a diagnostic tool.

Szafranska *et al.* (Szafranska et al., 2008) identified potential miRNA markers in EUS-FNA biopsies of pancreatic tissue. The results show that the combined expression pattern of miR-196a and miR-217 can differentiate PDAC cases from healthy controls and chronic pancreatitis in the FNA samples. Furthermore, miR-196a expression is likely to be specific to PDAC cells and is positively associated with the progression of PDAC.

The potential use of these miRNAs as biomarkers has been evaluated in pancreatic juices. Habbe *et al.* (Habbe et al., 2009) have observed significant overexpression of 10 miRNAs in IPMNs (n = 15). miR-155 and miR-21 show the highest relative fold-changes in the precursor lesions. The upregulation of both miR-155 and miR-21 in the subset of IPMN-associated pancreatic juices was observed.

Wang *et al.* (Wang et al., 2009) have studied plasma samples from patients with PDAC and found that four miRNAs (miR-21, miR-210, miR-155 and miR-196a) are able to differentiate pancreatic cancer patients from healthy controls with moderate accuracy (64% sensitivity and 89% specificity).

2.5.3.2 miRNAs as therapeutic targets in pancreatic cancer

Several studies have shown that the events leading to EMT are regulated by miRNAs (Gregory et al., 2008; Korpal & Kang, 2008; Wellner et al., 2009). Li *et al.* (Li et al., 2009) investigated the effects of let-7 and miR-200 on the morphological changes of EMT in gemcitabine-resistant pancreatic cancer cells (GRPCCs). They noted several observations: (1) the expression of miR-200 and let-7 is significantly downregulated in GRPCCs, which have EMT characteristics; and (2) transfection of GRPCCs with miR-200 rescues the epithelial phenotype by upregulating the epithelial marker E-cadherin and downregulating the mesenchymal markers ZEB1 and vimentin.

Oh *et al.* (Oh et al., 2010) have shown that upregulation of let-7a results in the attenuated expression of Kras and increased radiosensitization of pancreatic cancer cells. This suggests that miRNA could be used as a valuable therapeutic option in radioresistant tumors that have K-ras mutations.

Weiss *et al.* (Weiss et al., 2009) have shown that miR-10a expression promotes metastasis, and repression of miR-10a inhibits invasion and metastasis in xenotransplantation experiments using zebrafish embryos. These data also suggest new therapeutic applications for miRNA in patients with metastatic pancreatic cancer.

Moriyama *et al.* (Moriyama et al., 2009) showed that miR-21 could be a target for a therapeutic strategy for patients with chemoresistant pancreatic cancer. Ji *et al.* (Ji et al., 2009) showed that miRNAs, such as miR-34, can be a novel molecular therapy for human pancreatic cancer *via* inhibiting pancreatic cancer stem cell differentiation.

Overall, many reasearchers suggest that miRNA play an important role in pancreatic carcinogenesis. However, many questions about the function and clinical application need to be further answered for pancreatic cancer.

2.6 A multistep model that involves the accumulation of genetic alterations during the development of pancreatic cancer

We now know that the development of pancreatic cancer, like other malignant diseases, is a multistep process involving the accumulation of genetic and epigenetic mutations. Furthermore, it has been shown that some genetic alterations occur early in the disease and can be designated disease-promoting mutations, whereas others occur later and enhance the oncogenic potential of earlier mutations. Three different types of preneoplastic lesions have been identified in the pancreas: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasia (IPMN) and mucinous cystic neoplasms (MCN). Of these, PanIN lesions are the best characterized, both genetically and pathologically. A well-known progression model of pancreatic cancer development explains that normal pancreatic ductal cells progress from flat (PanIN-1A) and papillary lesions (PanIN-1B) without dysplasia to papillary lesions with dysplasia (PanIN-2) to carcinoma in situ (PanIN-3) and finally to invasive pancreatic cancer (Hruban et al., 2008).

There are two distinct genetic events that occur in the early stages of pancreatic cancer PanIN -1 lesions: telomere shortening and K-ras mutations (Hruban et al., 2000). Activating point mutations of K-ras occur in approximately 45% of PanIN-1 lesions (Hingorani et al., 2003). Telomere shortening is found in approximately 90% of PanIN-1 lesions and may contribute to global chromosomal abnormalities in PanINs (van et al., 2002). Inactivating mutations of CDKN2A/p16 begin to occur in PanIN-2 lesions, whereas inactivation of TP53, SMAD4/DPC4, and BRCA2 are generally associated with higher-grade PanIN lesions (PanIN-3) (Schonleben et al., 2008).

Furthermore, a recent study described a cell surface marker-mediated system for identifying pancreatic cancer stem cells. Pancreatic cancer cells share several features with embryonic pancreatic cells, including activation of the Notch and Hedgehog signaling pathways, which regulate the growth of many organs during embryogenesis and is aberrantly activated in pancreatic cancer cells (Hong et al., 2011; Wong & Lemoine, 2009). The Notch pathway is a critical regulator of pancreatic development and appears to be active in the early stages of pancreatic cancer initiation as well as in invasive cancers. Activation of this pathway leads to the proteolytic intramembrane cleavage of Notch receptors, which results in the release and translocation of their active intracellular domain to the nucleus. Moreover, the upregulation of several Notch target genes in invasive pancreatic cancer as well as preneoplastic lesions suggests that this pathway is an important contributing factor in the development of pancreatic cancer (Maitra & Hruban, 2008).

The activity of the Hedgehog pathway is another important pathway in the development of the gastrointestinal tract and has been implicated in the development and maintenance of

the pancreatic cancer phenotype. The Hedgehog family is composed of Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh). Many studies have shown that many of the components of the Hedgehog family show abnormal expression in pancreatic cancer and precursor lesions (Dosch et al., 2010). These studies indicate that Hedgehog signaling plays a role in the initiation and growth of pancreatic cancer (Kayed et al., 2006). Overall, multistep changes and pathway involves the development of pancreatic cancer.

3. Conclusion

As in colorectal cancer, two distinct tumor categories exist in pancreatic cancer, which are distinguishable by the predominant mutagenic mechanism. Most pancreatic cancers exhibit chromosomal instability (CIN), which causes numerous gross chromosomal changes that result in aneuploidy. A second category is characterized by microsatellite instability (MSI) (Vogelstein & Kinzler, 2004), which results in a drastically decreased fidelity of DNA replication and repair due to defects in the DNA mismatch-repair pathway. Therefore, MSI tumors exhibit frequent errors during DNA replication, which are particularly pronounced at repetitive sequences termed microsatellites.

In the past decade, major advances have been made in understanding the earliest histological and molecular changes that occur in precursor lesions and cancers of the pancreas (Hruban & Adsay, 2009). In addition, the identification of molecular signatures that mark the earliest changes of carcinogenesis may lead to the earlier detection of pancreatic cancer. Understanding the signature of molecular alterations that occur before the development of invasive pancreatic cancer may lead to improved detection and survival of pancreatic cancer patients.

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4

Pancreatic Cancer: Current Concepts in Invasion and Metastasis

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1. Introduction

Pancreatic cancer remains to be one of the most lethal solid tumours of the gastrointestinal tract with a 5-year survival rate lower than 5%. It is characterised by late diagnosis, aggressive local invasion, early systemic dissemination and resistance to chemo- and radiotherapy. At the time of diagnosis more than 85% of patients have already developed metastasis and are therefore not eligible for local treatments with curative intention such as surgery or radiotherapy. Chemotherapy with Gemcitabine is the mainstay of palliative treatment with modest antitumour effects in these patients.

The process of cancer initiation, progression and metastasis remains to be poorly understood. Little is known about the development of metastatic progression and the dissemination of cells from the primary tumour site into distant organs. A better understanding and thorough investigation of the biology behind pancreatic cancer invasion and metastasis is urgently needed. Current concepts and emerging fields of research in pancreatic cancer metastasis shall be discussed in this chapter.

2. Mechanisms of metastatic evolution

Metastasis has been conventionally viewed as the last step in a cumulative process of genetic alterations within cells of a primary tumour mass. For metastatic cells to progress, they have to acquire distinct properties such as loss of cell adhesion, acquisition of an invasive potential, ability of intravasation, transport through the circulation, extravasation, formation of micro-metastases, and finally the ability to induce an angiogenic switch to form macro-metastasis (Coghlin & Murray, 2010). This hypothesis has been recently challenged by several groups (Bernards & Weinberg, 2002; Weinberg, 2008; Klein, 2008). An alternative model is proposed that considers the genetic alterations accumulated at the initial stages of

tumour's evolution to be sufficient to promote the metastasis process. Two distinct models arising from this hypothesis are discussed in the following section.

2.1 Metastasis: Progression models

2.1.1 Late metastasis (linear progression) model

This model is based on Leslie Fould's description of a step-wise progression of morphological abnormalities accompanying cancer (Klein, 2009). It places selection of genetic and epigenetic modifications mostly inside the established primary tumour for competitive fitness. After multiple rounds, the cells may be able to proliferate relatively autonomously at a competitive rate and seed to secondary metastatic niche sites (Fig.1). Late disseminating cells are expected to be genetically identical to the parental cells of the primary tumour and should be subject to similar tumour cell targeting therapeutic modalities (Klein, 2008, 2009; Coghlin & Murray, 2010).

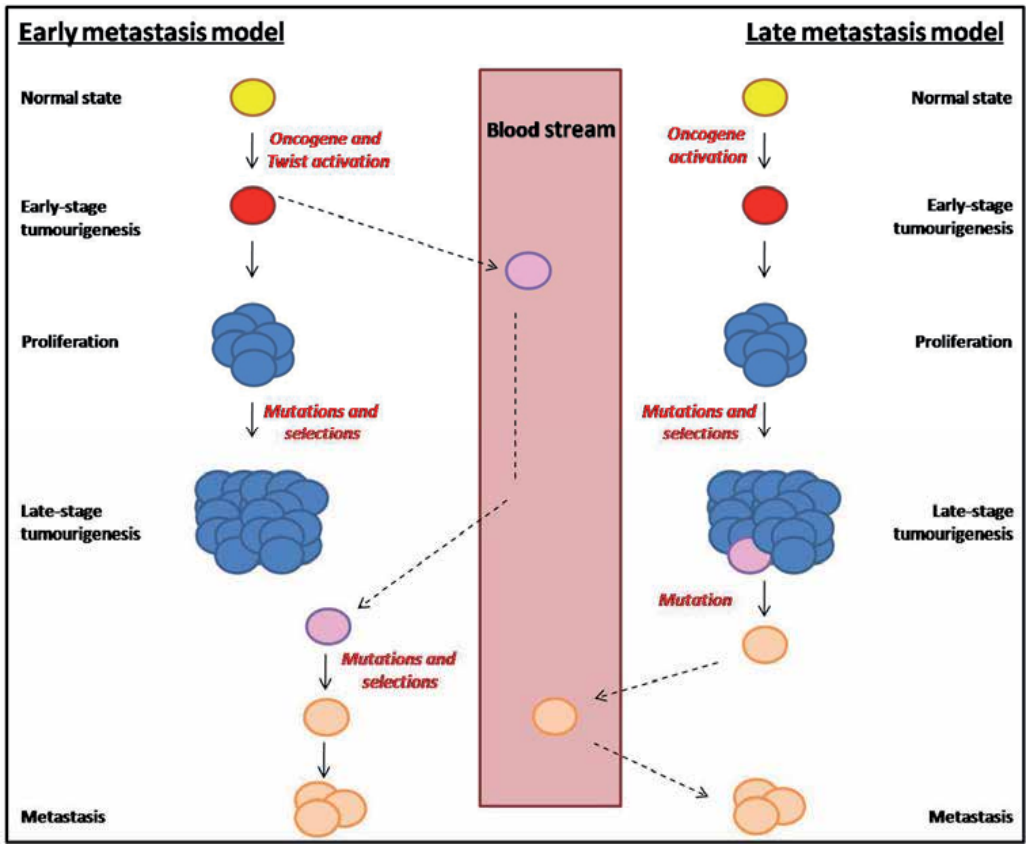

Fig. 1. Models of metastasis evolution: in the late metastasis model, cells acquire genetic and epigenetic modifications mainly in the primary tumour site of the organ. Mutated cells disseminate within the blood stream into the final metastatic niche. However, in the early metastasis model cells accumulate genetic alterations at distant sites and thus diverge from the primary tumours at both genetic and epigenetic levels.

2.1.2 Early metastasis (parallel progression) model

In contrast to the late metastasis model, here tumour cells are believed to leave the primary site quite early up-on activation of factors such as Twist (master regulator of embryonic morphogenesis) with a major role in metastasis during tumourigenesis (Yang et al., 2004) and to diverge genetically at ectopic sites whereby they generate a further cascade of metastatic cells (Fig.1). Owing to the different selection pressures at different niches (metastastic sites) and inherent genetic instability of tumour cells, parallel progression predicts greater variation among metastatic founder and primary tumour cells. Consequently they may respond differently to systemically administered drugs (Klein, 2008, 2009; Coghlin & Murray, 2010).

2.2 Progression of metastasis in pancreas cancer

Whether the dismal prognosis of patients with pancreatic cancer compared to those with other cancer types is a consequence of late diagnosis or early dissemination to distant organs is still debated. In a study performed by Yachida et al., data generated from sequencing of the genomes of seven pancreatic cancer metastases, were used to evaluate any clonal relationship between primary and their corresponding metastatic cancers. After development of the parental clone, clonal evolution continues within the parental site giving rise to distant metastases. They provided evidence that primary pancreatic cancers contain a mix of geographically and genetically distinct sub-clones, each harbouring large numbers of cells that are present within the primary tumour years before the metastases become clinically apparent (Yachida et al., 2010). Additional studies led to estimation of at least three time-scales associated with tumour progression: the time between tumour initiation to establishment of the founder cell of the parental clone (average 10 years, T1); the sojourn time between the arising parental clone and its acquisition of metastatic potential (average 6.8 years, T2); and the time from metastatic dissemination to patient's death (average 2.7 years, T3) (Yachida et al., 2010; Lubeck, 2010). Unfortunately, the vast majority of cancer patients are diagnosed within the last two years of tumour development. The great challenge would rather be the detection of these lesions during or shortly after T1, but before T2-T3 i.e., the seeding of metastases.

3. The host and the tumour micro-environment

Malignant cells do not exist in isolation, but are rather intensely communicating with the surrounding cells in their micro-environment such as microvessel endothelial cells, macrophages, fibroblasts, bone-marrow-derived cells etc. These interactions significantly contribute to tumour proliferation, local invasion and distant metastasis.

3.1 Pancreatic stellate cells: Role in pancreatic cancer metastasis

The transition of the pancreatic stellate cells (PSC) from a quiescent to an ''activated'' or ''myofibroblastic'' state plays a key role in various pathogenic disorders of the exocrine pancreas. In quiescent state, PSC express intermediate filament proteins desmin, glial fibrillary acidic protein (GFAP), vimentin and nestin. Up-on activation, induced by various stimuli such as injury, PSC attain other markers such as smooth muscle actin (SMA), and

interstitial collagen type I. Therefore, activated PSC constitute a major source for development of tissue fibrosis attributed to e.g. chronic pancreatitis and pancreatic adenocarcinoma (PDAC) (Omary et al., 2007; Hwang et al., 2008).

Intense fibrotic reaction referred to as tumour desmoplasia is a hallmark of pancreatic cancer (Cruickshank et al., 1986; Hwang et al., 2008; Bachem et al., 2008; He et al., 2007; Erkan et al., 2010) whereby infiltrating carcinoma cells get surrounded by a dense fibrotic stroma consisting mainly of collagen types I and III as well as fibronectin (Imamura et al., 1995; Bachem et al., 2008). PSC, attracted by pancreatic cancer cells, get activated by various paracrine stimulants and growth factors, such as platelet-derived growth factor (PDGF) rendering them motile and proliferative, fibrogenic mediators such as transforming growth factor beta (TGFβ) which stimulate matrix synthesis resulting in this desmoplastic reaction, cytokines such as interleukin Il-1, Il-6, Il-8, and tumour necrosis factor alpha (TNFα) (Omary et al., 2007; Bachem et al., 2008). Among the key implications of the desmoplastic reaction are the promotion of survival and prevention of apoptosis of pancreatic tumour cells through direct interaction with extra-cellular matrix (ECM), increased production of matrix metalloproteases (MMP) and serine proteases such as members of the plasminogen activator system (Vaquero et al., 2003; Edderkaoui et al., 2005).

Additionally, soluble factors produced by PSC themselves stimulate the proliferation and survival of pancreatic cancer cells. Hwang et al. have shown that co-injection of PSC along with pancreatic cancer cells increases tumour incidence, size, and metastasis in an orthotopic model of pancreatic cancer (Hwang et al., 2008). In addition, combined inoculation of human pancreatic cancer and stellate cells in a xenograft model was shown to promote tumour growth and progression as compared to inoculation of tumour cells alone (Bachem et al, 2008). In another study, significantly elevated expression of urokinase plasminogen activator (uPA) as well as fibroblastic uPA receptor (uPAR) was correlated with liver metastasis of human pancreatic cancers, indicating a possible role of stromal fibroblasts in promoting pancreas cancer dissemination. Co-culturing of peri-tumour fibroblasts with metastatic BxPC3 pancreas cancer cells activates matrix metalloprotease-2 (MMP-2) and up-regulates uPAR expression, along with elevated expression of integrin α6β1 in BxPC3 cells. This suggests a possible interaction between integrins of cancer cells and the uPAR of the stromal fibroblasts along the uPAR-uPA-MMP-2 cascade (He et al, 2007). Moreover, Buechler et al. demonstrate a *de novo* transcriptional regulation of uPAR mRNA by the hypoxia-inducible factor-1 (HIF-1), accompanied by an increased rate of hypoxia-induced metastasis (Buechler et al., 2009). Taken all together, pancreas cancer growth and progression are accelerated via an orchestrated functional interaction among carcinoma cells and stellate/stromal fibroblast cells.

3.2 Tumour-associated macrophages: Role in cancer metastasis

Tumour-associated macrophages (TAM) are bone-marrow-derived cells capable of promoting tumour invasion, angiogenesis, immune evasion, and migration (Allavena et al., 2008; Coghlin & Murray, 2010). In normal tissues, pathogenic challenge or wounding results in the local expression of a variety of growth factors e.g. colony stimulating factor 1 (CSF1) also known as macrophage CSF, granulocyte-macrophage CSF (GM-CSF), TGFβ1, in

addition to various chemokines such as CCL2, CCL7, CCL8, CCL3 CCL4. Such a milieu recruits circulating monocytes and stimulates their differentiation into macrophages (Pollard et al., 2004).

At the primary tumour site, hypoxia-related factors and oncogenic induction of pro-inflammatory mediators mentioned above also result in the recruitment of macrophages (TAM) as well as mobilisation of bone-marrow-derived progenitor cells. TAM may thereafter stimulate angiogenesis by expressing factors such as vascular endothelial growth factor (VEGF), and angiopoietin 1/2 (ANG1-ANG2). Moreover, they recruit hematopoietic cells (e.g., mast cells or neutrophils) which exert similar functions (Pollard et al., 2004).

Along with activated stromal cells, TAM act synergistically with malignant cells to degrade the ECM and release growth factors favouring invasiveness. TAM promote invasion by producing proteases that digest the basement membrane and remodel the stromal matrix. Additionally, they produce multiple growth factors which stimulate the growth of the tumour cells themselves (Pollard et al., 2004).

4. Local invasion and distant metastasis

4.1 The epithelial-to-mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) describes a biologic process in which polarised epithelial cell under-go various bio-chemical modifications and ultimately gain mesenchymal characteristics i.e., enhanced migration and invasiveness, increased resistance to apoptosis, as well production of ECM components (Kalluri & Neilson, 2003). A proposal has been made which classifies EMT into three different sub-types. Type 1 EMT is associated with implantation and embryonic gastrulation and gives rise to the mesoderm, endoderm and to mobile neural crest cells. On the other hand, type 2 EMT promotes organ fibrosis mediated by inflammatory cells. The last type of EMT, type 3, is associated with cancer progression and metastasis (Kalluri & Weinberg, 2009).

The EMT process is characterised by decreased expression of Epithelial cell-cell junction molecule E-cadherin along with increased expression of non-epithelial cadherins mostly N-cadherin and acquisition of mesenchymal cytoskeleton markers such as vimentin and SMA, accompanied by nuclear beta-catenin accumulation (Shintani et al., 2006; Yang & Weinberg, 2008; Coghlin & Murray, 2010). Tumour cells undergoing EMT are typically seen at the invasive fronts of primary tumours. These cells may eventually enter into subsequent steps of the invasion-metastasis cascade namely intravasation, transport through the circulation, extravasation, formation of micro-metastases, and colonisation (Fig. 2) (Thiery, 2002, 2003; Fidler & Poste, 2008; Brabletz et al., 2001).

EMT is initiated by extra-cellular signals e.g. hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and TGFβ which activate multiple EMT-inducing transcription factors notably Snail, Slug, zinc finger E-box binding homoebox (ZEB1) and Twist (Savagner et al., 2001; Lee et al., 2006; Shintani et al., 2006; Kalluri & Weinberg, 2009). Activation of EMT cascade also involves the disruption of cell-cell adherens junctions and integrin-mediated adhesion in the ECM.

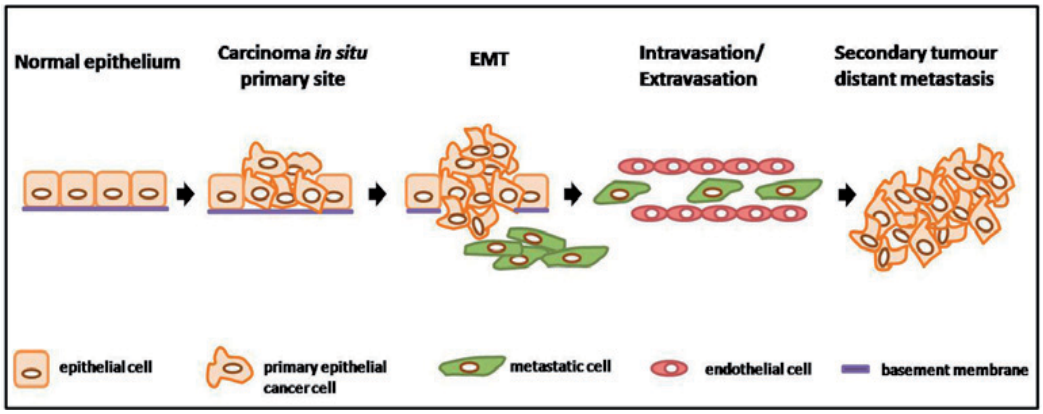

Fig. 2. The role of EMT in cancer metastasis: epithelial tumour cells lose their polarity and detach from the basement membrane. This situation is referred to as *carcinoma in situ*. During the EMT process, primary epithelial tumour cells undergo various modifications whereby they lose their epithelial markers and acquire mesenchymal properties, i.e. digest the basic membrane and invade the surrounding tissue. The onset of angiogenic switch and establishment of abnormal tumour neo-vasculature favours the intravasation of metastatic cells into the blood-stream. Only a small fraction of disseminated tumour cells (DTC) survive in circulation, extravasate into distant organs and form single cell or small tumour colonies also called micro-metastases. DTCs or micro-metastases could remain dormant for a prolonged period of time until they eventually switch to angiogenic macroscopic metastatic lesion and become clinically apparent (Folkman & Kalluri, 2004; Almog et al., 2009). Interestingly, metastatic lesions derived from carcinomas demonstrate the epithelial characteristic of their primary tumour of origin suggesting that according to this model mesenchymal to epithelial transition (MET) occur during tumour colonisation in the distant sites.

4.2 Matrix metalloproteases (MMP)

MMP are a family of enzymes involved in degradation of ECM components e.g. collagen and fibronectin (Jimenez et al., 2000). MMP of either malignant cells or induced fibroblasts at the invasive front are thought to mediate cancer invasion and metastasis via disruption of tumour cell adhesion molecules and degradation of basement membranes and other matrix components.

Immunohistochemical studies have demonstrated over-expression of active MMP-2, MMP-9 (Gress et al., 1995; Koshiba et al., 1998), MMP-7 and MMP-11 (Bramhall et al. 1997) in tumours with lymph node metastasis. The involvement of MMP-1 in promoting angiogenesis and metastatic spread has been recently linked to pancreatic cancer (Abdollahi et al., 2007). Using an orthotopic mouse model of pancreatic cancer, the MMP inhibitor Batimastat was shown to improve the survival and to reduce growth of tumours in mice implanted with HPAC, a moderately differentiated pancreatic cancer cell line (Zevros et al., 1997). Recent work in multiple carcinomas suggests a crucial role of transcriptional regulators Snail and Slug in the regulation of E-Cadherin expression (Gavert & Ben-Ze'ev, 2008). In pancreatic cancer, Slug is shown to promote the invasiveness of tumour cells

through up-regulating ECM proteins such as MMP-9 along with remodeling of the actin cytoskeleton (Zhang et al., 2011).

4.3 Growth factor expression patterns in pancreas metastasis

Among its pleiotropic effects TGFβ was shown to potently induce EMT in different tumours. TGFβ signalling in pancreatic cancer is often attenuated because of alterations in components of this pathway (Jonson et al., 2001). Using genetic array data, genes responsive to TGFβ in pancreas cancer cell line PANC-1 are depicted to be involved in ECM remodeling, cell motility, adhesion, angiogenesis, cell cycle, proliferation and apoptosis (Gaspar et al., 2006). Negative regulation of E-cadherin necessary for EMT has been observed in PANC-1 cultures (Halder et al., 2005), and has been associated with lymph node metastasis (Pignatelli et al., 1994). Regulation of *JAG1* by TGFβ on the array is interesting because its gene product Jagged1 (Notch ligand), is influenced by TGFβ in cultured epithelial cells. Notch signalling is an important cell signalling pathway involved in regulation of the balance between cell proliferation, differentiation, and apoptosis. Notch-1 has been reported to induce nuclear factor κB (NFκB) promoter activity (Jang et al., 2004). Down-regulation of Notch-1, and consequently of NFκB and MMP-9 inhibits invasion of pancreatic cancer cells through matrigel (Wang et al., 2006). Inhibition of TGFβ signalling reduces PDAC growth and invasiveness (Gaspar et al., 2006).

TGFβ signalling is altered in human pancreas cancer cells with one half of tumours showing allelic deletions or inactivating mutations of the Smad4 gene. These cells show an enhanced TGFβ-mediated EMT as determined by increased vimentin expression and decreased beta-catenin and E-cadherin expression. TGFβ-mediated invasion is suppressed in Smad4 intact cells *in vitro* which show reduced dissemination in an orthotopic pancreatic cancer model (Zhao et al., 2008). Interestingly, cells with an intact Smad pathway reveal reduced activation of signal transducer and activator of transcription (STAT3). STAT3 is constitutively activated in various carcinomas and plays a pivotal role in regulating cell growth, cell survival and angiogenesis (Yu & Jove, 2003; Abdollahi et al., 2004). Inhibition of STAT3 phosphorylation by Smad4 suppresses TGFβ-mediated invasion and metastasis of pancreatic cancer cells (Zhao et al., 2008). Therefore, reconstitution of Smad4 activity or suppression of STAT3 down-stream signalling constitutes attractive targets to inhibit TGFβ-induced EMT and pro-metastatic effects.

Other factors known to be frequently over-expressed in several human malignancies and to be associated with invasion of tumour cells are the insulin-like growth factor I receptor (IGF-IR) and HGF receptor c-Met. IGF-IR over-expression and excessive activation are associated with malignant transformation, tumour aggressiveness, and protection from apoptosis (Macaulay, 1992; Sell et al., 1995). Over-expression of IGF-IR, reported in pancreatic carcinomas, is regulated by AKT activation thereby promoting invasiveness of human pancreatic cells (Tanno et al., 2001). Furthermore IGF-IR and c-Met co-operate synergistically to induce migration and invasion of human pancreatic carcinoma cells (Bauer et al., 2006).

EGFR is over-expressed in approximately 90% of pancreatic carcinoma and is associated with poor prognosis. Blocking of EGFR signalling in various animal models reduces growth and spread of pancreas carcinoma (Baselga & Arteaga, 2005). Of note, low-molecular weight

tyrosine kinase inhibitors targeting EGFR such as erlotinib are the only class of targeted inhibitors so far demonstrating additional mild benefits when combined with gemcitabine versus gemcitabine mono-therapy in treatment of metastatic PDAC (Moore et al., 2007). Accordingly, erlotinib is the only targeted therapy currently approved for clinical use in PDAC. In extension of this work, dual inhibition of EGFR signalling using erlotinib (alone or in combination with gemcitabine) and of hedgehog signalling with cyclopamine were shown to inhibit tumour growth, increase apoptosis, and suppress the dissemination of pancreatic cancer cells (Feldman et al., 2007; Mimeault et al., 2005, Hu et al., 2007).

4.4 Hypoxia and angiogenesis

The presence of significant hypoxia in pancreatic cancers has been long suspected due to the relatively poor contrast agent enhancement of pancreatic cancer lesions suggestive of hypo-vascular regions in e.g. computer tomography (CTscans, Megibow, 1992). Koong et al. directly detected hypoxia in pancreas cancer by placement of intra-tumoural needles measuring tissue oxygen levels at the time of resection in seven operable pancreatic cancer patients (Koong et al., 2000). Hypoxia renders tumours more aggressive, and resistant to chemo- and radio-therapy (Garcea et al., 2006, Abdollahi et al. 2005). Therefore, the combination of desmoplastic reaction and strong intra-tumoural hypoxia synergistically contribute to the inherent resistance of pancreas cancer against cancer therapies such as chemotherapy.

For a tumour or any other tissue to grow above the size of $1mm^3$, recruitment of new vessels is required. This process is termed tumour angiogenesis (Folkman, 1971). The "angiogenic switch" is considered a hallmark of cancer and refers to the phenomenon in which the balance of pro- and anti-angiogenic factors is shifted towards the pro-angiogenic state (Hanahan & Folkman, 1996; Abdollahi et al., 2005; Hanahan & Weinberg, 2011). Nevertheless, the hypo-vascular phenotype of pancreatic tumours observed by contrast enhanced non-invasive imaging techniques misled the research in this field to precept that angiogenesis is not playing a key role in development of PDAC. The role of an angiogenic micro-environment in development of pancreatic cancer is only recently reported (Abdollahi et al., 2007). It is shown that the angiogenic state gradually switches from normal pancreas (off) to chronic inflammation (pancreatitis, intermediate) to primary pancreatic tumour and distant metastases (on). These data indicate that aberrant pro-angiogenic micro-environment might contribute to the 19-fold increased cancer risk in patients with chronic pancreatitis (Abdollahi et al., 2007). Although several angiogenic factors have been associated with PDAC, most of the studies have so far focused on VEGF reporting its pivotal role in stimulation of endothelial cell proliferation, migration, gene activation, and apoptosis evasion (Dvorak et al., 1995; Ferrara, 1999, Abdollahi et al. 2005). VEGF is a dimeric cytokine with members including VEGF-A (most common isoform), VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF-A exerts its effects on target endothelial cells via binding to its specific trans-membrane tyrosine kinase receptors VEGFR-1 and VEGFR-2 (Ferrara, 1999; Dvorak, 2002). Hypoxia has been shown to stimulate VEGF transcription in pancreatic carcinoma cell lines. This requires Src activation and leads to increased steady-state levels of HIF-1α and increased phosphorylation of STAT3. Expression of VEGF in STAT3 or HIF-1α dominant negative mutants is significantly reduced. Together, STAT3 and HIF-1α are both required for maximum transcription of VEGF mRNA following hypoxia (Gray et al., 2005).

4.4.1 Local invasion and lymph-angiogenesis

Tumour cell dissemination may follow several patterns e.g. local invasion, lymphatics, hematogenous spread, or direct seeding of body cavities or surfaces (Rubbia-Brandt et al., 2004). The recent discovery of lymphatic endothelium-specific markers such as VEGFR-3, LYVE-1 and lymph-angiogenic growth factors VEGF-C and VEGF-D has allowed better understanding of tumour-associated *de n*ovo lymph-angiogenesis – the generation of new lymphatic vessel - in the metastatic process (Kopfstein et al., 2007). The expression of VEGF-C and VEGF-D is reported in a variety of human tumours and is correlated with markers of lymphatic vessel density (LVD), lymph node metastasis and poor prognosis (Sleeman et al., 2009). Tumour-associated lymphatic vessels have been reported to occur both peri- and/or intra-tumourally with the latter case correlating with lymph node metastasis and prognosis. In contrast to most tumours, in pancreatic carcinoma the correlation between lymph-angiogenesis marker and worse prognosis is still controversially debated (Sleeman & Thiele, 2009).

VEGF-C and VEGF-D are the most extensively studied factors that enhance tumour-induced lymph-angiogenesis and lymph node metastsasis (Thiele & Sleeman, 2006). VEGF-C is highly expressed in pancreatic cancer tissue and cell lines, while its receptor VEGFR-3 is expressed on cancer stromal cells. Thus, local tumour growth is promoted via paracrine simulation of VEGFR-3 expressing stromal cells leading to the entry of cancer cells into peri-tumoural lymphatics (Schneider et al., 2006). Kofstein et al. have used Rip1VEGF-D transgenic mouse model of pancreatic β-cell carcinogenesis to investigate the functional role of VEGF-D in inducing lymph-angiogenesis and tumour progression. They show that VEGF-D expressing tumours exhibit peri-tumoural lymphangiogenesis along with lymphocyte accumulations and hemorrhages, with frequent lymph node and lung but not hepatic metastases (Kofstein et al., 2007). Similar to VEFG-D, transgenic expression of VEGF-C in Rip1VEGF-C model induces peri-tumoural, but not intra-tumoural lymph-angiogenesis, and promoting lymph node metastasis without affecting blood vessel angiogenesis (Mandriota et al., 2001).

Additional mechanisms are described to be involved in tumour lymph-angiogenesis e.g. insertion of endothelial cells into the existing lymphatic endothelium. The existence of lymphatic progenitor cells was attributed to the CD34+ CD133+ VEGFR-3+ expressing cells which could differentiate into cells expressing vascular and lymphatic endothelial cell markers (Salven et al., 2003). Moreover, expression of chemokine receptor CCR7 by tumour cells enables them to migrate to lymphatic endothelial cells expressing the cognate ligand CCL21. Interestingly, CCR7+ tumour cells could produce CCR7 ligands and migrate with the lymphatic fluid in a process referred to as autologous chemotaxis (Sleeman et al., 2009)

4.4.2 VEGF and liver metastasis

VEGF expression is closely related with micro-vessel density and seems to be a crucial indicator for liver metastasis and a poor prognosis in pancreatic adenocarcinoma (Seo et al., 2000). Elevated VEGF levels are correlated with tumour size (Itakura et al., 1997) and liver metastasis (Seo et al., 2000). Others have shown that the presence of TGFβ in the tumour micro-environment plays an important role in enhancing liver metastasis by modulating the capacity of angiogenesis and immunogenicity (Teraoka et al., 2001). Histological studies in

pancreatic cancer patients often show invasion into large veins and dissemination into the liver. This is accompanied by elevated expression of MMP-2 and MMP-9 (Nagakawa et al., 2002). VEGF levels were shown to be markedly elevated in liver metastases as compared to non-tumour bearing liver. However, no correlation was found in VEGF expression between liver metastases and primary pancreatic carcinoma (Tawada et al., 2008).

4.5 Role of chemokines in pancreas metastasis

Chemokines are low molecular-weight peptide ligands involved in the trafficking of leukocytes and other motile cells (Murphy et al., 2000; Mellado et al., 2001). Their receptors are cell-surface, seven trans-membrane G protein-coupled receptors. Many chemokines have more than one ligand and can activate more than one receptor.

4.5.1 Role of the chemokine receptor CXCR4 in pancreas metastasis

CXCR4 has been detected on many leukocytes such as lymphocytes, monocytes, natural killer cells, as well as on vascular smooth muscle cells, endothelial cells, and astrocytes (Caruz et al., 1998; Wegner et al., 1998; Zhang et al., 1998; Balkwill, 2004). The chemokine CXCL12, originally termed stromal derived factor 1 (SDF-1), is a ligand for CXCR4. In normal adult, the interplay between CXCR4 and CXCL12 is critical for homing and retention of hematopoietic progenitor cells in the bone-marrow (Richard & Blay, 2008). High levels of CXCR4 are expressed by these progenitor cells, which in turn get attracted to CXCL12 produced by stromal cells in specialised bone-marrow niches (Aiuti et al., 1997). Stem cells and some other differentiated cells in the pathological contexts of inflammation and tissue regeneration or repair are also influenced by the chemo-attractant potency of CXCL12. It is postulated that metastatic cancer cells subvert the physiologic function of CXCR4/CXCL12 in controlling cell migration and homing.

It has been shown that CXCL12/CXCR4 axis promotes progression and dissemination of various carcinomas. CXCR4 is over-expressed at high levels on cells of solid epithelial cancers including pancreas, and CXCL12 concentrates in fluid-filled cavities through which many cancers disseminate and at tissue sites where metastases develop (Richard & Blay, 2008). Most human pancreatic cancer tissues and more than 50% of pancreatic cell lines stain positively for CXCR4 and express CXCR4 protein, respectively. Chemotaxis induction of human pancreas carcinomas, as well as stimulation of their proliferation and survival is induced by CXCL12 (Figure 3) (Marchesi et al., 2004; Koshiba et al., 2000). Kayali and colleagues have shown in an interferon gamma-non-obese diabetic mouse model that CXCL12 stimulates the phosphorylation of AKT, mitogen-activated protein kinase (MAPK), and Src in pancreatic duct cells, and that it influences ductal cell migration. Blocking the CXCL12/CXCR4 axis in this model leads to a reduced proliferation and increased apoptosis of pancreatic ductal cells (Kayali et al., 2003). Moreover, CXCR4 small molecule antagonists, such as TN14003, were shown to inhibit migration of human pancreatic cancer cells *in vitro* via alteration of MAPK phosphorylation (Mori et al., 2004).

Recently, a study in 30 patients with pancreatic cancer was initiated to evaluate the expression of CXCL12 and CXCR4 in tumour tissues, normal pancreas, and regional lymph nodes. They report low CXCL12 levels in tumour tissues as compared to para-cancerous tissues, normal pancreas, and lymph nodes. On the other hand, levels of CXCR4 in tumour

tissues were markedly higher. Additionally, they depict a significant correlation among the expression of CXCL12/CXCR4 axis and that of lymph node metastases (Cui et al., 2010).

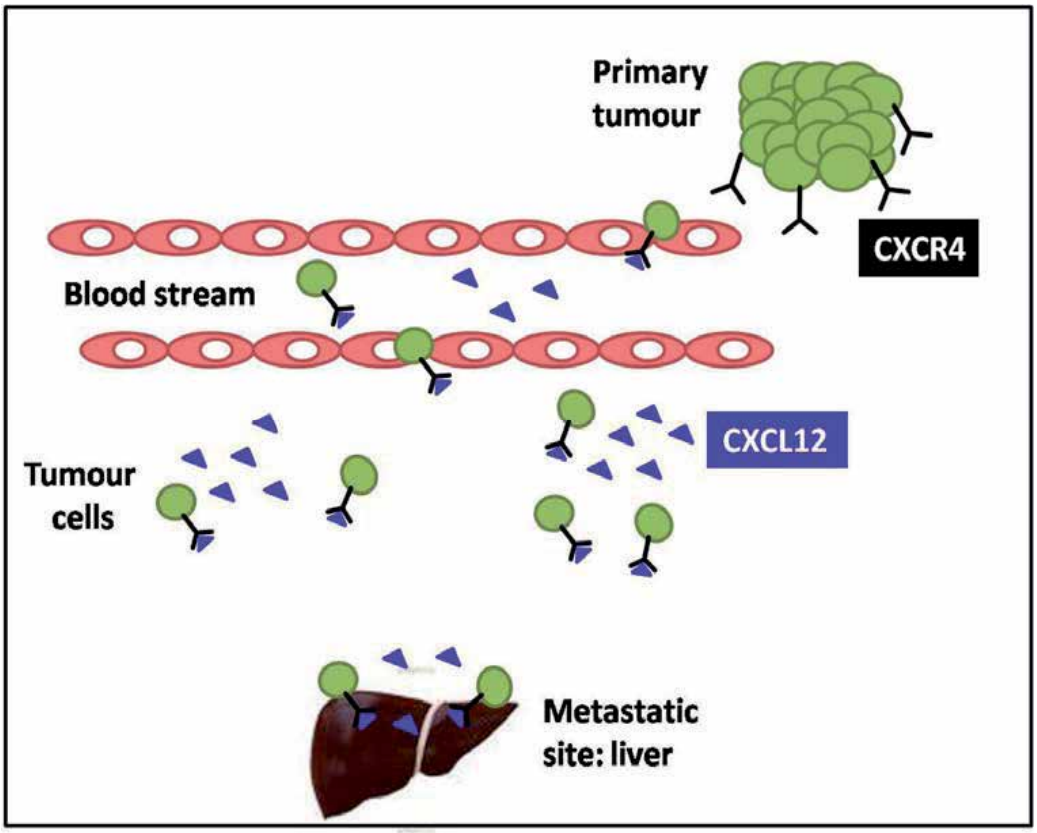

Fig. 3. Dissemination of tumour cells via the CXCL12/CXCR4 axis. Chemokine signalling enhances tumour invasion, tissue remodelling and tumour resistance to apoptotic stimuli. Moreover, pancreatic tumour cells expressing CXCR4 migrate towards the gradient of CXCL12 released by distant organs such as lymph nodes, lung, and liver. Hence, in addition to facilitating tumor invasion, the chemokine guidance plays a critical role in spread of tumour cells from primary sites to form distant metastases.

Several factors regulating the expression of CXCR4 in tumour cells reside within the tumour micro-environment. It has been shown that hypoxia via hypoxia-inducible factor 1 alpha (HIF1α) upregulates CXCR4 (Staller et al., 2003). Accordingly, CXCR4 expression was found to be enhanced in CXCR4-positive cell lines cultured under hypoxic conditions (Marchesi et al., 2004). As mentioned above matrix metalloproteases such as MMP-2 and MMP-9 have been associated with haematogenous tumour spreading. Chemokine and MMP activity seems to be intertwined as treatment of tumour cell lines with CXCL12 were shown to activate MMPs and trigger tumour cell invasion (Marchesi et al., 2004). Gao et al. proposed a

crucial role for PSC in promoting the invasion of human pancreatic cancer cells through the CXCL12/CXCR4 axis (Gao et al., 2010). In addition, CXCL12 was shown to protect CXCR4-positive pancreatic tumour cells from serum starvation-induced death or interleukin-1-induced damage via decreasing their rate of apoptosis (Marchesi et al., 2004). Together, these data suggest an important role for chemokine signalling in matrix-remodelling, tumour invasion and enhanced cell survival by evading apoptotic stimuli.

4.6 Influence of PPAR on pancreas metastasis

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors belonging to the super-family of nuclear hormone receptors (Isseman & Green, 1990). Three major sub-types have been described so far: PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-γ has been recently shown to be over-expressed in pancreatic cancers (Eibl et al., 2004). Troglitazone, a synthetic PPAR-γ agonist/ligand, leads to G1 cell cycle accumulation and inhibits cellular proliferation *in vitro (*Itami et al., 2001*)*. Implantation of PANC-1 tumours in nude mice shows significant inhibition of tumour growth treated with pioglitazone, another PPAR-γ agonist (Itami et al., 2001). In another study, both ciglitazone and 15d-prostaglandin J2 (15d-PGJ2) were shown to inhibit the growth of four tested pancreatic cancer lines. Treatment with 15d-PGJ2 significantly suppresses pancreatic cancer cell invasiveness which is accompanied by a reduction of MMP-2 and MMP-9 protein levels and activity (Hashimoto et al., 2002). The anti-tumour activity on pancreas cancer cell invasion was in part attributed to the influence of PPAR-γ ligands on the serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (Sawai et al., 2006). In addition, synthetic PPAR-γ agonists may impede metastasis formation via interference with chemokine signalling by decreasing CXCR4 expression levels (Richard & Blay, 2008). Of note, data generated with synthetic PPAR ligands known as PPAR "agonists" or "antagonists" are not always matching data generated by genetic knock-down studies suggesting that binding of synthetic ligands may induce additional or different effects, by e.g. release of endogenous ligands (Lee et al., 2003; Plutzky, 2003). In this context, anti-metastatic but not anti-proliferative effects were also reported after treatment of pancreas cancer with a synthetic PPAR-γ ligand (T0070907), known to be a specific PPAR-γ antagonist (Nakajima et al., 2008). In contrast, PPAR-γ agonists were shown to inhibit the growth of pancreas tumours via downregulation of VEGF and thus inhibition of tumour angiogenesis (Dong et al., 2009). This is in line with previous observations suggesting an involvement of PPAR-γ signalling in the angiogenesis process (Panigrahy et al. 2002). PPAR-α and PPAR-β/δ, the two other members of this family were also proposed to play a critical role in tumor growth and angiogenesis (Park et al., 2001, Abdollahi et al. 2007; Müller-Brüsselbach et al., 2007; Wang et al., 2006; Kaipainen et al., 2007; Panigrahy et al. 2008). In particular, PPAR-β/δ expression levels were shown to be gradually increased from normal pancreas to chronic pancreatitis to primary tumor and distant metastasis of pancreas cancer (Abdollahi et al. 2007). Moreover, PPAR-β/δ was found to play a central role within a network of genes that govern the angiogenic switch process. Accordingly, targeted removal of PPAR-β/δ in tumor microenviroment via implantation of wt-tumors in PPAR-β/δ knockdown mouse resulted in impaired tumor growth and angiogenesis (Abdollahi et al. 2007). This data are consistent with other studies reporting on impaired wound healing and reduced body fat; both processes known to be angiogenesis dependent (Peters et al., 2000; Michalik et al., 2001).

4.7 Genomic studies

Recent advances in high-through put sequencing analysis have improved our understanding of genetic alterations in pancreatic cancer. In 2008 Jones et al. reported on sequencing protein-coding exons from 20,735 genes in 24 pancreatic cancers. They found that pancreatic cancer contains an average of 63 genetic alterations, the majority of which were point mutations that could be assigned to a core set of 12 cellular signalling pathways being altered in 67% to 100% percent of pancreatic cancers. These include, apoptosis (100% affected), DNA damage control (83%), regulation of G1/S phase transition (100%), hedgehog signalling (100%), homophilic cell adhesion (79%), integrin signalling (67%), c-Jun N-terminal kinase signalling (96%), K-Ras signalling (100%), regulation of invasion (92%), small GTP-ase Ras-independent signalling (79%), TGF-β signalling (100%), and Wnt/Notch signalling (100%). Although these pathways partially over-lap in the majority of the patients tested, every individual tumour might reveal variations in the alterations observed in pathway components. This perspective likely applies to most of epithelial cancers, and explains the heterogeneity within individual genes and within individual tumours (Jones et al., 2008).

Shi et al. have established a highly metastatic pancreatic cancer line SW1990HM from intra-splenic injection of SW1990 tumor cells. Gene expression profiles of SW1990HM and SW1990 cells show 40 metastasis-related genes expressed with a 3-fold difference. From the 40 genes 32.5% are assigned to be adhesion and ECM-related genes, namely matrix metalloproteases (*MMP-10*, *MMP-9*, *MMP-7)*, E-cadherin tumour suppressor gene (*CDH1)*, and the golgi enzyme glycosyltransferase (*MGAT5).* Another 30% are found to be cell-growth and proliferation-related such as insulin growth factor 1 (*IGF1)*, interleukin 8 receptor beta (*IL8RB)*, integrin A7 (*ITGA7)*, murine double minute oncogene (*MDM2)*, mesenchymal epithelial transition factor (*MET)*, somatostatin receptor 2 (*SSTR2)*, and *VEGF* (Shi et al., 2009).

Thakur and colleagues have utilized Ela-c-myc transgenic mice, described previously to develop acinar carcinoma (50%) as well as mixed ductal and acinar cell carcinoma (50%), to show spontaneous metastasis to the liver (Liao et al., 2006, 2007; Thakur et al., 2008). Microarray analyses revealed up-regulation of genes involved in DNA replication, cell proliferation and cell cycle regulation, chromosome organization, and signal transduction. Many genes are related to the maintenance of chromosomal structure and integrity such as mini-chromosome maintenance 2 (MCM2), MCM5; MCM10; structural maintenance of chromosome 211 (SMC21l), SMC41l, SMC51l, RAD51, and BRCA1.

In alignment with these data expression analysis of two established cell lines (HPAC and PANC1) in terms of their patterns of invasiveness, reveals significant increase in the expression of DNA repair genes. DNA copy number of BRCA1 and RAD51 genes is also found to be increased in tissues isolated from metastatic pancreas cancer in comparison to normal tissue from the respective sites (Mathews et al., 2011).

Thakur et al also described elevated expression levels of IGFBP1 and Serpin1 in liver metastatic tissues as compared to primary pancreatic tumours and normal pancreas. Both genes are also known to be over-expressed in highly metastatic human pancreatic cell lines (PANC28, CoLo357fg, L3.6pl) in comparison to less metastatic cell lines (PANC1 and BxPC3) (Thakur et al., 2008).

5. Peri-neural invasion

Tumour peri-neural invasion (PNI), i.e., the neurotropism of pancreatic tumour cells and their metastasis into the peri-neural space of peripheral nerves constitutes a unique feature of pancreatic ductal adenocarcinoma (PDAC). PNI is associated with poor prognosis in patients due to the fact that tumour cells disseminating along nerve fascicles are spared by surgery and could therefore contribute to the local recurrence of pancreatic cancer (Marchesi et al., 2010; Pour et al., 2003). The human pancreas harbours a large amount of neural tissue and is innervated by the autonomic nervous system through plexi from the celiac and superior mesenteric artery ganglia. In majority of pancreatic cancer patients (~90%) tumor cells infiltrate intra-pancreatic nerves, with involvement of about 70% of extra-pancreatic nerves. Neural infiltration by cancer cells along with the accompanying ultimate nerve damage serve to cause the characteristic severe pain in pancreatic cancer patients (Pour et al., 2003). Morphologic changes at the migration front include characteristic increased neural density and hypertrophy and clustering of malignant cells around the neuritis (Ceyhan et al., 2008; Dai et al., 2007).

5.1 Mediators and molecular mechanisms of PNI

Investigation of many pathologic sections reports an increase in the size of nerve fibres in the vicinity of pancreatic tumours, suggesting the necessity of neurotropic factors, growth factors, and axonal guidance molecules as key players in this aspect (Chedotal et al., 2005; Chilton et al., 2006). Major neurotropic factors, such as neurotropins (NT) which include nerve growth factor (NGF), brain-derived nerve growth factors (BDNF), NT-3, NT-4, and NT-5, are over-expressed in tumour cells and intra-tumoural nerves (Ketterer et al., 2003). Other factors including hematopoietic colony stimulating factors (G-CSF) and their receptors (G-CSFR and GM-CSFRα) have also been shown to be expressed at high levels in pancreatic tumour micro-environment and to be associated with induction of pain. To this end, injection of anti-sera containing neutralising anti-bodies against G-CSF and GM-CSF receptors in a murine model of tumour-induced bone pain prevents hyperalgesia and reduces the number of nerves branching into the skin surrounding the tumour (Schweizerhof et al., 2009). In addition, myelin associated glycoprotein (MAG or Siglec-4a), expressed by Schwann cells bind to mucin 1 (MUC1) enriched on the surface of pancreas tumour cells (Swanson et al., 2007).

5.2 Chemokines and tumour PNI

CX3C chemokine receptor 1 (CX3CR1) also known as the fractalkine receptor or G-protein coupled receptor 13 (GPR13) are known to be involved in leukocyte adhesion and migration. Cells expressing this receptor bind to corresponding ligand CX3CL1 expressed on the surface of neurons, nerve fibres, and activated endothelial cells (Marchesi et al., 2010). In contrast to normal pancreas cells, tumour cells over-express CX3CR1 which in turn stimulates PNI. A large fraction (~90%) of pancreatic cancer biopsies are CX3CR1 positive and high receptor expression is associated with prominent PNI in pancreas cancer (Marchesi et al., 2008). A novel CX3CR1 antagonist has been recently developed and shown to block the cell adhesion along the CX3CL1/CX3CR1 axis (Dorgham et al., 2009). Thus, interference with CX3CL1/CX3CR1 signalling poses an attractive approach in prevention of PNI. Figure 4 illustrates some molecular mechanisms known to be involved in PNI.

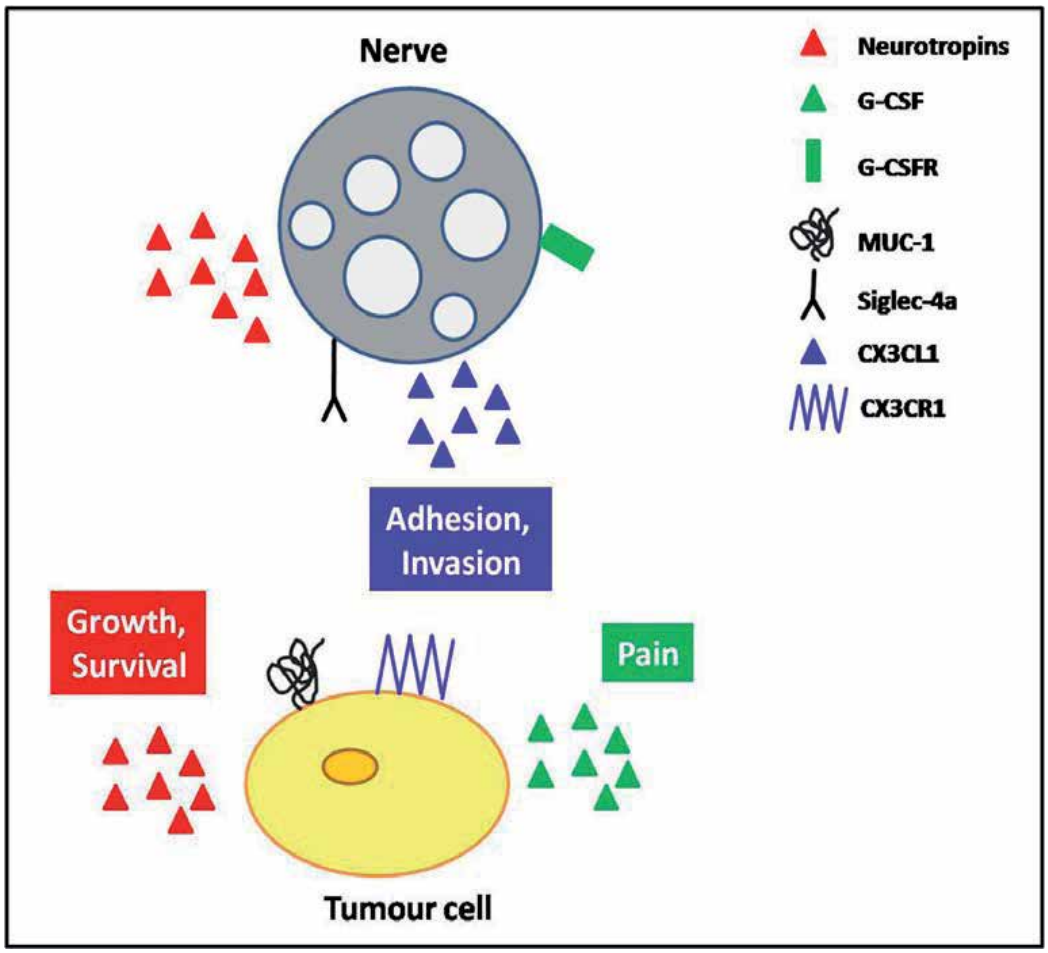

Fig. 4. Molecular mechanisms of PNI: Tumour cells expressing CX3CR1 and MUC-1 infiltrate the peri-neural space and adhere to CX3CL1 and Siglec-4a on the surface of neural cells. Both cell types secrete neurotropins which are crucial for sustaining growth and survival of both cell types. Pain perception is influenced upon the interaction between CSF secreted by tumour cells and their receptors CSFR on the surface of neural cells.

A novel method has been developed by Abiatari et al. to monitor *ex vivo* PNI of PDAC tumor cells into surgically resected rat vagal nerves. Genome-wide transcriptional analyses deciphered a set of differentially regulated genes in high versus low invasive pancreas tumour cells. Kinesin family member 14 (KIF14) and Rho-GDP dissociation inhibitor β (ARHGDIβ) are among two candidate PNI genes identified. Increased expression of both proteins was confined to tumour cells invading the peri-neural niche in pancreatic tumour patients. Finally, functional knock-down of KIF14 and ARHGDIβ resulted in altered PNI of tumour cells (Abiatari et al., 2009). These data indicate that a better molecular characterization of the PNI process is a prerequisite for development of targeted therapies aiming to inhibit the pancreatic cancer metastasis.

6. Pancreatic cancer stem cells

An emerging field of cancer research attempts to identify cellular hierarchies among the tumour cell population. Evidence are provided for tumor cells with self-renewing and stem-cell-like characteristics within solid tumours termed tumour-initiating cells (TICs) or cancer stem cells (CSC) (Reya et al., 2001; Al Hajj et al., 2003; Singh et al., 2004 O'Brien et al., 2007; Ricci-Vitani et al., 2007). It is hypothesised that CSC undergo EMT at the invasive front of primary tumours and migrate to colonise new tissue. The concept of ''migrating CSC'' describes a cancer stem cell which possess both an element of stemness and mobility (Brabletz et al., 2005). The relationship between CSC and pancreatic cancer progression was investigated by Li et al. (2007). They have chosen cancer stem cell markers based on previous work on breast cancer stem cells. These include the cell surface markers CD44, CD24, and epithelial-specific antigen (ESA). CD44+ CD24+ ESA+ pancreatic CSC demonstrate typical features observed in adult stem cells such as the ability of self-renewal, generation of differentiated progeny, and activation of developmental signalling pathways such as sonic hedgehog (Li et al., 2007). They further reported that only hundred human CD44+ CD24+ ESA+ pancreatic CSC are required to generate subcutaneous tumours in 50% of immuno-compromised SCID mice.

CD133 is yet another potential marker discussed to be characteristic for pancreatic CSC (Hermann et al., 2007). They showed that the capacity of cells to form primary tumours following orthotopic implantation in nude mice was exclusive to the CD133+ sub-population which also demonstrated inherent resistance to gemcitabine chemotherapy. Further studies on highly metastatic pancreatic cancer cell line L3.6pl identified two sub-sets of tumour cells based on the expression of CXCR4 receptor (Miller et al., 2008). Depletion of CXCR4 subset of CD133+ pancreatic CSCs precluded the formation of spontaneous liver metastases. In line with above mentioned data, CXCL12 appears to be the strongest inducer of migration in CD133+ cancer cells *in vitro*. A component of the therapeutic plant *Boswellia serrata,* acetyl-11-keto-β-boswellic acid (AKBA), has been shown to down-regulate CXCR4 expression in pancreatic tumour cells and suppress cancer cell invasion (Park et al., 2011). Negative staining of cytokeratin epithelial cell marker in the CD133+ (in contrast to CD133- CSC) indicated EMT phenotype, thereby explaining their invasive potential (Mani et al., 2008). Blocking of the CXCL12/CXCR4 interaction with the CXCR4 non-peptidic antagonist reduces the spread of CD133+/CXCR4+ invasive pancreas cancer cells (Hermann et al., 2007).

Correlation between CD133 expression and lymph node metastasis in pancreatic cancer was investigated by Maeda et al. Immunohistochemical assessment of samples from 80 patients with PDAC after surgery revealed <15% CD133+ tumours cells per tumour in only 60% of specimen (48/80) suggesting a low frequency of these cells in PDAC. However, if CD133+ cells were detected they were cytokeratin negative and were confined to the glandular structures in the periphery of tumours. CD133 expression significantly correlated with clinicopathological parameter including VEGF-C expression, lymphatic invasion and lymph node metastasis (Maeda et al., 2008).

7. Conclusion

A better comprehension of the processes governing the formation of metastases is critical towards development of more advanced cancer treatment modalities. Various theories of

metastatic cancer progression have lately emerged. In contrast to late dissemination model early dissemination and parallel evolution of tumours in primary vs. metastatic sites impact current perception of tumour heterogeneity and consequently will impact the development of targeted cancer treatment strategies. The emergence of novel sequencing and high-through put genetics methods will hopefully assist cancer research in defining the relevance of each model in pancreatic cancer development.

Acquisition of multiple genetic aberrations in the tumour cells is crucial for initiation of cancer. However, the communication of tumour cells with tumour-micro-environment is a prerequisite for successful tumour progression towards metastatic disease. Therefore, a better understanding of molecular mechanism underlying the orchestrated action between tumor cells and its micro-environmetal participants such as stellate cells, endothelial cells, pericytes, immune cells and bone-marrow derived cells are urgently needed. The contribution of angiogenesis, EMT, cytokine/chemokine axis, neurotropism, hypoxia and tissue remodelling in development of pancreatic cancer are still in an early stage. Further research is needed to elaborate the molecular characteristics of specific niches such as liver vs. lymph nodes in development of PDAC metastases. In contrast to dynamic models considering tumour cell plasticity as the pivotal force behind its ability to gain specific traits when exposed to e.g. EMT or hypoxia stimuli, the existence of deterministic hierarchies among pancreatic tumour cells as proposed by the emerging tumour stem cell community remain elusive. In conclusion, a concerted multidisciplinary effort is needed to identify novel targets, rationally design therapies and ultimately improve the treatment of this devastating disease.

8. Acknowledgments

This work was supported in part by the German Krebshilfe (Deutsche Krebshilfe, Max-Eder 108876), DFG National Priority Research Program: the Tumor-Vessel Interface "SPP1190" and the German Federal Ministry of Research and Technology (Bundesministerium für Bildung und Forschung - BMBF 03NUK004C).

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5

Nitric Oxide Regulates Growth Factor Signaling in Pancreatic Cancer Cells

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1. Introduction

Growth factor signaling plays a critical role in cancer proliferation and invasion. Therefore, molecules, involved in growth factor signaling have become the targets of cancer therapy, and many drugs targeting growth factor signaling pathways have been developed. Some of these drugs have been used clinically, while many more are being tested in clinical trials. However, to date, molecule-targeted therapies for pancreatic cancer have not been developed.

Nitric oxide (NO) was discovered two decades ago and was initially identified as an endothelial relaxing factor. Subsequently, NO has been shown to play key roles in the post-translational modification of proteins and the regulation of protein enzymatic activity. In this paper, we present evidence indicating that NO influences cancer proliferation and invasion, and discuss the mechanisms trough which NO is thought to exert these effects.

2. Production of NO in cells and tissues

NO is produced by three distinct genes products: neuronal and endothelial nitric-oxide synthases (nNOS and eNOS) and inducible nitric-oxide synthases (iNOS) (Palmer *et al.*, 1987). The activities of nNOS and eNOS are tightly regulated by calcium-dependent calmodulin binding, whereas iNOS does not require calcium ion or posttranslational modification for its activity. As a result, iNOS expression is associated with prolonged, exaggerated NO generation of up to > 1,000-fold greater than that generated by nNOS and eNOS. Although iNOS expression is increased in macrophages and endothelial cells by various stimuli, including acute inflammation, recent studies have revealed that iNOS is expressed even in normal conditions in many tissues, including skeletal muscle and cancer (Perreault and Marette, 2001; Xie and Fidler, 1998). The expression of iNOS protein has been reported in pancreatic cancer cells, colon cancer cells, gastric cancer, breast cancer, hepatocellular carcinoma, glioma cells, melanoma cells, and laryngeal squamous cell carcinoma.

3. NO-donors

Several types of reagents called NO-donors, are capable of releasing NO constitutively (Table 1). *S*-Nitrosothiols (RSNO), which break down to form NO and the corresponding disulphide (RSSR), are an important class of NO -donor drugs. NO-donors containing R-NO (S-NO), are unstable and release NO upon breakdown. Decomposition of these compounds is catalysed by Cu+ ions, which themselves can be formed by reduction of Cu^{2+} ions by thiols. Breakdown is accelerated by light at ultraviolet and optical wavelength and is influenced by PH.

Organic nitrates such as nitroglycerin, isosorbide dinitrate and mononitrate, which have long been used as vasodilators for the treatment of angina pectoris, release NO via both enzymatic and non-enzymatic pathways. Iron-nitrosyl complexes such as sodium nitroprusside (SNP), sydnonimine, and amine NONOate, all demonstrate NO donating capacity. NO -donating non-steroidal anti-inflammatory drugs (NO-NSAIDs), which were developed recently, are potential anti-cancer drugs (Gao *et al.*, 2005). NO-NSAIDs consist of a conventional NSAID to which an NO-releasing moiety is attached covalently. Glutathione S-transferase -activated NO-donors such as JS-K, have shown some therapeutic promise in cancer without hypotension (Weiss *et al.*).

NO-donor reagents offer a convenient source of NO for *in vitro* and *in vivo* experiments. Researchers can thereby avoid use NO gas but must consider intrinsic half-life, metabolites, and other activities derived from the unique moiety in choosing a NO-donor reagent.

S-Nitrosothiols
S-nitrosoglutathione (GSNO)
S-nitroso-N-acetylpenicillamine (SNAP)
Organ nitrates
Nitroglycerin (NTG)
isosorbide dinitrate (ISDN)
Iron-nitrosyl complex
sodium nitroprusside (SNP)
Sydnonimine
3-morpholino-sydnonimine (SIN-1)
Molsidomine
Diazeniumdiolate (NONOate)
Angeli's salt
Diethylamine
O2-(2,4-Dinitrophenyl)
1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K)
NO-donating NSAIDs
Nitric oxide-donating aspirin (NO-ASA)
NO-naproxen
NONO-ASA

Table 1. NO donors

4. Actions of NO *in vivo*

4.1 cGMP-dependent actions

Guanylyl cyclases (GC) are expressed in the cytoplasm of almost all mammalian cells and mediate a wide range of important physiological functions, including inhibition of platelet aggregation, relaxation of smooth muscle, vasodilation, neuronal signal transduction, and immunomodulation (Collier and Vallance, 1989). GCs have evolved to synthesize cGMP in response to diverse signals, such as NO. NO activates GC by binding directly to heme to form a ferrous-nitrosyl-heme complex. Endogenous and exogenous compounds, including autocoids, hormones, neurotransmitters, and toxins, produce cellular responses through cGMP. The specificity of cellular responses to cGMP is dictated by cGMP-binding motifs in target proteins PKA (cAMP-dependent protein kinase) and PKG (cGMP-dependent protein kinase) (Francis and Corbin, 1999), cyclic nucleotide-gated cation channels (Biel *et al*., 1999; Kaupp, 1995) and cGMP-regulated phosphodiesterases (Beavo, 1995).

4.2 cGMP-independent actions

The major cGMP-independent actions of NO are nitrosative post-translational modifications, including protein *S*-nitrosylation and tyrosine nitration. Post-translational modification of proteins by S-nitrosylation, attachment of nitrosonium ion (NO) to cysteine sulfhydryls, is a major mode of signaling in mammalian cells

Indeed, critical signaling molecules and transcription factors are primary targets of NO (Stamler *et al*., 2001). To date, over 100 proteins have been shown to be *S*-nitrosylated both *in vitro* and *in vivo*. In many of these proteins, *S*-nitrosylation leads to functional alterations. Signaling proteins that are directly modified by S-nitrosylation include Ras, Akt, JNK, PTEN, IκB kinase, and Bcl2. (Azad *et al*.; Lander *et al*., 1997; Numajiri *et al*.; Park *et al*., 2000; Reynaert *et al*., 2004; Yasukawa *et al*., 2005)

5. Roles for NO in cancer

Conflicting results have been reported regarding the roles of NO in cancer. Recent papers reported that endogenous NO promotes oncogenesis and angiogenesis in various cancers (Ambs *et al*., 1998; Camp *et al*., 2006). In contrast, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, *in vitro* and *in vivo* (Chawla-Sarkar *et al*., 2003; Jarry *et al*., 2004; Kalivendi *et al*., 2001; Kotamraju *et al*., 2007; Notas *et al*., 2006; Peshes-Yaloz *et al*., 2007; Wang *et al*., 2003). These studies suggest that NO can act either as a tumor suppressor or a tumor enhancer depending on cell type and the level of NO in the cells. However, the molecular mechanism underlying the inhibitory effects of NO on cancer viability, remains unclear.

5.1 Roles in carcinogenesis and cancer promotion

NO and reactive nitrogen species (RNOS) induce the formation of nitrosamines, which can cause cancers in a wide variety of animal species. Nitrosation of nucleic acid bases leads to deamination which in turn results in mutagenic or carcinogenic conversion cytosine to uracil, guanine to xanthine, methylcytosine to thymine and adenine to hypoxanthine (Caulfield *et al*., 1998; Wink *et al*., 1991). RNOS can cause both single- and double- strand breaks in DNA.

Furthermore, NO inhibits DNA repair proteins and poly- (ADP-ribose) polymerase (PARP), which regulates DNA repair and apoptosis (Sidorkina *et al.*, 2003). Thus, NO induces DNA damage that can lead to carcinogenesis.

NO can promote cancer by enhancing vascularization, which favors growth and metastasis , and by inhibiting apoptosis. NO induces the expression of VEGF in carcinoma cells and suppresses angiostatin and thrombospondin-1, inhibitors of angiogenesis, resulting in promotion of tumor vascularization (Cooke and Losordo, 2002; Dulak *et al.*, 2000). The growth of xenografted murine mammary adenocarcinoma which expresses iNOS is reduced by treatment with iNOS inhibitor (Thomsen *et al.*, 1997). Nitric oxide (NO)-mediated S-nitrosylation of Bcl-2 prevents its ubiquitination and subsequent proteasomal degradation, leading to inhibition of apoptosis. NO-mediated S-nitrosylation and stabilization of Bcl-2 protein was the primary mechanism involved in the malignant transformation of nontumorigenic lung epithelial cells in response to long-term carcinogen exposure (Azad *et al.*).

5.2 Anti-cancer effects

In contrast to the aforementioned effects of NO, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, *in vitro* and *in vivo* (Chawla-Sarkar *et al.*, 2003; Jarry *et al.*, 2004; Kalivendi *et al.*, 2001; Kotamraju *et al.*, 2007; Notas *et al.*, 2006; Peshes-Yaloz *et al.*, 2007; Wang *et al.*, 2003). Nitrosylcobalamin (NO-Cbl), an analog of vitamin B12 that delivers nitric oxide (NO) and xhibits anti-tumor activity; NO-Cbl increases the expression of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) and its receptors, resulting in apoptosis of human tumors. (Chawla-Sarkar *et al.*, 2003). The tumor suppressor P53 participates in numerous critical cellular functions including gene transcription, DNA repair, cell cycle control, genomic stability, and apoptosis (Gottlieb and Oren, 1996; Harris, 1996) . DNA damage, especially DNA double strand breaks caused by ionizing radiation or other exogenous mutagens, induces p53 protein accumulation and activation, leading to cell cycle arrest during G1/S transition (Huang *et al.*, 1996). High concentrations of nitric oxide (NO), inducing DNA damages, also triggers wild-type p53 protein accumulation and apoptosis (Messmer *et al.*, 1994). In addition, nitric oxide induces death of colon cancer cells through down-regulation of beta-catenin via proteasome-independent degradation (Prevotat *et al.*, 2006). Some report document specific effects of NO in pancreatic cancer. Decker et al. reported that human pancreatic cancer cells engineered to overexpress eNOS show down-regulation of liver metastasis and tumor growth in mice (Decker *et al.*, 2008). Wang et al. established a role of NOS2 in pancreatic cancer growth and metastasis in an animal model. They demonstrated that pancreatic cancer clones expressing low levels of NOS 2 produced tumors in the pancreas which metastasized to the liver, whereas those expressing high levels of NOS 2 did not (Wang *et al.*, 2003).

6. Growth signaling in cancer

Insulin/insulin-like growth factor (IGF) signals play a key role in cancer proliferation and invasion (Bergmann *et al.*, 1995; Furukawa *et al.*, 2005; Kim *et al.*, 2007). Insulin/IGF-I and IGF-II bind to insulin/IGF-I receptors, leading to tyrosine phosphorylation of the cognate

receptors. Insulin receptor substrate (IRS)-1, an adaptor protein, exists mainly in the cytosol, and binds to phosphorylated insulin receptor (IR) and IGF-I receptor (IGF-IR), resulting in the phosphorylation and activation of IRS-1. IRS-1 activates phosphatidylinositol-3 kinase (PI3K), which in turn activates further down-stream components, including Akt/PKB and glycogen synthase kinase (GSK)-3β. Alternatively, phosphorylated and activated IRS-1 can also bind to another adaptor protein, Grb-2, which activates mitogen-activated protein kinase (MAPK), another major insulin/IGF signaling cascade parallel to the PI3K-Akt/PKB pathway (Ito *et al.*, 1996; Tanaka and Wands, 1996). IRS-1 protein expression is detected in several types of cancer, including pancreatic cancer, breast cancer, and hepatic cell carcinoma (Asano *et al.*, 2005; Chang *et al.*, 2002). Thus, insulin/IGF signaling is thought to play a major role in not only metabolic actions, including stimulation of glucose uptake and synthesis of glycogen and protein, but also in cancer viability including proliferation and invasion. IRS-1 is a key molecule in insulin/IGF signaling that transduces a signal from IR/IGF-IR to both PI3K and MAPK pathways (Asano *et al.*, 2005).

Epidermal growth factor (EGF) signaling also plays a key role in cancer proliferation and invasion. EGF binds to EGF receptor (EGFR) and triggers tyrosine phosphorylation of the receptor. Phosphorylated EGFR activates phosphatidylinositol-3-kinase (PI3K), which activates further down-stream components, including Akt. Alternatively, phosphorylated EGFR can also activate the Ras/MEK/ERK pathway, another major EGF signaling cascade parallel to the PI3K/Akt pathway.

In the section to follow, we present our data showing effects of nitric oxide on growth factor signaling.

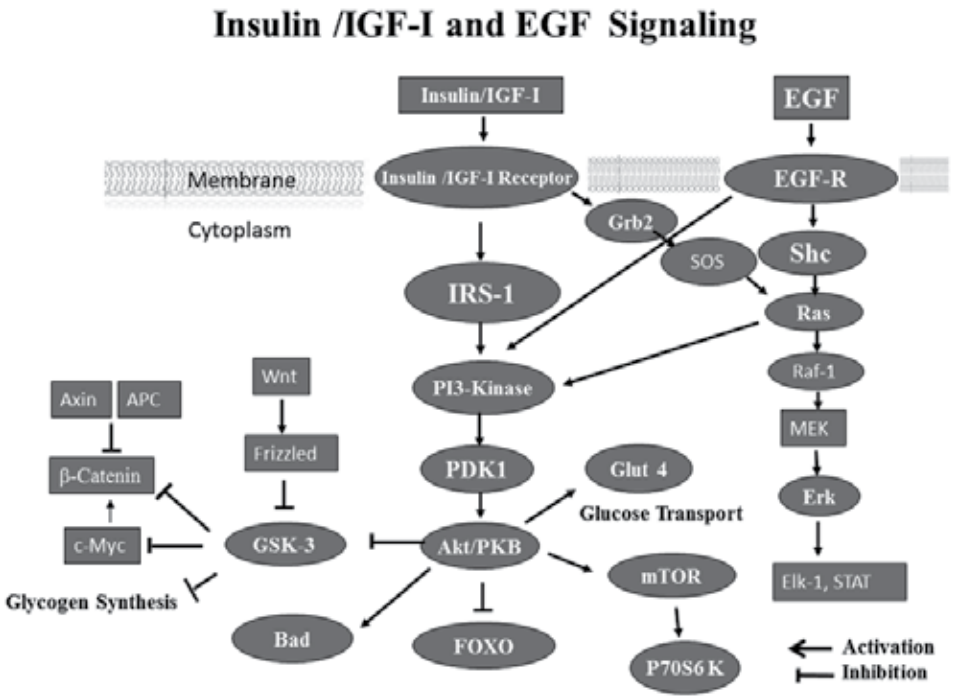

Fig. 1. Insulin/IGF-I and EGF signaling

7. NO regulates growth signaling

7.1 NO regulates insulin signaling in skeletal muscle

Expression of iNOS is elevated in skeletal muscle of patients with type 2 diabetes (Torres *et al.*, 2004) and in high fat diet-induced diabetic mice. Perreault and Marette showed that disruption of the iNOS gene protects against high fat diet-induced insulin resistance in mice (Perreault and Marette, 2001). Furthermore, we demonstrated that disruption of the iNOS gene reverses IRS-1 protein reduction in skeletal muscles of leptin deficient obese mice and NO-donor treatment induces proteasome-dependent IRS-1 degradation in skeletal muscle cells (Sugita *et al.*, 2005). Thus, NO inhibits insulin signaling and is associated with IRS-1 protein degradation, resulting in insulin resistance. This may explain the occurrence of insulin resistance in patients with inflammation or diabetes.

7.2 NO influences insulin/IGF signals in MIAPaCa-2 cells

We examined whether NO influences on insulin/IGF-I signaling in MIAPaCa-2 cells, a pancreatic cancer cell line. Protein expression and phosphorylation were detected by immunoblotting using specific antibodies. SNAP, a NO -donor, inhibited insulin-stimulated tyrosine phosphorylation of IR, IRS-1, the phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9}. In addition, SNAP inhibited IGF-I-stimulated tyrosine phosphorylation of IGF-IR and IRS-1, phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9}. Furthermore, SNAP reduced IRS-1 protein expression, although this did not alter the expression of other IGF signaling proteins, including IGF-IR, Akt/PKB, GSK-3β and Erk 1/2 or of β-actin protein. SNAP induced phosphorylation of Erk 1/2 without stimulation by insulin/IGF-I, and enhanced the insulin/IGF-1-stimulated phosphorylation of Erk 1/2; however, SNAP did not influence Erk 1/2 protein expression in MIAPaCa-2 cells (Figure 2A and B).

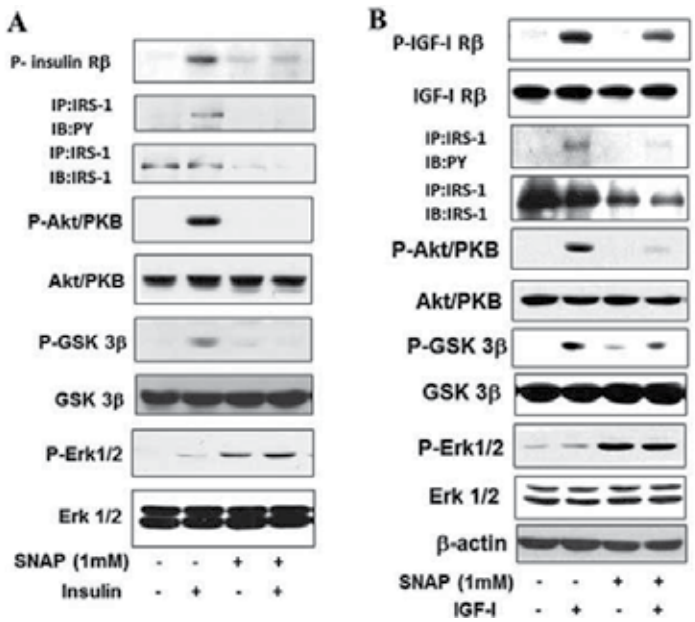

Fig. 2. NO influences IGF signals in MIAPaCa-2 cells

GSNO, a NO-donor, inhibited IRS-1 protein expression in MCF-7 as well as MIAPaCa-2 cells in a dose-dependent manner, but did not influence IRS-1 protein expression in MB 468 and Panc-1 cells, which exhibited less IRS-1 protein expression (Figure 3A). The proteasome inhibitor, MG132, completely reversed the reduction of IRS-1 protein expression by NO-donors in MIAPaCa-2 cells. Neither GSNO nor MG132 influenced GSK-3β and β-actin protein expression (Figure 3B). To further investigate IRS-1 protein degradation induced by NO-donor, cDNA constructs of IRS-1 full-length, IRS-1 DM1, IRS-1 DM2, and IRS-1 DM3 were produced and sub-cloned into mammalian expression vectors (Figure 3C). MIAPaCa-2 cells were transfected with these expression vectors. GSNO reduced IRS-1 full-length, IRS-1 DM1, and IRS-1 DM3 protein expression, although GSNO did not alter IRS-1 DM2 and β-actin protein expression (Figure 3D). Ubiquitination of wild-type and mutant IRS-1 was detected by immunoprecipitation using anti-Flag antibody followed by immunoblotting with anti-ubiquitin. SNAP induced the ubiquitination of IRS-1 full-length, IRS-1 DM1, and IRS-1 DM3, but did not induce the ubiquitination of IRS-1 DM2 (Figure 3E). These results indicate that NO-donor is capable of inducing ubiquitination at multiple sites in the carboxy-terminus of the IRS-1 protein.

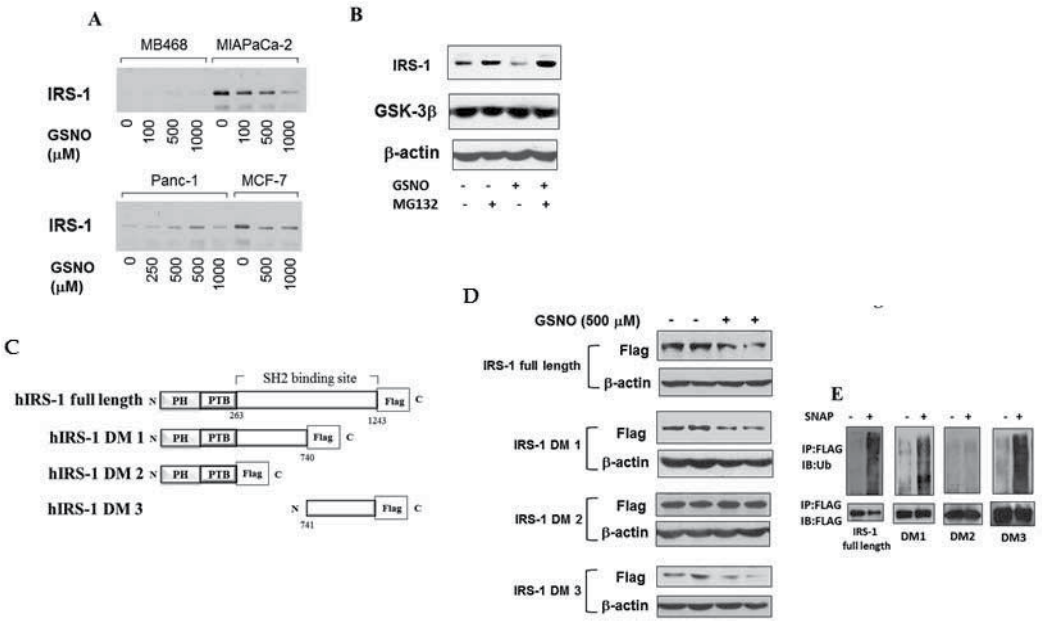

Fig. 3. NO donor downregulates IRS-1 protein expression through proteasome-mediated degradation in MIAPaCa-2 cells

iNOS protein was detected by immunoblotting in Panc-1 cells, a pancreatic cancer -derived cell line. IRS-1 protein expression was significantly increased by 1400 W, an iNOS specific inhibitor, in a dose-dependent manner. Expression of Akt/PKB, β-actin, and Erk 1/2 protein was unaffected by treatment (Figure 4A). GSNO inhibited IRS-1 protein expression, upregulated by 1400W (Figure 4B). Treatment of 1400W enhanced IGF-I-stimulated tyrosine phosphorylation of IRS-1, phosphorylation of Akt/PKB at Ser ^{473}, and GSK-3β at Ser ^{9} in

Panc-1 cells. In contrast, 1400 W did not alter IGF-I-stimulated phosphorylation of Erk 1/2 (Figure 4C). These results indicate that endogenous NO produced by iNOS plays a role in insulin/IGF-I signaling.

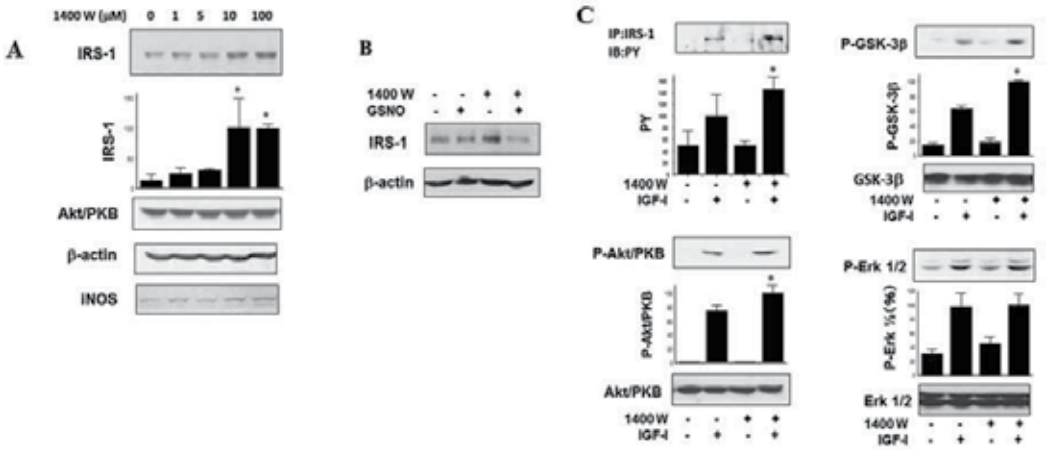

Fig. 4. iNOS inhibitor 1400W upregulates IRS-1 protein expression and IRS-1/Akt pathway in Panc-1 cells, a pancreatic cancer cell line

Mammalian expression vectors, pCMV Tag 4/IRS-1 full- length, pCMV Tag 4/IRS-1 DM2 and pCMV Tag 4A vector alone, were transfected into MIAPaCa-2 and incubated with G418 for the selection of protein-expressing cells for more than 14 days. Subsequently, the cells expressing high IRS-1 full-length protein or IRS-1 DM2 protein were cloned.

Proliferation of MIAPaCa-2 cells was elevated in a culture medium containing serum or IGF-I, while no proliferation was observed in a culture medium without serum or IGF-I. Proliferation of cells overexpressing full-length IRS-1 was greater than that of vector alone-transefected cells in the culture medium containing 10 % FBS. By contrast, the proliferation of cells expressing IRS-1 DM2 was attenuated compared to cells transfected with full-length IRS-1 or vector alone (Figure 5). The proliferation of cells transfected with full- length- IRS-1 was greater compared to that of vector alone-transfected cells in the culture medium containing 100 nM IGF-I without 10% FBS, while IGF-I-stimulated proliferation of IRS-1 DM2- transfected cell was not observed (Figure 5). GSNO (200 μM) significantly reduced the proliferation of vector alone-, IRS-1 full- length-, and IRS-1 DM2- transfected cells in culture medium containing 10 % FBS or IGF-I. To further investigate the role of iNOS in IGF-I-stimulated proliferation, we evaluated the effects of selective iNOS inhibitor, 1400W, in Panc-1 cells cultured with IGF-I in the absence of FBS. Proliferation in Panc-1 cells was not observed in the presence and absence of 1400W (100 μM), when cultured without serum or IGF-1 (Figure 6A). 1400W significantly enhanced the proliferation of Panc-1 cells when cultured with 10% FBS (Figure 6B). In the absence of 1400W, IGF-I failed to increase the cell numbers of Panc-1. The combination of IGF-I and 1400W, however, increased the number of Panc-1 cells (Figure 6C). These results provide further evidence for the involvement of downregulation of IGF-I signaling in NO-induced inhibition of cancer cell proliferation.

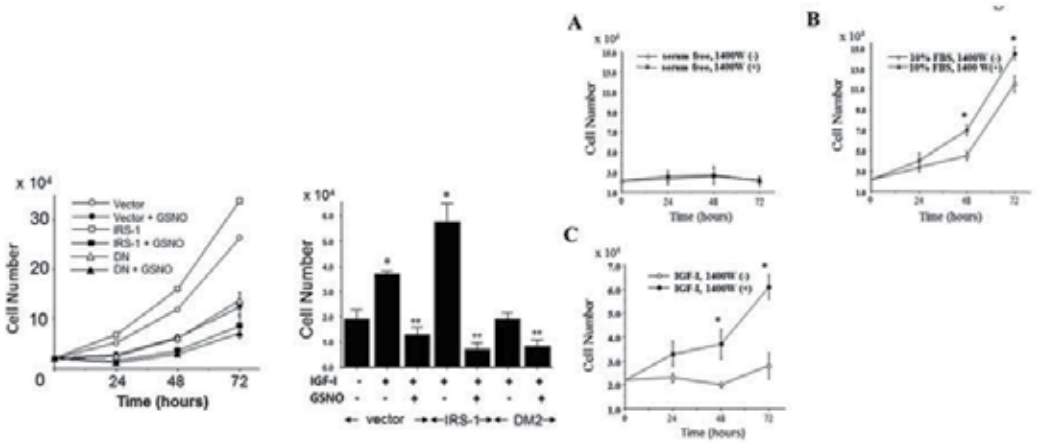

Fig. 5. and 6. NO inhibited the proliferation of cancer cell lines and IRS-1 protein expression was associated with cancer cell proliferation

In vitro invasive potential of MIAPaCa-2 cells and Panc-1 cells was determined using BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA). There was no difference between the invasion of vector alone-, IRS-1 full-length-, and IRS-1 DM2-transfected MIAPaCa-2 cells in the absence of the NO -donor. The addition of 200 μM GSNO markedly reduced invasion in vector alone- and IRS-1 full-length-transfected MIAPaCa-2 cells but did not alter invasion in IRS-1 DM2-transfected MIAPaCa-2 cells (Figure 7A). Invasion in Panc-1 cells incubated with 1400W (5 and 100 μM) was significantly greater than that of untreated cells (Figure 7B).

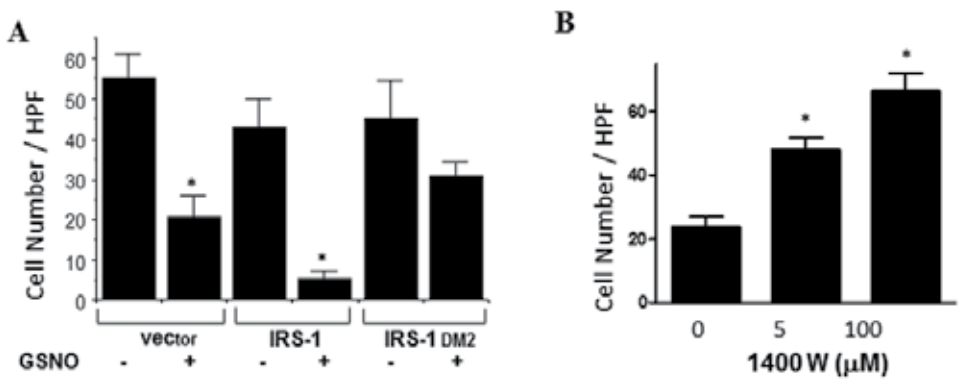

Fig. 7. Sensitivity of NO to invasion was dependent on IGF signaling

NO-donor treatment leads to several effects on insulin/IGF signaling in pancreatic cancer cells. NO-donor treatment reduced IRS-1 protein expression via proteasome-dependent degradation, and inhibited insulin/IGF-I-stimulated phosphorylation of Akt/PKB and GSK-3β, while enhancing phosphorylation of Erk 1/2 in pancreatic cancer cells (Figure 2, 3, 4). NO-donor inhibited IGF-I-induced phosphorylation of Akt/PKB and GSK-3β in MIAPaCa-2 cells transfected with IRS-1 wild-type or vector, but not in cells transfected with a dominant

negative carboxy-terminal deletion mutants (Tanaka and Wands, 1996) of IRS-1. This indicates the importance of IRS-1 in the inhibition of insulin/IGF signal by NO. IRS-1 expression and IGF-I signaling have important roles in the proliferation and invasion of MIAPaCa-2 cells and Panc-1 cells, consistent with previous reports on other cancer cells (Kim *et al*., 2007; Shi *et al*., 2007; Tanaka and Wands, 1996). NO donor inhibited IGF-I signaling, proliferation, and invasion in MIAPaCa-2 cells transfected with IRS-1 full-length or vector. In contrast, treatment with a selective iNOS inhibitor upregulated IRS-1 protein expression and insulin/IGF signaling, resulting in enhanced proliferation and invasion activity in Panc-1 cells. These results indicate that the expression of IRS-1 protein is regulated by endogenous NO production by iNOS as well as by exogenous NO, resulting in the downregulation of IGF-I signaling and the inhibition of cancer proliferation and invasion in MIAPaCa-2 and Panc-1 cells (Figure 4, 5, 6).

Furthermore, the carboxy-terminus as the site responsible for IRS-1 protein degradation by NO, which is located in SH2-containing molecule binding site next the phosphotyrosine binding (PTB) domain was detected. The observation of the ubiquitination and degradation of IRS-1 deletion mutants indicates the possibility that there may be at least two sites responsible for NO donor-induced ubiquitination in the IRS-1 protein. These data had been published in 2010 (Sugita *et al.*)

In addition, NO inhibits Akt activity directly through post-translational modification, (Yasukawa *et al*., 2005), which seems to contribute to NO-induced cancer inhibition.

Furthermore, we confirmed that NO-donors down-regulate EGF-stimulated phosphorylation of EGFR and Akt in colon cancer cells (data not shown).

8. Therapeutic prospects

The usefulness of cancer therapy using NO, including iNOS gene therapy and administration of NO-donor, was recently confirmed in animal models (Adams *et al*., 2008; Kiziltepe *et al*., 2007; Wang *et al*., 2004). Consequently, NO therapy has been focused on, and is currently undergoing clinical evaluation for cancer prevention (Ma *et al*., 2007). This should leads to clinical trials using NO -donors in the near future. Nitroglycerin, a NO-donor, has long been used as a vasodilating, and the safety of nitroglycerin therapies is well established. Nitroglycerin treatment on non-small cell lung cancer is currently planned as a phase II clinical trial. A promising novel class of drugs, nitric oxide-donating NSAIDs (NO-NSAIDs), has been found to be more active than classical NSAIDs against cancer (Rigas and Williams, 2008). The effects of the NO-donating aspirin derivative, NCX 4040, on three human pancreatic adenocarcinoma cell lines were recently described (Capan-2, MIA PaCa-2 and T3M4)(Rosetti *et al*., 2006). Clinical trials using NO-donors or NO-donating aspirin derivatives are urgently required.

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6

Kinase Activity is Required for Growth Regulation but not Invasion Suppression by Syk Kinase in Pancreatic Adenocarcinoma Cells

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1. Introduction

Syk (spleen tyrosine kinase) is a nonreceptor tyrosine kinase containing two tandem amino-terminal SH2 domains, followed by an extended linker region and a carboxy-terminal kinase domain (Sada et al., 2001). Tyrosine-352 (Y352) in the linker region is trans-phosphorylated by src family members, promoting the activation of syk (Kimura et al., 1996; Sada et al., 2001). Activation further involves the autophosphorylation of syk on tyrosines 525 and 526 (YY525/6) in the activation loop of the kinase domain, which promotes substrate-specific catalytic activity and is required for signaling by syk (Sada et al., 2001; Zhang et al., 1998); this modification is thus indicative of the functional enzyme. The active form of syk then localizes to appropriate substrates or bridging molecules primarily through interactions with its SH2 domains and linker tyrosines (Kimura et al., 1996; Sada et al., 2001).

Syk has been identified as a putative tumor suppressor in human breast cancer since reintroduction of syk retarded the growth of syk-negative breast cancer cells, and suppression of endogenous syk enhanced the tumorigenic phenotype of the resulting cells (Coopman et al., 2000). Loss of syk correlates with poor survival and metastasis of breast cancer in patients (Toyama et al., 2003), and syk regulates breast cancer cell mitosis (Zyss et al., 2005) and transcription (Wang et al., 2005). Previously we identified syk as being a pancreatic ductal adenocarcinoma (PDAC) tumor suppressor. Syk is uniformly expressed by normal pancreatic ductal epithelium and well-differentiated (grade1; G1) PDAC; however moderately-differentiated (grade2; G2) PDAC demonstrates progressive loss of syk, and high-grade (grade3; G3), poorly-differentiated lesions are essentially devoid of syk *in situ* (Layton et al., 2009). In fact, syk expression is a strong positive indicator of patient survival (Layton et al., 2009). Mechanistically, we demonstrated that syk is a central mediator of phenotypic changes regulating PDAC progression, including anchorage-independent growth, cellular invasion, and gene expression changes responsible for epithelial-mesenchymal transition (Layton et al., 2009). To assess the utilization of syk in pancreatic ductal cells, we examined phosphorylation states of syk that correspond to upstream

activation (tyrosine-352) and catalytic activity (tyrosines-525/6). We further examined the activity of syk and the requirement for syk kinase activity in regulating cell growth and invasion. Herein we show that although blockade of syk activity suppressed growth of endogenously syk-positive PDAC cells *in vitro*, kinase activity is not required for syk-dependent regulation of PDAC cell invasion. Phosphorylation of tyrosine-352 and tyrosines-525/6 is detectable in pancreatic ductal epithelial cells *in situ* and *in vitro*, and inhibition of syk kinase activity specifically retards the growth of endogenously syk-positive PDAC cells *in vitro*. In contrast to our previous demonstration that syk regulates the invasion of αvβ3-positive Panc1 PDAC cells by attenuation of the matrix metalloproteinase-2 axis (Layton et al., 2009), invasion of αvβ3-negative MIAPaCa2 and BxPC3 PDAC cells is metalloproteinase-independent and involves the urokinase/plasminogen system, which is regulated by syk in these cells. Thus we demonstrate that endogenous syk is active in pancreatic ductal epithelial cells, and that syk kinase activity is required for growth regulation, but not invasion suppression, by syk in this cell type.

2. Materials and methods

2.1 Cell lines and transfection

CAPAN2(G1), CFPAC1(G2), AsPC1(G2), BxPC3(G2), Panc1(G3) and MIAPaCa2(G3+) cells were originally from ATCC and cultured accordingly. pCDNA3.1/IH/syk^{wt} encoding myc-tagged human syk linked to a hygromycin phosphotransferase gene through an IRES was described previously (Layton et al., 2009). Kinase-dead (KD) syk was produced by site-directed mutagenesis of lysine-402 in the ATP-binding pocket of pCDNA3.1/IH/syk^{wt} using the following primers (K402R-FWD: 5′-GTGAAAACCGTGGCTGTGAGAATACTGAAAAACGAGGC-3′; K402R-REV: 5′-GCCTCGTTTTTCAGTATTCTCACAGCCACGGTTTTCAC-3′). MIAPaCa2 cells were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). BxPC3 cells were electroporated as described previously (Layton et al., 2009). Stable populations were hygromycin-selected and assessed for protein expression. For transient studies, pEF4-LacZ reporter (Invitrogen, Carlsbad, CA) was cotransfected, and cells stained with x-gal to identify and specifically quantitate transfected cells; average MIAPaCa2 transfection efficiency >95%. In all cases, serum-free (SF-) media consisted of all components except serum, as appropriate for the cell line, supplemented with 0.5% bovine serum albumin (BSA).

2.2 Antibodies and reagents

Anti-syk mAb 4D10 and anti-erk2 pAb C14 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-syk phospho-Y352 and anti-syk phospho-YY525/6 pAbs were from Cell Signaling (Beverly, MA). Anti-phosphotyrosine mAb 4G10 and anti-myc tag mAb 4A6 were from UBI (Lake Placid, NY). Function-blocking anti-integrin antibodies LM609 (αvβ3) and PIF6 (αvβ5) and the MMP inhibitor *N*-(*R*)-[2-(Hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-napthylalanyl-L-alanine, 2-aminoethylamide (TAPI-1) were from Chemicon/EMD (San Diego, CA). Function-blocking anti-uPA mAb 3689 was from American Diagnostica (Stamford, CT). HRP- and FITC-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Nonspecific mouse IgG1 antibody MOPC-21, purified rabbit IgG, piceatannol (3,4,3′,5′- tetrahydroxy-*trans*-stilbene), Crystal Violet and other chemicals were from Fisher Scientific (Pittsburg, PA).

2.3 Immunohistochemistry

Patient tissue samples were obtained under approved UCSD Institutional Review Board protocol from the UCSD Dept. of Pathology archives and stained essentially as described previously (Layton et al., 2009). Briefly, samples were deparaffinized with xylenes, rehydrated through sequential alcohols (100%, 95%, 70%, 50%), and incubated with 1% H_2O_2 to inactivate endogenous peroxidases. Slides were quenched with 50 mM glycine, and blocked with 2% horse serum/5% BSA/phosphate-buffered saline (PBS), pH7.4, before renaturing for 20min in a steamer using Target Retrieval Solution (DAKO North America; Carpinteria, CA). Slides were allowed to cool and then incubated with the appropriate primary antibody overnight at 4˚C. Slides were washed and biotinylated-anti-rabbit applied according to the VectaStain Elite ABC Kit (Vector Labs; Burlingame, CA, USA). Sections were developed with DAB, counterstained with hematoxylin, dehydrated through sequential alcohols and mounted. Brightfield images were acquired on a Nikon TE600 microscope with a Model 3.2.0 CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) using SpotBasic software at the Moores UCSD Cancer Center.

2.4 Immunoblotting

Immunoblotting was performed essentially as described previously (Chen et al., 2010). Briefly, cells were lysed on the plate in NP40 lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1% NP-40) containing Complete™ Protease Inhibitor Cocktail (Roche, Indianapolis, IN) supplemented with 10mM PMSF, 1mM NaF and 10mM Na_3VO_4. Samples were separated by SDS-PAGE under reducing conditions and electroblotted to a PVDF membrane. Membranes were blocked with 10% nonfat dry milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and incubated 2h to overnight in 2% milk/TBS-T with the indicated primary antibody. Primary antibody was detected with HRP-conjugated secondary antibody, and complexes were visualized by enhanced chemiluminescence with PS-3 (Lumigen, Inc.; Southfield, MI).

2.5 Flow cytometry (FACS)

FACS was performed on a FACScalibur (BD Biosciences, Bedford, MA) at the Moores UCSD Cancer Center Flow Cytometry Shared Resource as described previously (Chen et al., 2010). Cells were harvested with 0.1% trypsin/versene, inactivated with 0.1% soybean trypsin inhibitor and resuspended in FACS buffer (1mM $MgCl_2$, 1mM $CaCl_2$, 0.1% NaN_3 0.5% BSA in PBS pH 7.4) before sequential labelling with primary and FITC-conjugated secondary antibodies. Gates were set with secondary alone, and 5µg/ml propidium iodide was included to exclude dead and dying cells.

2.6 Proliferation assays

Cell growth was assessed with the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI), or as follows: cells ($5x10^2$/well) were seeded into a 48-well plate. After 24 hours (and every 72 hours thereafter), fresh growth medium was replaced and the initial time point fixed with 1% paraformaldehyde/PBS, pH7.4. Additional triplicate wells were fixed at 24 hour intervals. All wells were stained with 1% Crystal Violet, which was subsequently extracted with 10% acetic acid, quantitated at 550nm and

compared to a standard curve of cells. For proliferation assays in the presence of the syk inhibitor, piceatannol, a titration of cells was plated and allowed to adhere for 24 hours before fresh growth medium (full serum) containing the indicated concentrations of piceatannol or DMSO vehicle was replaced. Medium was replenished in the same manner every other day for a total of 3 treatments, and the cells were fixed and stained with Crystal Violet 24h after the last treatment. Dye was extracted with 10% acetic acid and quantitated at 550nm.

2.7 Anchorage-independent growth

Anchorage-independent growth was assessed as described previously (Layton et al., 2009). Briefly, a top layer containing $5x10^3$ cells in 0.5% agar/DMEM/10%FBS was seeded onto a base layer of 0.7% agar/DMEM containing 10% FBS in a 6 well plate. Cultures were incubated at 37°C, media was replaced every 3rd day, and the assay stopped on day 10. Cultures were stained with 0.01% Crystal Violet and colonies were enumerated on a Bio-Rad GelDocXR using QuantityOne Software (Sensitivity=8.1, Average=5).

2.8 Invasion assay

Invasion assays were performed as described previously (Layton et al., 2009) using BioCoat Growth Factor-Reduced Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA). Briefly, cells were applied in SF-media to the upper chamber with or without 15min preincubation with TAPI1 (40µg/mL), aprotinin (100µg/mL), anti-uPA monoclonal antibody 3689 (25µg/mL), nonspecific IgG_1 control antibody MOPC-21 (25µg/ml), or an equal volume of DMSO or PBS control included in both chambers. SF-media or growth media was provided in the lower chamber and cells were allowed to invade for 24h before removal of uninvaded cells, and enumeration of invaded cells.

2.9 Reverse Transcription (RT)-PCR

cDNA was synthesized from 1µg of total RNA using oligo-dT primer. PCR was performed on 1µL of resulting cDNA using primers described previously (Layton et al., 2009; Leissner et al., 2006). GAPDH primers were from Stratagene (San Diego, CA) and served as internal controls. Densitometry was performed on unadjusted images using NIH *Image* 1.61 software and GAPDH as reference control.

2.10 Statistics

Experiments were performed in triplicate and independently repeated at least twice. Data shown are mean $\pm$ standard deviation unless otherwise indicated. Colony-formation and cellular invasion were analyzed by two-tailed Students *t*-Test.

3. Results

3.1 Endogenously–expressed syk is functional in pancreatic ductal cells in situ and in vitro

Previously we demonstrated the expression of syk in normal pancreatic ductal epithelial cells *in situ* (Layton et al., 2009). In order to assess whether this endogenous syk is

functionally active, we performed immunohistochemistry with antibodies specific for the phosphorylated form of Y352, which is representative of syk activation by upstream mediators, or YY525/6, which is indicative of substrate-specific activity of the syk kinase domain (Kimura et al., 1996; Sada et al., 2001; Zhang et al., 1998). These antibodies react with both isoforms of syk and do not recognize more distantly related src family members (Cell Signaling Technology, Beverly, MA). Strong staining for both phospho-Y352 (Fig.1A-C) and phospho-YY525/6 (Fig.1D-F) was observed in individual cells of independent ducts as well as ductules associated with acinar clusters. No staining was observed in the absence of primary antibody or when purified rabbit IgG was used as a control (not shown).

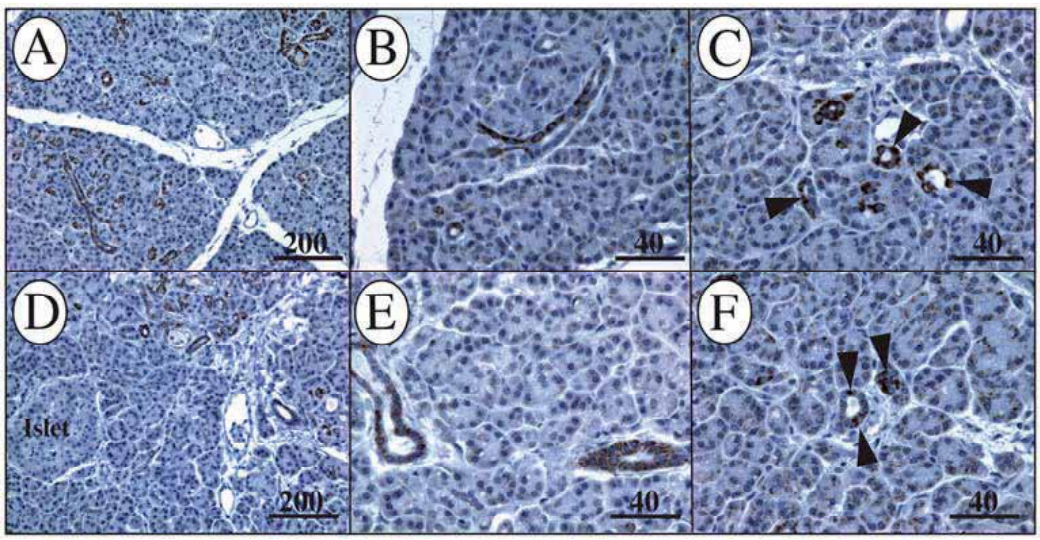

Fig. 1. **Syk is functional in pancreatic ductal cells *in situ*.** Immunohistochemical staining (brown-black) of normal pancreas with phospho-Y352 **(A-C)** and phospho-YY525/6 **(D-F)**. Arrowheads in (**C,F**) denote nuclear staining. Scale bars in μm.

Nuclear staining was observed in a subset of the duct cells (arrowheads in Fig.1C,F), consistent with the detection of nuclear syk in pancreatic ductal cells *in situ* (Layton et al., 2009) and previous reports of syk nuclear translocalization (Wang et al., 2003) and regulation of cell division (Zyss et al., 2005) and transcription (Wang et al., 2005). Of the 20 samples analyzed, all showed evidence of syk activation in a subset of ductal cells and none showed widespread constitutive phosphorylation at either site, demonstrating the active regulation of syk activation and activity in ductal cells of the normal human pancreas and suggesting an active role for syk in regulating the phenotype of this cell type.

We also previously established the expression of syk in well- to moderately-differentiated PDAC cells (CAPAN2-G1, CFPAC1-G2, BxPC3-G2, AsPC1-G2), to the exclusion of poorly-differentiated PDAC cells (Panc1-G3, MIAPaCa2-G3+)(Layton et al., 2009). In order to establish the utilization of syk by these endogenously syk-positive PDAC cells, we analyzed the activation surrogate readout of Y352 and YY525/6 phosphorylation in BxPC3 cells. Serum-starved cells were stimulated with insulin and then lysates immunoblotted with the phospho-syk-specific pAbs used for IHC. Interestingly, both Y352 and YY525/6 are

constitutively phosphorylated under starvation conditions, and dephosphorylated in response to insulin (Fig.2). Similar results were obtained with serum stimulation (not shown), and with endogenously syk-positive CAPAN2 cells (not shown). These data demonstrate the active regulation of syk activity in response to specific signalling pathways in endogenously syk-positive, well-differentiated PDAC cells, consistent with the restricted syk activation observed *in situ* and suggesting that syk is an active participant in regulating the phenotype of these cells.

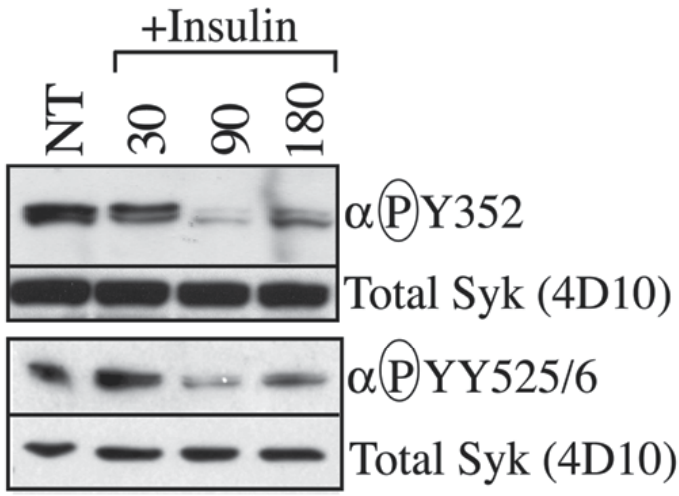

Fig. 2. **Syk is functional in PDAC cells *in vitro*.** Immunoblot of phospho-Y352 and phospho-YY525/6 in BxPC3 cells that had been serum-starved for 24h prior to stimulation with 10ng/ml insulin for the indicated times. NT, no treatment. Total syk levels determined with the 4D10 mAb are shown as a control.

3.2 Syk-dependent regulation of cell growth requires syk kinase activity

Syk regulates proliferation of immune cells in response to specific receptor activation events (Kimura et al., 1996; Sada et al., 2001; Wieder et al., 2001), and ectopic expression of an RFP-tagged syk caused anomalous cell division and mitotic catastrophe in breast cancer cells, where it was observed to interact with γ-tubulin of the mitotic spindle (Zyss et al., 2005). We observed localization of syk to the perinuclear region of dividing PDAC cells (not shown), therefore we assessed whether syk might be involved in regulating the proliferation of endogenously syk-positive PDAC cells in a manner analogous to that described in breast cancer (Coopman et al., 2000). Consistent with such a role, the *in vitro* growth of endogenously syk-positive CAPAN2, CFPAC1, BxPC3 and AsPC1 cells was suppressed in a dose-dependent manner by piceatannol, a phytochemical that inhibits the kinase activity of syk, suppressing downstream phosphorylation events by a largely unknown mechanism (Geahlen et al., 1989; Ferrigni et al., 1984; Wieder et al., 2001)(Fig.3A). Significantly, the only G1 cells in this group, CAPAN2, were completely eradicated by the highest dose of inhibitor, suggesting cytotoxicity in addition to any cytostatic effect of this compound in these highly differentiated cells.

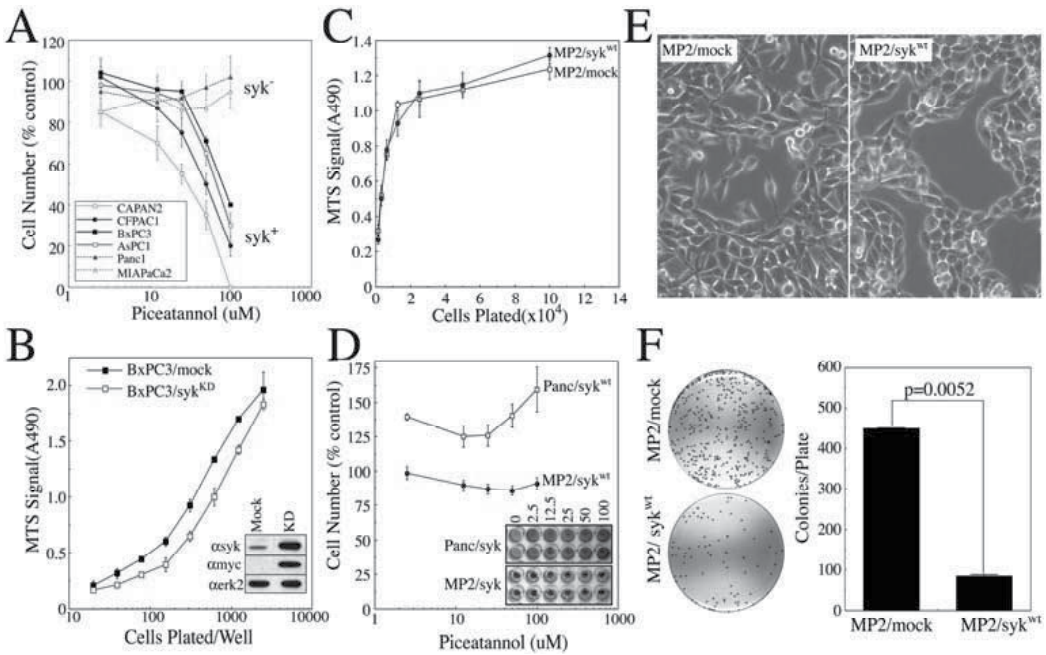

Fig. 3. **Kinase activity is required for syk-dependent regulation of cell growth. (A)** Cell growth of syk-positive versus syk-negative PDAC cell lines after 7 days of piceatannol treatment, plotted as percent of untreated control. **(B)** Proliferation of stable BxPC3/mock and BxPC3/syk^{KD} cells after 5 days. **(B,inset)** Immunoblot of total syk (syk) and the myc-tag (myc) of syk^{KD}. Erk2, loading control. **(C)** Proliferation of MP2/mock and MP2/syk^{wt} cells after 5 days. **(D)** MP2/syk^{wt} and Panc1/syk cell growth assay after 7 days of piceatannol treatment, as in (A). **(E)** Phase contrast images demonstrating the effect of stable syk expression on MIAPaCa2 cell morphology. Both populations were maintained under identical culture conditions including maintenance doses of the selectable marker hygromycin. **(F)** Effect of syk on anchorage-independent growth of MIAPaCa2 cells. A representative example of replicate plates is shown with quantitation points superimposed.

The specificity of this effect to the inhibition of syk is demonstrated by the following facts. First, *trans*-stilbene, which is the base molecule from which piceatannol is derived, had no effect on the proliferation of any of these cells (not shown). Second, syk-negative MIAPaCa2 and Panc1 cells demonstrated no growth response to piceatannol in this assay (Fig. 3A). Third, although piceatannol has been reported to inhibit FAK, src, PI3K and IκBα/NF-κB kinases (Ashikawa et al., 2002; Choi et al., 2010; Law et al., 1999), inhibition of these kinases has been shown to retard the proliferation of both syk-positive and syk-negative lines used in this study (Hering et al., 2007; Hochwald, et al., 2009; Ito et al., 2003; Perugini et al., 2000). Together, these points demonstrate that the antiproliferative effect of piceatannol reported here is likely restricted to the inhibition of syk itself. It should be noted that the concentrations of piceatannol used in this study are based on prior established parameters designed to minimize non-specific effects (Ashikawa et al., 2002; Choi et al., 2010; Geahlen et al., 1989; Law et al., 1999; Seow et al., 2002; Wieder et al., 2001).

To extend these pharmacological inhibitor studies, we stably transfected endogenously syk-positive BxPC3 cells with a kinase-dead syk construct harbouring a point mutation (K402R) in the ATP binding site of the kinase domain (BxPC3/KD cells). This construct has been shown to act as a dominant-negative in endogenously syk-positive cells through an incompletely understood mechanism (Coopman et al., 2000). BxPC3/KD cells demonstrated a consistently reduced growth rate versus mock-transfectants (Fig.3B). Stable expression of wildtype syk did not affect the proliferation rate of these cells (not shown). It should be noted that these engineered cells are non-clonal populations, being the result of a bicistronic system that links transgene expression to drug resistance through an IRES sequence. Moreover, repeated generation of stable populations using this system resulted in stable "lines" that behaved similarly, demonstrating that these data are not artifactual in nature.

Since stable reexpression of syk in G3 Panc1 cells reduced their growth rate *in vitro* (Layton et al., 2009), we questioned whether stable reexpression of syk in MIAPaCa2 (MP2/syk^{wt}) cells would have a similar effect on these G3+ PDAC cells. Unlike Panc1 cells, no growth rate difference was observed between MP2/mock and MP2/syk^{wt} cells in culture (Fig.3C). Moreover, piceatannol did not suppress the growth of MIAPaCa2 or Panc1 cells stably reexpressing syk (Fig.3D), suggesting that PDAC cells that have progressed to syk-negative status are able to bypass the growth requirement for endogenous syk when it is ectopically reintroduced. It should be noted, however, that piceatannol was functional in this assay, since it caused a slight dose-dependent increase in the growth rate of Panc1/syk cells (Fig.3D) that is commensurate with overcoming the minor growth rate reduction that resulted from the original stable reexpression of syk in these cells (Layton et al., 2009). In contrast, however, we did observe an effect of stable syk reexpression on overall MIAPaCa2 morphology similar to that observed in Panc1/syk cells (Layton et al., 2009). As such, MP2/syk^{wt} cells demonstrate increased cell-cell interactions, resulting in more of a traditional monolayer characteristic of epithelial cells in culture, and a reduced propensity for cells to remain separate in between cell clusters (Fig.3E). This suggests the reestablishment of a more differentiated phenotype by syk in these cells. Accordingly, MP2/syk^{wt} cells demonstrate a dramatically reduced ability to grow in an anchorage-independent growth assay (Fig.3F), exhibiting a plating efficiency of 1.7%, versus 9.0% for MP2/mock cells, less dramatic but similar to the effect observed previously in Panc1/syk cells (Layton et al., 2009).

3.3 Stable expression, but not kinase activity, is required for syk-dependent invasion suppression

We previously observed a negative regulation of Panc1 invasion by stable reexpression of syk (Layton et al., 2009). Therefore, we assessed the *in vitro* invasion capabilities of MP2/mock and MP2/syk^{wt} cells. Stable MP2/syk^{wt} cells exhibit 90% less invasion towards serum-containing media compared to MP2/mock, and essentially no invasion in the absence of serum attractant (Fig.4A). To determine whether the kinase activity of syk is required for this effect, we stably expressed the K402R kinase-dead (KD) mutant syk in MIAPaCa2 cells (MP2/syk^{KD}); both wildtype and KD proteins were expressed at equal levels (Fig.4A, inset). Importantly, stable MP2/syk^{KD} cells exhibit nearly identical invasion suppression to MP2/syk^{wt} cells (Fig.4A), demonstrating that kinase activity is not required for syk's invasion-suppressor function in these cells. Consistent with this finding, 48h

pretreatment with piceatannol did not affect the invasion of either endogenously syk-positive BxPC3 cells or stable MP2/syk^{wt} cells (Fig.4B). Additionally, transient transfection with syk^{wt} or syk^{KD} did not retard MIAPaCa2 invasion (Fig.4C), suggesting that long-term expression is required for syk to influence invasion, likely through gene expression changes as described previously in Panc1 cells (Layton et al., 2009).

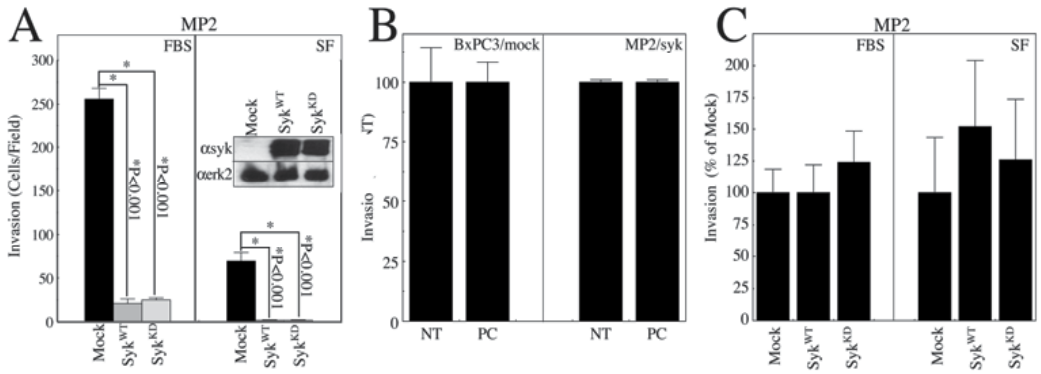

Fig. 4. **Stable expression, but not kinase activity, is required for invasion suppression by syk. (A)** The indicated stable MIAPaCa2 populations were provided serum-containing (FBS) or serum-free (SF) medium in the lower compartment of invasion chambers and allowed to invade for 24h before removal of uninvaded cells and enumeration of invaded cells. **(A,inset)** Immunoblot of ectopic syk expression with the 4D10 mAb (αsyk). Erk2, loading control. **(B)** Cells were pretreated for 48h with 25μM piceatannol (PC) or DMSO vehicle (NT) prior to seeding into invasion chambers with serum-containing media in the lower compartment. **(C)** MIAPaCa2 cells were transiently cotransfected with *lac*Z reporter and empty vector (mock), or wildtype (WT) or kinase-dead (KD) syk and 48h later seeded into invasion chambers. After 24 hours cells were stained with x-gal and invaded transfected cells (blue) were enumerated and plotted as percent of mock.

3.4 MIAPaCa2 invasion is regulated by urokinase–type plasminogen activators

We previously demonstrated that Panc1 invasion is dependent upon the matrix metalloproteinase (MMP)-2 axis, and that syk specifically attenuates the expression of MMP2 and its inhibitor, TIMP2, in these cells (Layton et al., 2009). In contrast to Panc1 cells, the MMP inhibitor TAPI1 did not suppress MIAPaCa2/mock invasion (Fig.5A), and RT-PCR (Fig.5B) and zymography (not shown) showed that MIAPaCa2 cells do not produce MMP2 in culture. MMP2 and MMP9 products were detected in parallel reactions run at the same time on unrelated samples (not shown), demonstrating that lack of signal in these samples is not the result of failed amplification reactions. These results demonstrate that MIAPaCa2 invasion is MMP-independent *in vitro*.

Another key regulator of epithelial cell invasion is the urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) axis (Leissner et al., 2006; McMahon and Kwaan, 2009), whose components are expressed by MIAPaCa2 cells in culture (Fig.5B). Consistent with the potential involvement of this system, MP2/mock invasion was completely suppressed by the serine protease inhibitor aprotinin (Fig.5C). This effect was not due to toxicity, as

aprotinin-treated cells remained 100% viable after 24h (Fig.5C, inset), which is the duration of the invasion assay. Specifically demonstrating the involvement of the uPA/uPAR axis, MP2/mock invasion was almost completely inhibited by a function-blocking anti-uPA antibody (Fig.5C). An isotype-matched (IgG_1) control antibody (MOPC-21) had no effect (not shown). Importantly, densitometry demonstrated >75% reduction in uPA mRNA levels by both syk^{wt} and syk^{KD} in MIAPaCa2 cells (Fig.5B), suggesting a potential mechanism for syk's effect on MIAPaCa2 invasion. Interestingly, expression of the uPA inhibitor, PAI-1, was also suppressed >30% by both wildtype and kinase-dead syk (Fig.5B). However, the uPA receptor, uPAR, was actually increased ~50% by syk^{wt}, but suppressed by >60% by syk^{KD}, suggesting that kinase activity may be required for appropriate regulation of uPAR, but not uPA or PAI-1.

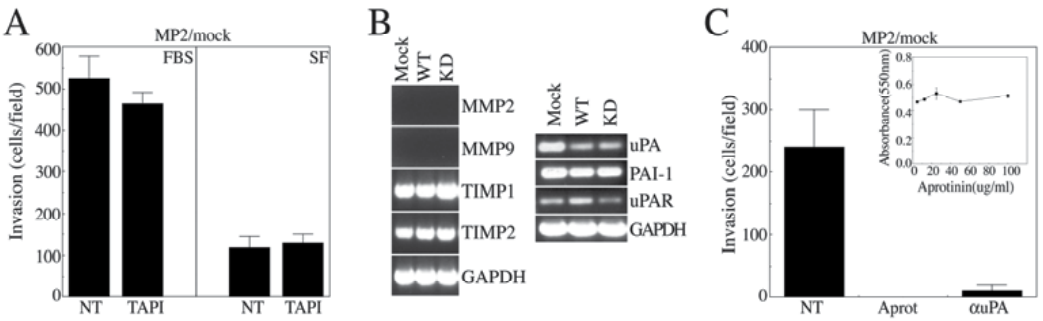

Fig. 5. **MIAPaCa2 invasion is mediated by the uPA/uPAR axis. (A)** MP2/mock invasion towards serum-containing (FBS) or serum-free (SF) medium +/- the MMP inhibitor TAPI1**. (B)** Semi-quantitative RT-PCR from the indicated stable MIAPaCa2 transfectants for the indicated products. GAPDH, control. **(C)** MP2/mock invasion +/- the serine protease inhibitor aprotinin or function-blocking anti-uPA antibody. (**C,inset**) Viability of aprotinin-treated cells after 24h.

Previous studies have demonstrated the regulation of the MMP2 axis by the αvβ3 integrin (Deryugina et al., 2001; Nisato et al., 2005), and an association of the uPA/uPAR axis with the αvβ5 integrin (Yebra et al., 1995; 1996). Moreover, MMP2 activation has been linked to αvβ3 in PDAC (Hosotani et al., 2002), and αvβ3 engagement suppresses the expression of uPA/uPAR components (Hapke et al., 2001). Therefore, to assess whether the different protease dependencies observed in this and our former study (Layton et al., 2009) are related to differential integrin expression, FACS analysis was performed on live cells from standard culture. Consistent with MMP2-dependence, Panc1 cells proved to be strongly αvβ3-positive (Fig.6A). In contrast, MIAPaCa2 cells are αvβ3-negative (Fig.6B), commensurate with their MMP2-independent/uPA-dependent phenotype. These results on cell surface integrin expression were corroborated at the total expression level by immunoblotting of whole cell lysates (not shown). Further demonstrating the integrin-dependent phenotypes of these cells, MIAPaCa2 invasion could be fully suppressed by a αvβ5 integrin-specific function-blocking mAb, while Panc1 invasion also involves αvβ3 and could only be fully blocked by combination of αvβ5- and αvβ3-specific function blocking mAbs (Fig.6C).

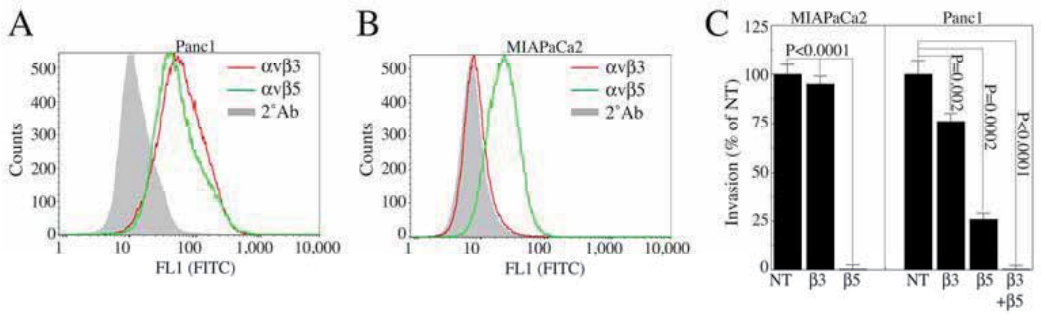

Fig. 6. **The invasion mechanism of MIAPaCa2 and Panc1 cells is related to their integrin expression profiles. (A,B)** FACS analysis of integrin αvβ3 and αvβ5 surface expression in Panc1 (**A**) and MIAPaCa2 cells (**B**). **(C)** Invasion of MIAPaCa2 (left) and Panc1 (right) cells in the presence or absence of 50μg/ml of function-blocking antibodies directed against the indicated integrins.

To further test the role of syk in regulating PDAC invasion, we examined the BxPC3/KD cells, which stably express the dominant-negative kinase-dead syk in addition to their endogenous wildtype syk. While BxPC3/mock cells show marginal invasion towards serum-containing media and essentially no invasion in the absence of serum attractant, stable BxPC3/KD cells demonstrate a >75% increase in invasion towards serum-containing media and significant *de novo* invasion in the absence of serum attractant (Fig.7A). FACS demonstrated that BxPC3 cells are also αvβ3-negative (not shown) and, consistent with this finding, the MMP inhibitor TAPI1 had no effect on serum-free BxPC3/KD invasion (Fig.7B). However, both aprotinin and anti-uPA mAb treatment reduced serum-free BxPC3/KD invasion significantly (Fig.7B), demonstrating a role for the uPA/uPAR axis in the syk-regulated invasion of these endogenously syk-positive PDAC cells. An isotype-matched (IgG_1) control antibody (MOPC-21) had no effect (not shown). It should be stressed that BxPC3/mock cells do not invade under these conditions, thereby allowing us to assess the role of uPA in a phenotype that was dependent upon syk inhibition and allowing us to exclude confounding issues derived from the endogenous phenotype.

4. Discussion

Previously we identified syk as being expressed not only in normal pancreatic ductal epithelium, but also in well- to moderately-differentiated PDAC *in situ* (Layton et al., 2009). The expression of syk in normal ductal epithelium of the breast has also been reported, as has a role for syk as a tumor suppressor in that tissue (Coopman et al., 2000). As such, loss of syk expression in primary breast tumors is associated with a poor prognosis (Toyama et al., 2003). We found that syk similarly correlates with patient survival in PDAC patients (Layton et al., 2009). However, we observe consistent loss of syk expression in poorly-differentiated PDAC, and have not observed loss of syk in well-differentiated PDAC samples. This differs from breast cancer, where syk is absent from a subset of well-differentiated lesions and expressed normally in many poorly-differentiated samples (Toyama et al., 2003), suggesting potentially dramatic differences in the regulation or function of syk in the ductal epithelium of these two glandular tissues. Indeed, we present evidence that syk functions to facilitate growth of PDAC cells that express it. This is perhaps

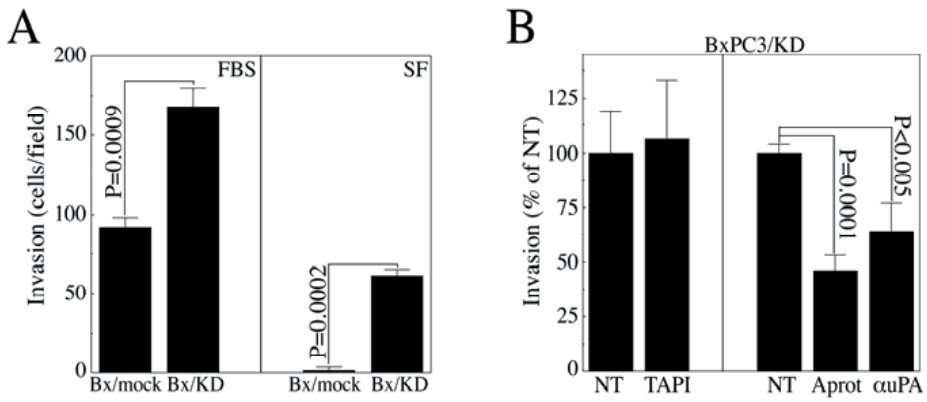

Fig. 7. **BxPC3 invasion is regulated by syk and mediated by the uPA/uPAR axis**.
(A) Invasion of stable BxPC3/mock (Bx/mock) and BxPC3/KD (Bx/KD) cells towards serum-containing (FBS) or serum-free (SF) medium. **(B)** Serum-free BxPC3/KD invasion +/- TAPI1 (left), or aprotinin or function-blocking anti-uPA antibody (right). BxPC3/mock cells were not assessed as they exhibit no invasion in the absence of serum in the lower chamber.

not entirely unexpected since syk promotes immune cell proliferation in response to antigenic stimulation (Kimura et al., 1996; Sada et al., 2001), and vascular defects observed in syk-deficient mice are attributable to a reduction in endothelial cell number (Yanagi et al., 2001). Indeed, syk was subsequently shown to be required for endothelial cell proliferation *in vitro* (Yanagi et al., 2001).

Mechanistically, syk has been shown to regulate mitosis through direct interactions with γ-tubulin and catalytic activity within the centrosome (Zyss et al., 2005). Morphological examination of piceatannol-treated PDAC cells stained with DAPI revealed that aberrant/dysfunctional mitosis may be responsible for the reduced cell numbers observed in this assay (not shown). This finding is consistent with prior ectopic expression studies that demonstrated localization of a carboxy-terminally RFP-tagged syk to centrosomes (Zyss et al., 2005); this chimera caused aberrant mitosis that likely resulted from steric interference of the RFP molecule on the directly adjacent kinase domain, further supporting our contention that syk kinase activity is required for syk-dependent regulation of cell division, and hence growth.

Similarly, since pharmacological inhibition of syk does not affect BxPC3 invasion, but stable expression of syk^{KD} does, this suggests that this effect of syk^{KD} is not due to direct suppression of endogenous syk signaling in these cells. As such, this construct may function by sequestering binding partners that would normally be phosphorylated by catalytically active syk, or a similar mechanism, since this construct can still be phosphorylated by upstream mediators such as src. Indeed, phosphorylation of Y352 couples syk to binding partners such as phospholipaseC and vav through the SH2 domains of these proteins (Sada et al., 2001). Mutation of Y352 impairs signaling in immune cells (Sada et al., 2001), demonstrating that Y352 is an important regulator of syk function in these cells. The presence of phosphorylated Y352 in pancreatic cells *in situ* and *in vitro* suggests that these or similar pathways are functional in syk-mediated processes in this cell type as well.

Proteolytic degradation of the extracellular matrix during tumor progression often involves uPA, its inhibitor, PAI-1, and its receptor, uPAR (Binder and Mihalay, 2008; McMahon and Kwaan, 2009). This system regulates the activation of the serine protease plasmin, and in the pancreas uPAR has been shown to regulate PDAC phenotype through interaction with integrin αvβ5 and PKC signalling (Yebra et al., 1995; 1996). More importantly, studies have shown a correlation between uPA and PAI-1 expression and tumor aggressiveness (Hansen et al., 2003), and PAI-1 has been shown to be necessary for tumor invasion (Binder and Mihaly, 2008). We recently reported that stable reexpression of syk dramatically reduced the invasion of endogenously syk-negative Panc1 cells at least partly by attenuating the MMP2 axis (Layton et al., 2009). Herein, we demonstrate that MIAPaCa2 and BxPC3 invasion is MMP-independent and mediated by the urokinase/plasminogen system, and that syk attenuates the expression of both uPA and PAI-1. This difference in protease utilization may be due to the differential expression and/or involvement of specific integrins since Panc1 cells are αvβ3-positive while MIAPaCa2 and BxPC3 cells are αvβ3-negative. Integrin αvβ3 suppresses uPA/uPAR expression in ovarian cancer cells (Hapke et al., 2001) and, as noted previously, the MMP2 axis has been associated with expression of the αvβ3 integrin and αvβ3 is required for MMP2 activation in some cell types (Deryugina et al., 2001); Nisato et al., 2005), including PDAC (Hosotani et al., 2002). Reciprocally, the uPA/uPAR axis is linked to αvβ5 (Yebra et al., 1995; 1996) and other integrins including α3β1 (Zhu et al., 2009), which is also expressed by both MIAPaCa2 and BxPC3 cells (S. Silletti, unpublished data). Importantly, the differential integrin utilization by these cells extended to the regulation of invasion as well. Therefore, this differential use of protease pathways in an integrin-specific manner by PDAC cells may be useful from a diagnostic and/or prognostic standpoint if further studies bear out this relationship in this tumor type.

In summary, we have further characterized the activity of syk in the growth and invasion regulation of pancreatic ductal epithelial cells. The expression of syk in these cells is associated with phosphorylation of tyrosines indicative of syk activation and activity, both *in vitro* and *in situ*, and syk activity is involved in regulating the proliferation/survival of syk-positive PDAC cells *in vitro*. This effect may be related to syk's role in transducing signals from growth factor receptors, or from syk's regulation of the mitotic spindle during mitosis. Irrespective, there has been a recent surge of interest in inhibitor-based strategies to target syk for conditions such as asthma, rheumatoid arthritis, and other immune disorders (Scott, 2011; Ulanova et al., 2005; Wong et al., 2004). Therefore, we propose that, aside from being a potential regulator of PDAC biology and biomarker of more differentiated PDAC tumors *in situ*, syk may be a viable target for therapeutic intervention in the clinic, since all grades of PDAC are uniformly fatal (NCI PDQ Database [http://www.cnacer.gov/cancertopics/pdq]; NCI-PANC-PRG) and syk expression is maintained in G1 and G2 PDAC *in situ* (Layton et al., 2009).

5. Conclusion

In this report we demonstrate that upstream activated and catalytically active syk kinase states can be detected in pancreatic ductal epithelial cells, both *in vitro* and *in situ*. The activity of syk is further demonstrated with regard to the regulation of cellular growth and invasion. Importantly, we have found that syk-dependent regulation of invasion is via modulation of the urokinase/plasminogen system in αvβ3-negative PDAC cells. This is in

contrast to the MMP-dependent invasion-suppressor effect of syk in αvβ3-positive PDAC cells, which are the exception, rather than the norm in this tumor type. These data provide further evidence of a central role for syk in regulating pancreatic ductal epithelial cell phenotype, and support the potential utility of targeting syk as a potential therapeutic modality in well- to moderately-differentiated PDAC patients, which are the majority of clinical cases. The significance of this is highlighted by the fact that PDAC is characterized by extensive dissemination at the time of diagnosis, irrespective of grade of disease.

6. Acknowledgement

S.Silletti is an American Cancer Society Research Scholar supported by ACS RSG-05-116-01-CSM and NIH grants CA130104 and CA109956. Funding agencies had no role in study design/performance or manuscript preparation. Moores UCSD Cancer Center is an NCI-sponsored Comprehensive Cancer Center supported by NCI Specialized Support Grant P30 CA23100.

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7

New Targets for Therapy in Pancreatic Cancer

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth cause of cancer-related death in the US, accounting for an estimated 37.000 in 2010 (Jemal et al., 2010). Only about 10-15% of newly diagnosed PDAC are potentially resectable, while the majority of patients present with locally advanced or metastatic cancer. The median survival of non operable patients treated with standard chemotherapy ranges between 3 and 10 months, with less than 20% alive at 1 year (Van Cutsem et al., 2004a). Survival rates have not substantially improved during the past 25 years, and gemcitabine, currently considered as the standard for the treatment of patients with advanced PDAC, only offers a limited advantage over 5-fluorouracil. Moreover, in these patients the tolerance of chemotherapy is often limited, due to the frequent occurence of pain and poor performance status. Given the limited efficacy of conventional chemotherapy, there is an urgent need of new treatment options for this disease. It is now clear that development and progression of PDAC is a complex process involving alterations of a core set of signalling pathways implicated in the regulation of multiple processes such as proliferation, cell cycle, migration, invasion, metastatization, metabolism, angiogenesis and resistance to apoptosis (S. Jones et al., 2008).

This chapter will overview the more relevant cellular pathways involved in the development and progression of PDAC, and the results obtained in preclinical models and clinical trials with the use of novel agents specifically targeting them.

2. Tyrosine kinases

Proteins endowed with tyrosine kinase activity (TKs), i.e. able to transfer the terminal phosphate of ATP to the hydroxyl group of tyrosine on acceptor molecule, have long been established as key regulators of multiple cellular processes including cell growth, proliferation, migration, invasion and resistance to apoptosis. TKs include receptor tyrosine kinase (RTKs) and non-receptor TKs (NRTKs) (Natoli et al., 2010).

2.1 RTKs

RTKs are structurally divided in 20 subfamilies of single transmembrane alpha-helic proteins, with the exception of the IGFR family that retains a permanent dimeric

conformation. The binding of a specific ligand to the extracellular domain of the receptor induces receptor dimerization and autophosphorylation at a regulatory tyrosine within the intracellular activation loop of the kinase. This triggers a cascade of intracellular reactions that mainly follows the p42/p44 mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) pathways, and culminates with the activation of genes involved in cell proliferation and survival.

2.1.1 MAPK and PI3K/AKT pathways

In the MAPK pathway a pivotal role is played by Ras proteins (k-Ras, N-Ras and H-Ras), enzymes with intrinsic GTPase activity (Ramos, 2008). Ras proteins are attached to the plasma membrane by virtue of farnesyl or geranylgeranyl chains covalently linked to their C-terminal end. These post-translational modifications are essential for membrane-anchorage and function of Ras.

Following RTKs activation, a variety of proteins are recruited nearby the plasma membrane. The tyrosine phosphorylation of RTKs creates a specific binding site for the SH2 domain of adaptor proteins, such as Grb2, that in turn allows the recruitment of Guanine Nucleotide Exchange Factors (GEFs), such as SOS1. GEFs directly activate Ras by promoting the release of GDP and the binding of GTP. Activated (GTP-bound) Ras induces the activation of the Raf/MEK/MAPK signaling, and eventually of downstream transcription factors such as Jun/Fos.

The key regulator of the PI3K/AKT pathway is PI3K, an enzyme that phosphorylates the membrane lipid phosphatidyl inositol 4,5-P2 (PIP2) in 3,4,5-P3 (PIP3). PIP3 is then responsible for AKT activation (Osaki et al., 2004). A negative regulator of this pathway is the Phosphatase and Tensin homolog (PTEN), that switchs off the signalling by dephosphorylating PIP3 in PIP2.

PI3K is recruited to the plasma membrane by activated RTKs directly through its SH2 domain, or indirectly through adaptor proteins such as Grb2/Gab1, or IRS1/2 in the case of IGFR. Once PI3K is activated and PIP3 is produced, the serine-threonine kinase AKT and the phosphoinositide-dependent kinase 1 (PDK1) are co-recruited to the plasma membrane, resulting in the phosphorylation and activation of AKT by PDK1. Activated AKT is able to phosphorylate multiple downstream targets, such as BAD, MDM2 and mTOR. In particular, phoshorylation of mTOR activates many biological processes essential for angiogenesis, cell metabolism and proliferation (Dowling et al., 2010). AKT activates mTOR complex 1 (mTORC1) via the small GTPase, Rheb. In basal condition, Rheb activity is suppressed by the TSC1/TSC2 complex, a GTPase activating protein. AKT determines TSC2 phosphorylation and inhibition of TSC1/TSC2 function, changes that unleash Rheb activity and mTOR signalling. Once activated, mTOR increases mRNA translation via two major downstream targets: the eIF4E-binding proteins (4EBPs) and the S6 kinases (S6K1 and S6K2). The 4EBPs are suppressors of the initiation translation factor eIF4E. After phosphorylation by mTOR, 4EBPs release eIF4E and make it available for the assembling of the eIF4F initiation complex that activates mRNA translation. In addition, the phosphorylation of S6K determines the subsequent phosphorylation of the ribosomal protein S6, a component of the 40S ribosomal subunit, that further facilitates mRNA translation. Proteins encoded by "eIF4E-sensitive mRNAs" include VEGF, cyclins, c-Myc and Bcl-xl, molecules involved in angiogenesis, cell proliferation and survival.

2.2 NRTKs

Based on structure, at least 10 subfamilies of NRTKs have been identified (Natoli et al., 2010). ABL and Src families have been particularly investigated for their implication in hematological and non-hematological malignancies. Physiologically, these proteins play a critical role in relaying intracellular signalling to the nucleus, regulating RTKs downstream signals, MAPK and PI3K/Akt, and several other key pathways, such as focal adhesion kinase (FAK) and signal transducers and activator of transcription (STAT transcription factors). NRTKs are maintained in an inactive state by intramolecular autoinhibition or by cellular inhibitor proteins or lipids. They are activated by diverse intracellular signals, including recruitment to RTK, dissociation of inhibitors, and trans-phosphorylation by other kinases. Activated NRTKs critically participate in the regulation of cell proliferation, differentiation, migration, adhesion, angiogenesis, invasion, and immune function.

2.3 Dysregulation of TKs signalling in PDAC

Given the central role of TKs in the regulation of cell growth and survival, it is not surprising that TKs and/or their downstream signalling mediators are aberrantly activated in different types of cancers, including PDAC.

In PDAC different RTKs have been shown to be frequently overexpressed, such as Epidermal Growth Factor Receptor (EGFR) in 90% (Lemoine et al., 1992), HER2 in 45-70% (Yamanaka et al., 1993b), Insulin-like Growth Factor-1 Receptor (IGF-1R) in about 50% (Bergmann et al., 1995), and cMET in 70% of the cases (M. Ebert et al., 1994). Overexpression is often reported to be associated with enhanced tumor growth, motility, invasion and drug resistance (M. Ebert et al., 1994; Freeman et al., 1995; Yamanaka et al., 1993a).

Consistent with RTKs overexpression, the downstream MAPK and PI3K/AKT pathways are often activated in PDAC. Activation of the MAPK pathway has been shown to be responsible for the malignant transformation of pancreatic cells (Matsuda et al., 2002). However, activation of Ras in PDCA is mostly RTK-independent, since activating mutations of this oncogene occur in about 90% of cases in advanced disease (Hruban, 2001). Because of an impaired GTPase activity, mutated Ras maintains a GTP-bound state, resulting in a continuous activation of the downstream Raf/MEK/MAPK signalling. Activating mutation of Raf has also been described in pancreatic cancer (Hruban, 2001).

Activation of PI3K/AKT pathway has been described in 50% of PDAC cases and is associated with a worse prognosis (Schlieman et al., 2003). PI3K has been shown to stimulate proliferation and to be involved in drug resistance of pancreatic cancer cells (Perugini et al., 2000), while overexpression of AKT promotes invasion (Cheng et al., 1996) and expression of IGF-IR (Tanno et al., 2001).

Among NRTKs, aberrant Src activation has been described in multiple malignancies, including pancreatic cancer (Dehm & Bonham, 2004), and shown to be related to increased cell motility and invasiveness (Shah & Gallick, 2007).

The importance of TKs signalling in the maintenance of the neoplastic phenotype is emphasized by the fact that inhibition of these pathways induces cell-cycle arrest and apoptosis in preclinical models of pancreatic cancer (Asano et al., 2005; Bondar et al., 2002; Ng et al., 2002; Yip-Schneider & Schmidt, 2003).

2.4 TKs therapeutic targeting

Preclinical studies have shown dependency of pancreatic tumor cell proliferation upon TKs activity and tumor regression by its blockade.

In the last decade a number of molecules have been developed to inhibit TKs signalling. These agents can be divided into two major groups: small molecules that inhibit the catalytic activity of the kinase by interfering with the binding of ATP or substrates, and antibodies against RTKs or their ligands. In addition, other agents have been designed to block TKs' downstream signalling molecules, in particular Ras, Raf, MEK, AKT and mTOR. The most relevant anti-TKs strategies applied in PDCA are detailed below, along with ongoing clinical trials.

2.4.1 Anti-EGFR therapies

Cetuximab, a chimeric monoclonal antibody that targets the extracellular domain of human EGFR, has been tested in a phase III randomized trial by the Southwestern Oncology Group (SWOG trial S0205) in combination with gemcitabine in patients with unresectable, locally advanced or metastatic PDAC. The study showed no significant improvement in overall or progression-free survival with the addition of cetuximab, with a minimal although significant advantage in time-to-treatment-failure (Philip et al., 2010). A similar study design was used to test the efficacy of panitumumab, a humanized anti-EGFR antibody (clinicaltrial.gov: NCT00613730). The study was early terminated based on the results of the S0205 trial. A randomized phase II trial of panitumumab, erlotinib and gemcitabine vs. erlotinib and gemcitabine in patients with untreated, metastatic PDAC (clinicaltrial.gov: NCT00550836) has been recently completed and results are awaited.

Erlotinib, an EGFR tyrosine kinase inhibitor, has been tested in combination with gemcitabine in a randomized phase III trial (Moore et al., 2007). A very modest benefit in OS (median 6.24 *vs* 5.91 months, P=.038) was observed compared with gemcitabine alone. Even if approved for the treatment of advanced PDAC, erlotinib is not routinely used because of increased toxicity and cost. Erlotinib is currently studied in the adjuvant setting (RTOG-led study 0848).

Other EGFR tyrosine kinase inhibitors, including gefitinb and lapatinib, have been tested in pilot studies, but failed to demonstrate a clinically significant activity (Brell et al., 2009; Ignatiadis et al., 2006; Safran et al., 2008).

2.4.2 Anti-HER2 therapies

Trastuzumab, a recombinant humanized antibody directed against HER2 and largely used in the treatment of HER2-positive breast cancer, has been tested in combination with gemcitabine in 34 PDAC patients with HER2-overexpressing tumors. No significant survival benefit was observed (Safran et al., 2004).

2.4.3 Anti-IGF-1R therapies

Several trials testing the safety and activity of inhibitors of IGF-1R are ongoing.

IMC-A12, an anti-IGF-1R monoclonal antibody, is currently being used in a randomized phase II study (SWOG 0727) in combination with chemotherapy and erlotinib in advanced

PDAC. The dual block of IGF-1R and EGFR has a strong preclinical rationale since the inhibition of IGF-1R might reverse resistance to anti-EGFR agents (Camirand et al., 2005).

A study with the same design, carried out at the MD Anderson Cancer Center, is testing MK-0646, a humanized monoclonal antibody against the IGF-1R (clinicaltrial.gov: NCT00769483).

Finally, AMG-479, another anti-IGF-1R antibody, is being used in combination with gemcitabine in a three-arm randomized phase II study. Preliminary results have been presented showing a trend toward longer PFS in the combination arm (Kindler et al., 2010a).

2.4.4 Anti-c-MET therapies

AMG-102, a fully humanized monoclonal antibody against c-MET, has been tested in a phase I study in patients with advanced solid tumors, including pancreatic cancer (Gordon et al., 2010). The drug was safe and well tolerated and it is going to be tested in a phase II study, in monotherapy or in combination with other agents.

2.4.5 Anti-MAPK therapies

Tipifarnib, a Ras inhibitor, has been studied in combination with gemcitabine in a randomized phase III clinical trial (Van Cutsem et al., 2004b). The combination has an acceptable toxicity profile, but does not prolong overall survival in advanced PDAC as compared with single-agent gemcitabine.

In a phase II study, Sorafenib, a TK inhibitor targeting Raf, VEGFR and PDGFR, has been tested in combination with gemcitabine in patients with advanced PDCA (Kindler et al., 2010b), but failed to demonstrate clinical benefit. Other Raf inhibitors are currently tested in clinical trials (W. W. Ma & Adjei, 2009).

CI-1040, an oral MEK inhibitor, has been used in a pilot phase II study in advanced tumors, including PDAC. Although generally well tolerated, CI-1040 demonstrated insufficient antitumor activity to warrant further development (Rinehart et al., 2004).

2.4.6 Anti-PI3K/AKT therapies

Clinical experience with inhibitors of the PI3K/Akt pathway in PDAC is mostly limited to mTOR or AKT inhibitors. Everolimus and enzastaurin failed to demonstrate significant clinical activity when tested in gemcitabine-refractory (Wolpin et al., 2009) or advanced PDAC (Richards et al., 2011), respectively.

2.4.7 Anti-Src therapies

Among NRTK-inhibitors, AZD0530, a Src kinase inhibitor, is currently being tested in a phase II trial in gemcitabine resistant patients and in a phase I/II trial in combination with gemcitabine in unresectable disease (clinicaltrial.gov: NCT00735917).

3. Angiogenesis

Angiogenesis, the process by which new blood vessels are formed from pre-existing ones, is critical for the growth, progression and metastatization of solid tumors, including PDAC.

This multistep process is tightly controlled by a fine-tuned balance between positive and negative regulators that emanate from cancer cells, endothelial cells, stromal cells, blood and the extracellular matrix.

Although several positive regulators of angiogenesis have been described, vascular endothelial growth factor (VEGF or VEGF-A) represents the prototypical pro-angiogenic factor (Ferrara et al., 2003). VEGF belongs to a gene family which includes placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D. When released mainly by tumor cells, VEGF binds two specific receptor tyrosine kinases, VEGFR-1 (de Vries et al., 1992) and VEGFR-2 (Terman et al., 1992). This triggers receptor autophosphorylation and initiates a series of downstream signalling that promotes proliferation, survival and migration of endothelial cells.VEGFR-3 is also included in the same family of RTKs, but binds VEGF-C and VEGF-D (Karkkainen et al., 2002).

Overexpression of VEGF has been associated with tumor progression and poor prognosis in several tumor types. The increasingly recognized importance of VEGF signalling in promoting tumor angiogenesis has led to the development and clinical validation of several agent that selectively target this pathway. Today, inhibition of angiogenesis is considered a valid treatment approach in a number of solid tumours, although with limited efficacy (Carmeliet & Jain, 2011).

Agents affecting the VEGF pathway include drugs targeting VEGF itself (antibodies or "traps") or the extracellular domain of VEGFR, small molecules targeting intracellular domains of VEGFR and those of other tyrosine kinases, and drugs inhibiting the intranuclear production of VEGFR mRNA, such as angiozyme.

Bevacizumab, a humanized monoclonal antibody with a high binding affinity for circulating vascular endothelial growth factor A (VEGF-A), has been demonstrated to enhance the response rate, progression-free and overall survival of patients with advanced cancer when added to various chemotherapeutic regimens. The cellular mechanisms of action of bevacizumab are multifactorial and include inhibition of vascular neogenesis, vascular regression and normalization of tumour vasculature (Ellis & Hicklin, 2008). Several studies have shown that VEGF and its receptors are overexpressed in pancreatic cancer tissue and associated with liver metastases and poor prognosis (Kuehn et al., 1999; Seo et al., 2000). In PDAC, antiangiogenic therapy is still in an experimental phase with rare promising results. In the pre-clinical setting, inhibition of secreted VEGF by an antisense oligonucleotide led to decreased neoangiogenesis in a mouse xenograft model of pancreatic cancer resulting in reduced tumor growth and metastasis (Hotz et al., 2005). In the clinical setting, two randomized phase III trials of bevacizumab and gemcitabine (with or without erlotinib) failed to show any significant improvement of overall survival (Kindler et al., 2010c; Van Cutsem et al., 2009), and in one of them (Van Cutsem et al., 2009) only a marginal gain in progression-free survival was observed. The modest clinical benefit so far observed might be, at least in part, dependent on the development of resistance. Upregulation of compensatory angiogenic signalling pathways, such as those modulated by platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), has been suggested as a potential mechanism of resistance to anti-VEGF therapy (Casanovas et al., 2005).

As opposed to isolated VEGF/VEGFR inhibition, multitargeted antiangiogenic TK inhibitors may more completely inhibit angiogenesis by blocking overlapping pathways

(Erber et al., 2004). A number of multitargeted antiangiogenic TK inhibitors are being tested in PDAC, including dual VEGFR/PDGFR and VEGFR/FGFR inhibitors (i.e. Sunitinib), as well as triple VEGFR/PDGFR/FGFR inhibitors (i.e. sorafenib), but limited activity in phase II trials ended further investigations on these agents.

Axitinib, an oral and selective inhibitor of VEGFR 1,2 and 3 and PDGFR-β, has been investigated in a phase II randomized trial in PDAC, associated with gemcitabine versus gemcitabine alone (Spano et al., 2008). The trial showed a small, non-statistically significant gain in overall survival for the combination arm. A randomized double-blind phase III trial with a similar design has been recently completed (Clinicaltrials.gov: NCT00471146) and results are awaited.

The persistent failure of anti-VEGF/VEGFR targeting in pancreatic cancer raises questions and concerns about choice of agents, trial design and our understanding of the biology of this disease.

4. The hedgehog pathway

The Hedgehog (Hh) signalling controls the proper tissue formation or "patterning" during normal embryonic development, modulating cell migration, proliferation and differentiation (Ingham & McMahon, 2001).

The Hh signalling is mediated by three secreted proteins with high degree of homology, known as Sonic Hh, Desert Hh and Indian Hh. These proteins act in an autocrine/paracrine manner by binding the cellular transmembrane receptor Patched 1 (PTCH1). In the absence of the Hh ligand, PTCH1 inhibits the activity of another transmembrane protein, Smoothened (Smo), preventing its localization to the cell surface. After Hh binding, PTCH1 is internalized and degraded, allowing Smo to translocate to the cell membrane where it initiates a downstream signalling responsible for the release of the glioma-associated (Gli) proteins from the inhibitor complex Suppressor of Fused (SUFU). The subsequent activation and nuclear translocation of Gli transcriptional factors results in increased expression of genes encoding for growth factors, cell cycle regulators, cell adhesion molecules, matrix proteins, other transcription factors, and inhibitors of the Hh pathway itself (Cohen, 2010; Ruiz i Altaba et al., 2002).

A negative regulator of Hh pathway is Hh-interaction protein (HIP), a transmembrane protein that binds all 3 Hh proteins and prevent ligand inactivation of PTCH1 (Chuang & McMahon, 1999).

Increased expression of the Hedgehog pathway components has been demonstrated in PDAC and its precursor lesions, indicating a role of this pathway during early stages of tumorigenesis (Morton et al., 2007). Moreover, reduced or absent expression of HIP is related to Hh signalling activation in pancreatic cancer cell lines (Martin et al., 2005). In a recent microarray study, nineteen altered genes of the Hh pathway were identified and 100% of tumors had alterations in at least one of the Hh pathway genes (S. Jones et al., 2008).

Given the major role of Hh signalling in cancer development, the targeting of this pathway is considered a promising approach in the treatment of patients with PDCA. Indeed, in preclinical models, the inhibition of Hh signalling increased tumor cell apoptosis, decreased metastases, and significantly extended animal survival (Feldmann et al., 2008).

Currently, a phase I/II clinical trial is evaluating IPI-926, a small molecule inhibitor of Smo, in combination with gemcitabine in patients with previously untreated metastatic PDAC. (ClinicalTrials.gov: NCT01130142). Other agents targeting various components of the Hh pathway are in preclinical or early clinical development, including Hh antagonist and Gli inhibitor.

5. DNA repair mechanisms

Mammalian cells are constantly exposed to exogenous (e.g. ultraviolet or ionising radiation and genotoxic chemicals) and endogenous (e.g. cellular metabolism and free radical generation) stresses responsible for DNA damage (Hoeijmakers, 2001). In order to preserve genomic integrity, cells are equipped with several DNA repair mechanisms, including nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways. Loss of function in one of these pathways determines an increased rate of chromosome breakage and, as a consequence, activation of oncogenes by translocations, inactivation of tumor suppressor genes by deletions and amplification of drug resistance genes, thus fostering tumor progression and drug resistance.

NER, BER and MMR pathways are involved in DNA single strand breaks (SSBs) repairing process, where the complementary DNA strand is intact and serves as a template (Hoeijmakers, 2001).

In particular, NER acts on damaged nucleotides that distort DNA helix, such as pyrimidine dimers induced by UV or adducts formed by chemotherapeutic agents. Interestingly, this pathway may confer resistance to platinum-based chemotherapy.

BER acts on nitrogenous bases damaged by reactive oxygen species or by spontaneous depurinations (Lindahl, 1993). Key enzymes in this pathway are PARP1 and PARP2, two nuclear proteins that catalyze the transfer of ADP-ribose units from intracellular NAD+ to nuclear acceptor proteins with the formation of ADP-ribose polymers.

MMR removes mispaired nucleotides derived from replication error (Li, 2008) and is involved in the repair of DNA adducts such as those resulting from platinum-based chemotherapy (Kinsella, 2009). MMR pathway includes proteins encoded by different genes, such as MSH2, MSH3, MSH6, MLH1, MLH3, PMS1, and PMS2. It is noteworthy that an intact MMR pathway is required for cisplatin sensitivity, indicating a role for MMR proteins in the apoptotic signalling (Pani et al., 2007).

On the contrary, HR and NHEJ pathways are recruited in the case of DNA double-strand breaks (DSBs), which are devoid of a viable chromatid template (Hoeijmakers, 2001). HR acts using sister chromatid as template, and therefore it functions only during the late S and G2 phases of the cell cycle, when a homologous region of DNA is available. HR repairs DNA DSBs caused by reactive oxygen species, ionizing radiation and certain antineoplastic drugs, such as bleomycin and anthracyclines. HR is initiated by the MRN complex which, thanks to its 3′-5′ exonuclease activity, exposes the 3′ ends on either side of the DSB (Zhong et al., 1999). Another complex, RPA, binds to the exposed single strand DNA to avoid degradation. Then, RAD51 is recruited at the site of DNA damage to initiate repair (Yu et

al., 2003). BRCA1 and BRCA2, known for their implication in familial breast and ovarian cancers, play an important role in this pathway. BRCA1 is required to retain RPA at the sites of DSBs (Durant & Nickoloff, 2005), while BRCA2 is required for RAD51 translocation (Yang et al., 2005).

NHEJ repairs DSBs during G1/S phase of the cell cycle. This pathway is activated by the DNA-dependent protein kinase Ku70/Ku80 and requires additional proteins, including the artemis protein and DNA ligase IV, for proper conclusion (Meek et al., 2004).

PDAC cells frequently harbor defects in DNA repair pathways, in particular as a result of BRCA2 or MMR gene mutation/deletion. BRCA2 mutation carriers have a 3-10-fold increased lifetime risk of developing PDAC (Shi et al., 2009). Germline BRCA2 gene mutations are responsible for approximately 10% of familial pancreatic cancer, whereas somatic mutations have been associated with 7–10% of sporadic PDAC (Shi et al., 2009).

The discovery that FANCD1, one of at least 13 Fanconi anemia (FA) proteins interacting in a common pathway involved in HR, is identical to BRCA2, resulted in a search for mutations in other FA genes as possible pancreatic-cancer risk genes. Recently, a pancreatic cancer susceptibility gene, PALB2 (FANCN), has been found to encode for a BRCA2-stabilizing protein (Tischkowitz et al., 2009) and a germline deletion in the PALB2 gene has been described in a patient with familial PDAC (S. Jones et al., 2009).

Alterations in the MMR genes have also been described in pancreatic cancer (Dong et al., 2011). A direct consequence of MMR impaired function is microsatellite instability that makes the genome vulnerable to other specific genetic alterations. However, tumors of the pancreas with microsatellite instability are relatively rare as compared to other malignancies of the digestive tract, and represent only 5% of PDAC.

Although abnormal DNA repair mechanisms critically contribute to tumor development and progression, they also provide a weakness that can be exploited therapeutically. In fact, tumors harboring defective DNA repair mechanisms might be particularly sensitive to DNA-damaging agents. For example, BRCA deficient cancer cells show increased sensitivity to agents causing DSBs, such as irradiation, mitomycin C, adriamycin, and cisplatin (Sonnenblick et al., 2011; van der Heijden et al., 2005). It has been shown that HR-deficient tumor cells, including those with defects in BRCA1/2, are highly sensitive to blockade of the BER pathway via inhibition of the PARP enzymes (Bryant et al., 2005; Farmer et al., 2005). In fact, PARP inhibitors (PARPis) lead to accumulation of SSBs that degenerate into stalled replication forks and, eventually, in DSBs, damage preferentially repaired by HR. Exposure of BRCA2-deficient murine tumors to PARPis resulted in a marked decrease in tumor growth and survival (Hay et al., 2009). A human pancreatic cancer cell line with defective BRCA2 function, CAPAN-1, has been shown to be very sensitive to the potent PARPis KU0058684 and KU0058948 (McCabe et al., 2005). The potential clinical application of PARP inhibition in BRCA2-related pancreatic cancer is encouraged by a recent published case report. A patient with a germline BRCA2 mutation affected by pancreatic cancer demonstrated a complete pathologic response after treatment with iniparib (BSI-201), a PARPi (Fogelman et al., 2011).

As expected, PARPis have also been shown to significantly enhance the cytotoxicity of chemotherapeutic agents, such as cisplatin and 5-fluorouracil (De Soto & Mullins, 2011), and of radiotherapy (Tuli et al., 2011) in pancreatic cancer cell lines.

A phase I/II study of the PARPi veliparib (ABT-888) in combination with chemotherapy (modified FOLFOX-6), is currently being conducted in patients with metastatic pancreatic cancer (Pishvaian et al., 2011). Another phase I study is testing the safety and tolerability of the PARPi olaparib (AZD2281) in combination with gemcitabine in PDAC (clinicaltrials.gov: NCT00515866). The same drug is being used as monotherapy in a phase II trial in patients with advanced cancers, including PDAC, with confirmed genetic BRCA1 and/or BRCA2 mutation (clinicaltrials.gov: NCT01078662).

6. Histone deacetylase (HDACs)

This large family of enzymes is composed of 18 members which are grouped into four classes according to their primary homology to similar yeast HDACs (de Ruijter et al., 2003). Classes I, II, and IV are structurally similar to the yeast proteins Hda1/Rpd3 and are zinc-dependent for their catalytic activity (de Ruijter *et al.*, 2003), while class III HDACs include 7 different members of the sirtuin (SIRT) family and require NAD+ for their catalytic activity (Blander & Guarente, 2004). These families differ for subcellular localization, catalytic activity and susceptibility to different inhibitors. HDACs are known to play a key role in the epigenetic regulation of gene expression (P. A. Jones & Baylin, 2007). The process of deacetylation of histones by HDACs results in a closed chromatin structure, decreased access of transcription factors to promoter regions and repression of gene transcription leading to the silencing of tumor suppressor genes involved in cancer cell growth, differentiation and/or apoptosis (de Ruijter et al., 2003). Apart from regulating histone modification, HDACs also regulate the post-translational acetylation status of many non-histone proteins involved in cancer cell proliferation, such as transcription factors, nuclear receptors and cytoskeletal proteins (Glozak et al., 2005).

Dysregulation of HDACs has been detected in hematological malignancies and several different types of solid tumors (Federico & Bagella, 2011), including pancreatic cancer (Ouaissi et al., 2008; Ouaissi et al., 2011).

During the 1990s, a clear link between the suppression of tumor cell growth and survival and the inhibition of HDAC activity was established (Xu et al., 2007), so that histone deacetylase inhibitors (HDACIs) have recently emerged as promising antineoplastic agents (Elaut et al., 2007). HDACIs can induce cell-cycle arrest, promote differentiation, and stimulate tumor cell death. Dimethyl sulfoxide (DMSO) and sodium butyrate (NaBu) are the first chemical agents identified as HDACIs (Friend et al., 1971)

The preferential toxicity of HDAC inhibitors in transformed cells (Burgess et al., 2004; J. H. Lee et al., 2010) and their ability to synergistically enhance the anticancer activity of many chemotherapeutic agents (Sigalotti et al., 2007) has generated a great deal of interest in developing new HDACIs for cancer therapy. Indeed, several compounds, including pan-HDAC inhibitors and class-selective or isoform-selective HDACIs, have been synthesized and tested in phase I, II and III clinical trials in cancer patients, either alone or in combination with other chemotherapeutic agents.

Currently, two HDACIs are available for cancer treatment, suberoylanilide hydroxamic acid (SAHA or Vorinostat) (Vrana et al., 1999) and romidepsin (Khan et al., 2004) which have been approved by FDA for the treatment of cutaneous T-cell lymphoma. Vorinostat as well

as other HDACI such as Entinostat (MS-275) (Gojo et al., 2007) and MGCD0103 (Kell, 2007) - both of them in clinical trials (Kell, 2007; Tomillero & Moral, 2010) - have been shown to exert proapoptotic effects on pancreatic tumor cells and to chemosensitize them to gemcitabine (Arnold et al., 2007; Iwahashi et al., 2011; Sung et al., 2011). These drugs are now being tested in phase I-II trials recruiting advanced pancreatic cancer patients.

7. Checkpoint kinase-1 (Chk1)

The Chk1, downstream the nuclear protein kinase ataxia-telangiectasia mutated -ATM- (Kurz & Lees-Miller, 2004), arrests cells in the S- and G2-phases of the cell cycle in the presence of replication stress or DNA double-strand breaks, thus allowing cells to repair the damage (Koniaras et al., 2001). When this mechanism is defective, cells accumulate DNA damages which lead to apoptosis. p53-deficient cancer cells, rely on CHK1 for the damage response and agents able to disrupt this pathway could induce a specialized form of cell death known as mitotic catastrophe.

Inhibitors of the Chk1 are currently in development with the aim to improve the efficacy and selectivity of a variety of DNA-damaging agents or antimetabolites (Bolderson et al., 2009; Du et al., 2011; C. X. Ma et al., 2011). Recently, novel Chk1 inhibitors, such as AZD7762, have been shown to be able to sensitize pancreatic cancer cells and tumors to gemcitabine and are now in Phase I clinical trials (Morgan et al., 2010; Parsels et al., 2011)

8. microRNAs

Two main classes of RNAs are known: messenger RNAs (mRNAs), which are translated into proteins, and non-protein-coding RNAs (ncRNAs), whose function has been related to the regulation of intracellular and extracellular signalling involved in cell differentiation and development (Amaral & Mattick, 2008; Dinger et al., 2008).

The following molecules have been included in the group of ncRNAs:

- long ncRNAs, longer than 100 nucleotides, with a positive effect on regulation of gene expression (Orom et al., 2010);
- small nucleolar RNAs (snoRNAs), able to modify or guide the pseudouridylation or the methylation of other RNAs (Mallardo et al., 2008);
- small interfering RNAs (siRNA), regulating gene expression post-transcriptionally, by silencing specific mRNA molecules (J. Wang et al., 2010);
- piwi-interacting RNAs (piRNA), involved in the silencing of retrotransposons in germ cells
- riboswitches, a part of RNA that binds small molecule ligands (Siomi et al., 2011);
- microRNAs (miRNAs), small (18–24 nucleotide in lenght) single stranded RNA molecules that negatively regulate gene expression (Bartel, 2004).

Among all these regulatory ncRNSa, miRNAs are now acquiring a major relevance as potential therapeutic targets. miRNA were first discovered in *Caenorhabditis elegans* (R. C. Lee et al., 1993) and subsequently shown to be evolutionarily conserved genes (Moss, 2007), more than 1000 miRNAs being so far identified (miRBase database, release 17).

The biogenesis pathway of miRNA in animal cells is a complex multi-step process starting in the nucleus and (through many post-transcriptional modifications) ending in the cytoplasm to generate the mature, single stranded miRNA (Huang et al., 2011). miRNAs silence gene expression by binding to the 3'UTR of the target mRNAs with imperfect complementarity, thus causing repression of translation or inducing target mRNA molecule degradation, with consequent reduction or loss of the protein product (Carthew & Sontheimer, 2009; Huntzinger & Izaurralde, 2011).

miRNAs have been shown to be missing or down-regulated in a variety of medical conditions including cancer, diabetes, and cardiovascular diseases (Medina & Slack, 2008; Z. Wang et al., 2011). For their role in cancer initiation and/or progression, miRNAs have been included in the family of genes recognized as tumor suppressors and oncogenes, with the name of *oncomiRs* or *anti-oncomiRs* (Iorio & Croce, 2009; Kent & Mendell, 2006). Moreover, because of these critical functions in the regulation of signalling pathways, miRNAs are regarded as new promising therapeutic targets for cancer treatment (Bader et al., 2011).

Direct therapeutic approaches are based on the development of new drugs able to modulate miRNA expression levels, including:

- *miRNA mimics*, to inhibit the expression of target protein-coding genes, for miRNAs whose expression is reduced in diseases (Kota et al., 2009)
- *miRNA inhibitors*, with antisense constructs like *antagomiRs*, to increase gene expression (M. S. Ebert et al., 2007)

However, there are still many problems to be solved, such as stability, effective in vivo delivery systems, and selectivity.

These therapeutic strategies could be of high relevance for pancreatic cancer treatment. Recently, deregulation of miRNAs expression has been correlated to diagnosis, prognosis and chemotherapy resistance of pancreatic cancer (Chakraborty et al., 2011; Dhayat et al., 2011). Among the several miRNAs reported to be up regulated in pancreatic cancer such as miR-21, miR-221/222, miR-25, miR-27a, miR-210, miR-200b, miR-148a,b, miR-196a-2, miR-155, and members of the miR-17-92 family (Chakraborty et al., 2011; Liffers et al., 2011; Nana-Sinkam & Croce, 2011; Zhang et al., 2011), the *oncomiR* miR-21 appears of high relevance as potential therapeutic target in pancreatic cancer, regulating proliferation, invasion, apoptosis and chemosensitivity (Ali et al., 2010; Giovannetti et al., 2010; Hwang et al., 2010; Park et al., 2009). miR-21 mimics, transfected into MIA PaCa-2 pancreatic cancer cells, have been shown to upregulate Bcl-2, to downregulate Bax expression, to decrease chemosensitivity to gemcitabine and to increase proliferation as compared with control cells. Transfection with miR-21 inhibitors had opposite effects, decreasing cell proliferation (J. Dong et al., 2011).

Further, miRNA-based biomarkers have a significant impact on the development of treatment strategy that combines therapeutics with diagnostics, a concept known as theranostics (Pene et al., 2009) and are highly relevant for drug development and personalized medicine.

9. Conclusion

In the last decade, a variety of molecularly targeted agents potentially useful in the treatment of PDAC have been developed. However, in contrast with the encouraging results

obtained in animal models, results in humans have been quite disappointing. The still limited knowledge of the molecular pathogenesis of PDAC, the inappropriateness of the experimental models, the suboptimal combinations and schedules selected for clinical trials can be suggested as possible reasons for this failure. Given the complexity of the molecular pathogenesis of PDAC, it is very likely that a substantial improvement of clinical outcome can derive from the rational targeting of more than a single altered pathway.

10. References

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8

Failure of Pancreatic Cancer Chemotherapy: Consequences of Drug Resistance Mechanisms

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1. Introduction

Pancreatic cancer is one of the most lethal forms of cancer and it is estimated that there will be about 44,000 new cases in US in the year 2011. With 37,600 estimated deaths in 2011, pancreatic cancer is the fourth leading cause of cancer related deaths in US (*American Cancer Society, 2011*). In spite of numerous efforts, the 5-year survival rate for pancreatic cancer has not improved much for the last few decades. The suggested reasons for low survival among the pancreatic cancer patients include late disease diagnosis, highly invasive and metastatic nature, lack of effective therapies, and acquisition of resistant characteristics (*American cancer Society 2007; Moore, et al., 2003; NIH 2007*). Only two drugs – gemcitabine (GEM) and 5-fluorouracil (5FU) – have been shown to improve the survival of patients consistently. 5FU was the first drug to be approved as adjuvant therapy for pancreatic cancer (*Kalser and Ellenberg, 1985; Moertel, et al., 1981*). Since then, GEM has been used as the first line chemotherapeutic drug for pancreatic cancer. However, GEM treatment does not always provide extended survival benefits. A study found that in post-operative patients, GEM treatment increased the survival by merely 6 months (*Shore, et al., 2003*). 5FU is also widely used as an adjuvant and neoadjuvant chemotherapeutic agent to treat pancreatic cancers (*Ahlgren, 1996; Blaszkowsky, 1998; Snady, et al., 2000*).

Although the cell death mechanisms induced by GEM and 5FU are well understood, their efficacy is limited due to the acquisition of drug-resistant characteristics by the cancer cells. Various molecular mechanisms have been suggested to play a role in development of resistance against these drugs. Upregulation of Akt (protein kinase B), NFκB, MDR (p-glycoprotein) and hypoxia have been shown to impart resistance against GEM (*Bergman, et al., 2002; Galmarini, et al., 2002; Garcia-Manteiga, et al., 2003; Nakano, et al., 2007; Yokoi and Fidler, 2004*). Similarly, modulation of thymidylate synthetase (TS), dihydropyrimidine dehydrogenase (DPDY), MAPK, p53 and *src* imparts 5FU resistant characteristics to

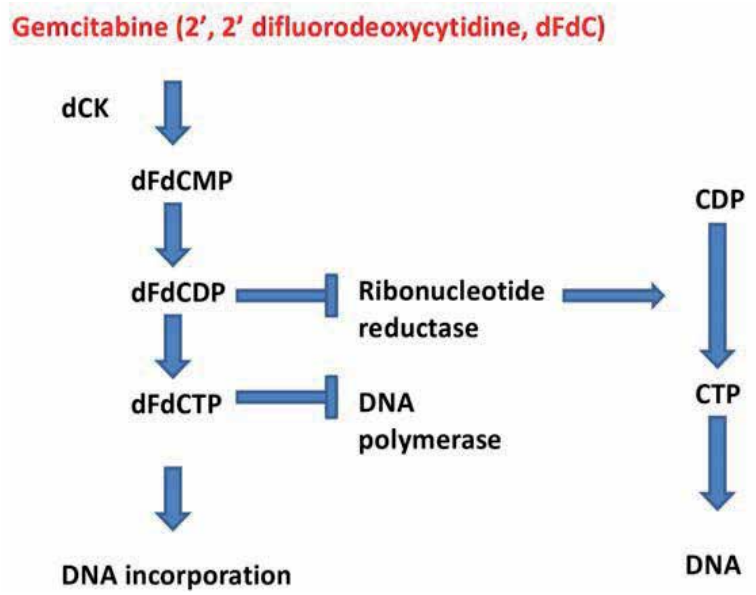
pancreatic cancer cells (*Eisold, et al., 2004; Kang and Saif, 2008; Zhang, et al., 2008; Zhao, et al., 2006b*). Epidermal growth factor receptor (EGFR) is a mitogenic receptor which has also been shown to provide cancer cells with proliferative and anti-apoptotic advantage (*Arteaga, 2001; Citri and Yarden, 2006*). EGFR is found to be upregulated in pancreatic cancer patients' tumors and the levels of EGFR correlate with aggressiveness and poor prognosis of the disease (*Yamanaka, et al., 1993*).

2. Pancreatic cancer chemotherapy

In spite of numerous efforts, the 5-year survival rate for pancreatic cancer has not improved much for last few decades. One reason contributing to this is the lack of chemotherapeutic agents which would effectively improve the survival of patients (*American Cancer Society, 2011; Moore, et al., 2003*).

2.1 Gemcitabine

Gemcitabine (2′, 2′- difluorodeoxycytidine) is a difluoro analog of deoxycytidine and is the first line chemotherapeutic agent used in the treatment of pancreatic cancer cells. In 1997, a randomized trial found gemcitabine to have better clinical benefit response of 23.8% to 4.8% when compared to 5-fluorouracil. In the same study, the median survival for gemcitabine treated patients was 5.65 months versus 4.41 months for 5FU treated subjects. Comparative 12-month survival was also increased in gemcitabine patients (18% to 2% for 5FU) (*Burris, et al., 1997*). Gemcitabine is used either alone or in combination with other agents in the treatment of pancreatic cancer.



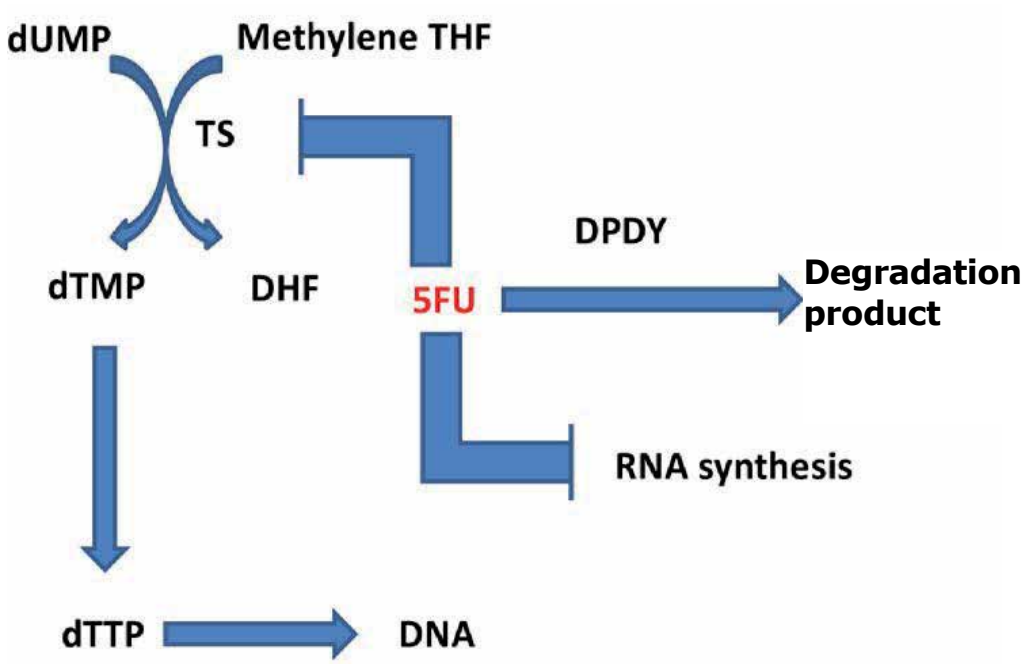
dCK: deoxycytidine kinase; dFdCMP: difluorodeoxycytidine monophosphate; dFdCDP: difluorodeoxycytidine diphosphate; dFdCTP: difluorodeoxycytidine triphosphate; CTP: cytidine triphosphate; CDP: cytidine diphosphate.

Fig. 1. Gemcitabine mechanism of action

Gemcitabine mainly acts by three mechanisms as shown in figure 1. First, it forms dFdCTP (di-fluorodeoxycytidine triphosphate) by the action of enzyme deoxycytidine kinase (dCK). dFdCTP competes with cytidine triphosphate (CTP) to get incorporated into the DNA. Secondly, its diphosphate metabolite (dFdCDP) inhibits ribonucleotide reductase, further preventing the formation of triphosphate nucleotide. Thirdly, triphosphate metabolite (dFdCTP) inhibits DNA polymerase which is important for DNA repair (*Huang, et al., 1991; Kang and Saif, 2008*). Gemcitabine enters the cell via human equillibrative nucleotide transporter 1 (hENT1) (*Mackey, et al., 1998*). Patients with detectable expression of hENT had significantly longer survival than patients with low levels or absence of this protein (*Spratlin, et al., 2004*).

2.2 5-Fluorouracil (5FU)

5FU belongs to the antimetabolite class of chemotherapeutic drug and is structurally similar to the uracil molecule with an additional fluorine atom at position 5. The drug 5FU gets mis-incorporated into DNA and RNA and also prevents nucleic acid synthesis by inhibiting the enzyme thymidylate synthase (TS). 5FU was the first drug to be approved as an adjuvant therapy for the treatment of pancreatic cancer. Combination of 5FU to radiation therapy increased the survival (10 months vs 6 months, no drug treatment) of pancreatic cancer patients with locally unresectable cancer (*Kalser and Ellenberg, 1985; Moertel, et al., 1981*).



dUMP: deoxyuridine monophosphate; THF: tetrahydrofolate; dTMP: deoxythymidine monophosphate; DHF: dihydrofolic acid; DPDY: dihyropyrimidine dehydrogenase; dTTP: deoxythymidine triphosphate; TS: thymidylate synthetase; 5FU: 5-fluorouracil.

Fig. 2. 5FU mechanism of action

Due to structural similarity, 5FU enters the cell using the same facilitator transporter as uracil (*Diasio and Harris, 1989; Santi, et al., 1974; Wohlhueter, et al., 1980*). Once inside the cell (Fig. 2), it forms various metabolites. Among these metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) are the active metabolites which disrupt RNA synthesis and inhibit TS. 5, 10-Methylenetetrahydrofolate (CH2THF) acts as the methyl donor for the conversion of dUMP to dTMP. 5FU binds to TS resulting in depletion of deoxythymidine triphosphate (dTTP) which further causes depletion in the levels of dATP, dCTP and dGTP. Imbalance in the ATP/dTTP ratio leads to disruption of DNA synthesis and repair (*Diasio and Harris, 1989; Howell, et al., 1981; JL, 1996; Santi, et al., 1974*).

3. Anticancer drug specific resistance

3.1 Gemcitabine resistance

Resistance to chemotherapeutic agents is observed commonly in mammalian tumors. Resistance can arise *de novo* or can be acquired after drug exposure. The vast genetic heterogeneity of cancer cells is considered to be the reason for the acquired resistance (*Casa, et al., 2008*). Cancer cells can acquire resistance against a drug through various mechanisms. It can prevent a drug's entry into the cell or increase its exit from the cell. Once the drug is inside the cell, it can be degraded into inactive metabolites by over-expression of the catabolic enzyme or by inhibiting the activity of the enzyme responsible for converting the pro-drug into an active agent (*Gottesman, 2002*). Drug resistance is one of the important factors responsible for low survival rate of pancreatic cancer patients. Numerous studies over the past two decades have suggested that pancreatic cancer is associated with various genetic alterations which contribute to its resistant characteristics (*Almoguera, et al., 1988; Feldmann, et al., 2007; Hruban and Fukushima, 2007*). Some of these mechanisms are listed in table 1:

Mechanisms	5-Fluoruracil (Santi, McHenry et al. 1974; Wohlhueter, McIvor et al. 1980; Howell, Mansfield et al. 1981; Diasio and Harris 1989; Lowe, Ruley et al. 1993; JL 1996; Ahnen, Feigl et al. 1998; Lenz, Hayashi et al. 1998; Bunz, Hwang et al. 1999; Eisold, Linnebacher et al. 2004; Zhang, Yin et al. 2008)	Gemcitabine (Bergman, Pinedo et al. 2002; Galmarini, Clarke et al. 2002; Garcia-Manteiga, Molina-Arcas et al. 2003; Yokoi and Fidler 2004; Nakano, Tanno et al. 2007)
MOA related	Thymidylate synthetase	Ribonucleotide reductase
Transporters	MRP 3 and 5	MDR1
Molecular	Akt, *src*	Akt, NF κB, *src*

Table 1. Suggested mechanisms for drug resistance in pancreatic cancer

Some of the markers of gemcitabine resistance include decreased expression of dCK, increased levels of competing dCTP, low levels of hENT, and alteration of PI3K/Akt/NFκB pathway, FAK, and hypoxia (Almoguera, et al., 1988; Feldmann, et al., 2007; Hruban and Fukushima, 2007)

3.2 5FU resistance

Resistance to 5FU induced cytotoxicity or cancer cells' response to 5FU treatment is controlled/characterized by various factors (*Kang and Saif, 2008; Zhang, et al., 2008*), as discussed below.

3.2.1 Thymidylate Synthase (TS)

TS controls several aspects of a tumor's response to 5FU therapy. Increase in the TS level can reduce the accumulation of activated metabolites of 5FU and hence reduce toxicity. Also, mutation of the enzyme can decrease 5FU's affinity to TS. TS overexpression is considered as a major mechanism responsible for 5FU resistance. Decreased levels of reduced folate substrate, 5,10-methyltetrahydrofolate, also reduces 5FU response.

3.2.2 Dihydropyrimidine dehydrogenase (DPDY)

Increase in the activity of DPDY can increase the catabolism of 5FU leading to its inactivation. Other studies have shown that low-DPDY tumors are more responsive to 5FU treatment.

3.2.3 Slow down of cell cycle

This mechanism can prevent the incorporation of 5FU metabolites in the cells and provide the cells with sufficient time to correct the misincorporated nucleotides.

3.2.4 Human Equilibrative Nucleoside Transporters (hENT)

These transporters are important for delivery of 5FU from the extra-cellular space into cells. Levels of hENT1 has been correlated with pancreatic cancer cell sensitivity to 5FU response and few studies have suggested that hENT plays an important role in 5FU resistance. hENT1 mRNA levels are suggested to be a useful marker to predict 5FU sensitivity (Kang and Saif, 2008; Zhang, et al., 2008)

3.2.5 Other factors causing 5FU resistance

Mutated p53

This tumor suppressor gene, which is mutated in 30-70 % of pancreatic cancer cases, can affect 5FU response in pancreatic cancer cases. Wild type p53 expression is required for 5FU-induced apoptosis and p53 status of tumor cells can determine the response to 5FU-based chemotherapy (*Ahnen, et al., 1998; Bunz, et al., 1999; Lenz, et al., 1998; Lowe, et al., 1993*). Another study found that transfection of pancreatic cancer cells with wild type p53 synergistically enhances the cytotoxicity of 5FU both *in vivo* and *in vitro*. The same study also showed that pancreatic cancer cell line with wild type p53 status was more sensitive to 5FU as compared to the p53 mutated line (*Eisold, et al., 2004*).

Mutated EGFR-Ras-MAPK cascade

This signaling pathway is important for growth, survival and proliferation of cells. The signaling cascade is found to be mutated at various levels in pancreatic cancer, leading to

over-activation of the pathway and hence increased growth and proliferation of the cancer cells (References). Protein expression of members of this pathway can modulate the cytotoxic effect of 5FU on pancreatic cancer cells. Blockade of EGFR increases the cytotoxicity of 5FU in both *in vivo* and *in vitro* conditions (*Overholser, et al., 2000*). Furthermore, activation of MAPK reduces the sensitivity of pancreatic cancer cells to 5FU treatment *in vitro* (*Wey, et al., 2005*). K-ras mutation is the hallmark of pancreatic cancer and occurs early during the development of pancreatic neoplasia. Ras-mutated pancreatic cells have shown to respond better to 5FU treatment as compared to their non-ras-transformed counterparts (*Hiwasa, et al., 1996*).

4. Relationship of resistance to cellular processes

The mechanisms involved in drug resistance in pancreatic cancer are different for different drugs. In a broad sense, drug resistance in pancreatic cancer can be linked to modulation of enzymes, receptors, DNA repair and other processes. These are discussed in depth in this section. For drugs to be effective, they should be transported successfully into the cells, reach their respective effective concentrations and should form their active forms before they are transported out of cells (*Longley, et al., 2003; Plunkett, et al., 1995*)

4.1 Enzyme linked mechanisms

The expression levels of the enzyme DCK determines the patient survival and the sensitivity of a tumor to gemcitabine (*Hagmann, et al., 2010a; Plunkett, et al., 1995*). HuR is a RNA-binding protein that modulates the translation of DCK mRNA and multiple other proto-oncogenic proteins in cancer cells (*Williams, et al., 2010*). Modulation of mRNA is dependent on stress conditions and includes the presence of therapeutic agents. Expression of HuR increases in pancreatic cancer cells treated with gemcitabine and an increased level of HuR in the cytoplasm is a marker of gemcitabine sensitivity (*Costantino, et al., 2009*). pp32 is a protein phosphatase and tumor suppressor gene that regulates the post- transcriptional activity of mRNA to which the HuR protein binds. Although the exact mechanism by which pp32 regulates HuR is yet to be unveiled, researchers have cited possible ways by which pp32 regulates the post-transcriptional changes of transcribed mRNA. The possibilities include: a) disrupting the interaction of HuR with mRNA in the nucleus, b) inhibiting translocation of the HuR-bound mRNA into cytosol and thereby inhibiting translation of oncogenic proteins, and other possible mechanisms (References). Overexpression of pp32 can result in inhibition of dCK mRNA translation and hence poor gemcitabine efficacy (*Williams, et al., 2010*).

Thymidylate synthetase (TS) controls a tumor's response to 5FU therapy. Increase in the TS level can reduce the accumulation of activated metabolites of 5FU and hence reduce toxicity. Additionally, mutation of the enzyme can decrease its affinity to TS. TS overexpression is considered as a major mechanism responsible for 5FU resistance. Decreased levels of reduced folate substrate, 5,10-methyltetrahydrofolate, also reduces 5FU response *(Zhang, et al., 2008)*.

Increase in the activity of dihydropyrimidine dehydrogenase (DPDY) can increase the catabolism of 5FU leading to its inactivation. Few studies have shown that low-DPDY tumors are more responsive to 5FU treatment.

5FU resistance can result from induction of TS levels upon administration of 5FU which brings about the activation of autoregulatory feedback pathway where the TS protein regulates the translation of its mRNA. The salvage pathway involving the enzyme thymidine kinase, the biochemical reaction in which thymidylate is derived from thymidine could be one of the ways the cells acquire resistance to 5FU. (*Zhang, et al., 2008*)

4.2 Receptor linked mechanisms

4.2.1 Drug uptake

Due to structural similarity to the nucleosides, a drug can enter the cells through uptake by the concentrative nucleoside transporters (CNT's) and equilibrative nucleoside transporters (ENT's). CNT1 and CNT3 have high affinity for gemcitabine whereas ENT1 and ENT2 have lower affinity for gemcitabine. Pancreatic tumor cells exhibit higher expression levels of ENT1 but low to negligible levels of CNT 3. This phenotype affects the transport of gemcitabine into the cancer cells and ultimately gemcitabine's action on DNA and RNA synthesis. The expression levels of ENT1 and CNT3 provide an index of patient survival after gemcitabine treatment (*Hagmann, et al., 2010b*).

In the case of 5FU, resistance was found to be imparted due to overexpression of ENT1. A study using 7 pancreatic cancer cell lines (AsPC1, BxPC3, MiaPaCa-2, PSN1, Panc1, PCI6, and KMP-4) reported an increased expression of ENT1 mRNA, which correlated with the IC_{50} of 5FU in the AsPC1 cell line, a cell line most resistant to 5FU among the cell lines tested *(Tsujie, et al.; 2007)*. Thymidylate synthase (a target of 5FU) and dihydropyrimidine dehydrogenase (DPDY), which metabolizes 5FU, were not overexpressed with simultaneous overexpression of ENT1: this phenotype implies that the toxicity of 5FU was countered by increased uptake of nucleosides and nucleotide bases through the salvage pathway (*Tsujie, et al., 2007*). It is therefore widely accepted that ENT1 overexpression serves as a marker of 5FU resistance (*Huber-Ruano and Pastor-Anglada, 2009; Tsujie, et al., 2007*).

4.2.2 Drug efflux

Drug efflux is one of the potential means by which cancer cells exhibit chemoresistance: it is mediated by a family of proteins, ATP binding cassette (ABC) proteins, which involve ATP utilization (*Wu, et al., 2008*). The human genome encodes 49 members of this protein family and about 15 proteins are implicated in cancer chemoresistance. These transporters have an intracellular nucleotide binding domain that hydrolyses ATP and results in conformational change in its structure leading to the transmembrane domain forming a channel-like structure through which the drug is effluxed to the extracellular space(*Santisteban, 2010*). P glycoprotein, multidrug resistance associated protein (MRP1) and breast cancer resistance protein (BCRP) constitute the universal drug efflux transporters (*Santisteban, 2010; Wu, et al., 2008*).

The efflux of gemcitabine and its triphosphate metabolite is mediated by multidrug resistant protein 5 (MRP5), which is a member of the ATP binding cassette (ABC) family of proteins. Evidences in support of the role of MRP3, MRP4 and MRP5 in the efflux of etoposide, 5FU and gemcitabine suggest that these MRP's are directly linked with the resistance phenotype (*Hagmann, et al., 2010a; Hagmann, et al., 2010b*). Transcriptional regulation of MRP's by nuclear factor like 2 protein (Nrf2) is an important target to overcome resistance because

overexpression of Nrf2 is associated with increased resistance of cells towards chemotherapy-induced cell death (*Hagmann, et al., 2010a*).

Evidences in support of overexpression of these transporters in cancer stem cells and failure of the classical concept of direct inhibition of transporters in resistant cells using the first generation inhibitors like verapamil and quinidine and second generation inhibitors like valspodar and biricodar have led researchers to focus on the pathways that may be involved (*Santisteban, 2010*).

4.3 Role of hedgehog pathway in resistance

Hedgehog signaling between the tumor cells and stromal cells brings about a desmoplastic reaction where the stromal fibroblasts secrete collagen in higher amounts and result in fibrosis of the surrounding stromal tissue. Hedgehog signaling also has a key role in promoting epithelial mesenchymal transition and the acquisition of mesenchymal phenotype is associated with over expression of ABC transporters in breast cancer and in pancreatic adenocarcinoma (*Santisteban, 2010*). Studies using KPC (Kras and p53 mutant) mice with PDAC (Pancreatic Ductal Adenocarcinoma) highlighted the role of hedgehog pathway in resistance aided by the desmoplastic reaction where the mean vascular density to the tumor tissue decreased and resulted in decreased delivery of gemcitabine to the tumor tissue (*Olive, et al., 2009*). Use of a pathway inhibitors like cyclopamine derivatives, that inhibit the protein smoothened, which is downstream of hedgehog signaling prevents transcriptional activation of target genes that bring about resistance by promoting overexpression of ABC transporters and by preventing desmoplasia (*Olive, et al., 2009; Santisteban, 2010*).

4.4 Role of MAPK in resistance

Three types of mitogen activated protein kinases (MAPK) have been identified in humans, including the extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases and the p38 MAP kinases and all these act by serine/threonine phosphorylation of target proteins (*Wagner and Nebreda, 2009*). Interestingly, ERK pathway activation promotes survival while activation of JNK and p38 MAPK pathways induce apoptotic cell death as they are activated under stress conditions (*Wagner and Nebreda, 2009; Zhao, et al., 2006a*). Involvement of MAPKinase pathways in acquired chemoresistance has been studied by researchers but a clear idea of the mechanisms involved has yet to be established. ERK pathway which is downstream of EGFR signaling, promotes cell survival through its pro-survival signals which may be responsible for chemoresistance. Employing the SW 1990 cell line, Zhao *et al* demonstrated that ERK signaling regulates chemoresistance depending on the chemotherapeutic agent. Resistant cell lines exhibit a higher level of ERK activity as compared to sensitive cell lines and that inhibition of ERK pathway resulted in 5FU sensitivity but increased GEM resistance. 5FU acts by activating intrinsic apoptotic pathway whereas GEM induces cell death by activating extrinsic apoptotic pathway (*Zhao, et al., 2006a*).

4.5 Role of PI3K/Akt pathway in resistance

As opposed to earlier notions that drug resistance arises by increased drug metabolism or efflux or decreased transport of the drugs into the cells, *Ng et al.*(2000), using PK1 and PK8 cell lines, demonstrated that the anti-apoptotic advantage of cells towards gemcitabine is

conferred not by the classical resistance mechanisms alone but also by the activation of PI3K/Akt pathway when the intracellular concentration of gemcitabine was found to be effective to affect DNA and cell cycle (*Ng, et al., 2000*). PI3K/Akt pathway activation stems from phosphorylation of receptor tyrosine kinases and the regulatory subunit of PI3K, the p85 interacts with the active tyrosine kinase domains for activation. Activation of PI3K results in phosphorylation of its substrates which includes phosphoinositides and protein kinase B (PKB), otherwise called Akt. Phosphorylation of Akt and its subsequent nuclear translocation results in transcriptional activation of genes that promote cell survival (*Hennessy, et al., 2005; Ng, et al., 2000*). Apart from this mechanism, the phosphorylated Akt is also shown to inactivate the pro-apoptotic protein BAD by phosphorylating it and thereby giving the anti-apoptotic advantage to the cells. In addition, the activation of PI3K can also occur by interaction of the catalytic subunit of PI3K (p110 subunit) with constitutively active membrane bound Ras (*Ng, et al., 2000*).

4.6 Role of Zeb-1 in resistance

Zeb1 is a transcriptional suppressor of E-cadherin which is involved in cell-cell adhesion and is the marker of epithelial cells. By doing so, it promotes epithelial-mesenchymal transition (EMT) upon which the cells metastasize and form secondary tumors (*Wellner, et al., 2010*). Epithelial mesenchymal transition is a process by which the tumor cells with epithelial lineage origin acquire the mesenchymal phenotype. This process gives them an advantage of migrating from the primary tumor site into the blood stream and develop secondary tumors at various sites depending on the availability of suitable microenvironment. The whole process is called metastasis which is dependent on EMT. During EMT, the epithelial cells lose epithelial cell surface markers, express mesenchymal markers and undergo cytoskeletal remodeling in which the cell polarity, a characteristic of the epithelial cells, is lost and the cells acquire an invasive phenotype. Downregulation of E-cadherin and upregulation of mesenchymal markers vimentin, smooth muscle actin, gamma-actin, beta-filamin, talina and extracellular matrix components like fibronectin and collagen precursors are key features of EMT (*Christiansen and Rajasekaran, 2006; Kalluri and Weinberg, 2009*).

Recent studies on the role of Zeb-1 by *Arumugam et al.* (2009) confirmed the role of Zeb-1 not only in promoting metastasis but also in drug resistance. This group found that the cancer cell lines which are sensitive to gemcitabine (L3.6pl, BxPC3, CFPAC, SU86.86) are more sensitive to 5FU and cisplatin as compared to the gemcitabine-resistant cancer cell lines (PANC-1, Hs766T, AsPC-1, MIAPaCa-2, MPanc96). When Zeb-1 activity was silenced using siRNA in PANC1, MIAPaCA-2 and Hs766T cell lines, there was increased apoptosis in these cell lines on treatment with gemcitabine, 5FU or cisplatin, separately. This finding suggests an important role for Zeb-1 in drug resistance. Erlotinib is an EGFR inhibitor that is used in combination with gemcitabine to treat pancreatic cancer. Resistance to EGFR inhibition is a hallmark of EMT which can be reverted by silencing the activity of Zeb-1, which in turn increases sensitivity of the cells to EGFR inhibition (*Arumugam, et al., 2009*).

4.7 Role of NFκB in inducing gemcitabine resistance

Nuclear factor kappa light chain enhancer of activated B-cells (NFκB) is a complex involved in important cellular processes like inflammation, apoptosis regulation and stress

adaptation. In the cell, it is present tightly bound to inhibitory proteins like IkBa and is released in response to stimuli that bring about activation of IkB kinase (IKK). The free cytosolic NFκB then translocates into the nucleus where it regulates gene expression. The role of NFκB in gemcitabine resistance has been demonstrated. *Arlt et al.* (2003), using gemcitabine resistant (BxPC3 and Capan1) and gemcitabine sensitive (T3M4 and PT45-P1) cell lines, found that the autocrine loops for generation of NFκB play an important role in gemcitabine resistance. Employing NFκB inhibitors (MG132 or sulfasalazine), they found basal NFκB levels confer resistance and the basal NFκB levels are not affected by the activated or inactive state of PI3K/Akt pathway (*Arlt, et al., 2003*).

4.8 Role of notch signaling in chemoresistance

Notch signaling is a developmental pathway which is implicated in organogenesis, development of nervous and vascular systems and hematopoietic stem cell generation in adults (*Chiba, 2007*). Notch signaling is important for self-renewal of stem cell and along with Wnt signaling, it prevents terminal differentiation of cells (*Katoh, 2007*). Overactivity of Notch signaling is observed in various hematopoietic and solid tumors leading to proliferation, and inhibition of differentiation and apoptosis. In pancreatic cancer cells, Notch signaling is implicated in drug resistance. Wang et al. found that components of Notch signaling pathway are upregulated in gemcitabine resistant pancreatic cancer cells and are associated with increase invasiveness (*Bao, et al., 2011b; Wang, et al., 2009*). Yao and Qian (2010) observed that inhibition of Notch3 by the siRNA approach increases gemcitabine-induced cytotoxicity in pancreatic cancer cell via affecting the PI3K/Akt pathway. The studies mentioned above strongly suggest that cancer stem cell signaling pathways could be attractive targets for increasing their sensitivity to chemotherapeutic agents (*Bao, et al., 2011b*).

4.9 Miscellaneous other mechanisms

A role of for the glycoprotein, mucin MUC4, in pancreatic cancer cell resistance to the first line chemotherapeutic agent, gemcitabine has recently emerged. Mucin MUC4 is overexpressed on the membrane of pancreatic cancer cells but not normal pancreatic cells (*Santisteban, 2010*). Studies on this glycoprotein's involvement in gemcitabine resistance have revealed the interaction of this glycoprotein with the HER2 receptor and the subsequent activation of ERK pathway and phosphorylation of the pro-apoptotic protein *BAD* which inhibits apoptosis induced by gemcitabine (*Ponnusamy, et al., 2008; Santisteban, 2010*).

5. Overall strategies to overcome resistance

5.1 miRNA

miRNA (miR) are 18-24 nucleotide-long RNA molecules which can regulate the translation of mature RNA into protein. They are synthesized as a 60-80 nucleotide-long, hairpin-shaped RNA molecule which is transported to the cytoplasm where it undergoes processing to form 18-24 nucleotide-long double stranded RNA molecule. One of the strands then interacts with RNA-induced silenced complex (RISC) and targets RNA translation. Various miRNAs have been demonstrated to play a role in development and cancer progression. Dysregulation of miRNAs has also been observed in pancreatic cancer tissues and cell lines. Bloomston *et al*. (2007) found that the levels of miRNAs can be used to differentiate between

pancreatic tumor, chronic pancreatitis and benign pancreatic tissue. They also found that the expression of six miRNAs can predict the survival of the pancreatic cancer patients (*Bloomston, et al., 2007*). Zhang et al. (2009) profiled the levels of 95 miRNAs in pancreatic tumors, pancreatic cancer cell lines, pancreatic tissues and pancreatic ductal epithelial cells. They found that the expression of 8 miRNAs were significantly upregulated in pancreatic cancer tissues and cancer cell lines compared to the pancreatic tissues and pancreatic ductal cells (*Zhang, et al., 2009*).

Deregulated miRNA levels could serve as attractive targets for treatment of pancreatic cancer. Moriyama et al. (2009) found that the level of miR21 is upregulated in pancreatic cancer cells and its inhibition decreases proliferation, invasion, chemoresistance and induces cell cycle arrest and apoptosis in pancreatic cancer cells. On the other hand, Banerjee et al., (2007) found that the expression of miRNAs can be modulated by natural compounds which reduce EMT and chemoresistance in pancreatic cancer cells. These studies strongly suggest that deregulated levels of miRNA in pancreatic cancer can be exploited as putative therapeutic targets for overcoming pancreatic cancer drug resistance.

5.2 Stem cell signaling

Some studies have suggested that a tumor comprises of heterogeneous populations of cells rather than just homogenous cell types. One subset is suggested to be distinct cells that have limited proliferative capacity but are responsible for initiation, progression and differentiation of cancer cells. Due to their ability to self-renew and differentiate like stem cells, these cells are termed as "cancer stem cells." There have been attempts with some successes to isolate the cancer stem cells so that they can be employed to elucidate genotypic and phenotypic characteristics as well as develop effective therapies to target to them because of their ability to self-renew and their resistance to conventional chemo- and/or radiation therapies.

Li et al. (*2007*) isolated pancreatic cancer cells which were highly tumorigenic and had the ability to self-renew based on their cell surface markers. These cells (CD44*CD24* ESA*) comprises of 0.2-0.8% of all pancreatic cancer cells and were able to produce differentiated progeny. Similarly, Herman et al. (2007) isolated pancreatic cancer stem cells which were CD133+.

The cancer stem cells are known to be resistant to conventional chemo-radiation therapies. Michor et al. showed that a subpopulation of chronic myeloid leukemia stem cells were resistant to imatinib (*Michor, et al., 2005*). In glioblastoma, enrichment of CD133+ cells was observed after treatment with ionizing radiation. These cells activate DNA damage response upon irradiation and therefore are resistant to ionizing radiation (*Bao, et al., 2006*). Similarly, enrichment of cells with stem cell characteristics is observed on treatment of pancreatic cancer with radio- or chemotherapy (*Hermann, et al., 2007; Li, et al., 2007*).

5.3 Natural compounds for reversing resistance

Dietary habit of individuals has been correlated with development of pancreatic cancer. High cholesterol diet increases the risk of pancreatic cancer (*Baghurst, et al., 1991; Ghadirian, et al., 1991; Howe, et al., 1992; Stolzenberg-Solomon, et al., 2002*). Diet rich in fruits and vegetables is associated with reduced risk while intake of red meat is associated with

increased risk of developing pancreatic cancer (*Boyle, et al., 1989; Inoue, et al., 2003; Ohba, et al., 1996; Tavani, et al., 2000*).

Various natural compounds have been tested for their anti-pancreatic cancer properties in laboratory settings. Kunnumakkara et al. (2001) showed that curcumin inhibits NFκB activation and increases the cytotoxicity of gemcitabine *in vitro* and *in vivo*. That study also found that curcumin decreases the microvascular density thereby decreasing angiogenesis (*Kunnumakkara, et al., 2007*). NFκB is involved in mediating resistance against gemcitabine and TRAIL, which may explain the increase in cytotoxicity. Curcumin also increased the accumulation of MRP5 substrate intracellularly in MRP5 positive cells; however, in the absence of MRP5, drug accumulation was not observed. Additionally, curcumin increased the cell's sensitivity to 5FU (*Li, et al., 2010*). Furthermore, hydroethanolic extract of curcumin (Tumeric Force) was more effective than curcumin in this effect.

Another group of natural compounds which are currently being investigated are isoflavones. One of the most well studied isoflavones, genistein has been tested extensively in pancreatic cancer. Various groups have shown that treatment with genistein controls proliferation, mitogenic signaling, invasion, migration and induces apoptosis in pancreatic cancer. Benerjee et al (2007) showed that genistein augments *in vitro* and *in vivo* efficacy of cisplatin in pancreatic cancer (*Banerjee, et al., 2007*). Previously, genistein was known to affect the activation of NFκB via the Akt pathway(*El-Rayes, et al., 2006*), which have been shown to be involved in pancreatic cancer drug resistance. Natural compounds (e.g., genistein, curcumin) have also been shown to inhibit the hedgehog and Notch signaling which provide the cancer cells with stem-cell like property of self-renewal and resistance (*Slusarz, et al., 2010*). A recent study noted upregulation of FOXM1, increased EMT, and cancer stem cell phenotype in pancreatic cancer (*Bao, et al., 2011a*). Treatment with natural compounds also reduced the levels of FOXM1 in pancreatic cancers.

Apart from the above mentioned approaches and based on the literature cited in this chapter, there are some other strategies to overcome resistance including potential targets and areas for drug discovery like developing agents that regulate Nrf2 activity selectively in tumor cells, agents that inhibit interaction between mucin MUC4 and HER2, selective inhibition of hedge hog pathway in the tumor cells, selective inhibition of PI3K/Akt pathway in tumor cells, combination of anti-NFkB agents with gemcitabine and Zeb1 silencing. Targeting sphingolipid metabolism is another approach to overcome resistance to gemcitabine (*Guillermet-Guibert, et al., 2009*). Recent renewed interests in the metabolic phenotypes of pancreatic and other cancers have raised possibilities of metabolic pathway(s) as drug targets for new anti-cancer drug discovery.

6. Conclusions

Although these strategies to overcome resistance to drugs are crucial for improving the outcome of chemotherapy, there is an urgent need to achieve early detection of pancreatic cancer. Finding novel biomarkers for detecting pancreatic cancer should be emphasized. Recent renewed interests in the metabolic phenotypes of pancreatic and other cancers have raised possibilities of metabolic pathway(s) as targets for strategies for developing agents for early tumor detection to addition to exploiting them as targets for new anti-cancer drug discovery.

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9

Prevention of Pancreatic Cancer

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1. Introduction

It is an intriguing problem whether human cells and/or bodies have the physiological function which supervises and selects survival and death fate in response to various kinds of stressors such as radiation. For example, what are the physiological effects of X-ray irradiation for examination of breast conditions including the risk of genetic mutation and cancer occurrence (Fig. 1A).

Based on a protease-activation signal in *E.Coli* SOS response we have attempted to search for human SOS response, particularly signals which protect stability of genetic information (Fig.1B) by estimation of plasminogen activator-like protease (PA) activity in peripheral blood-derived lymphocytes and cultured human RSa cells with hyper-mutability and their variant hypo-mutable cells (Fig.1C) (Suzuki *et al.,* 2005). Levels of PA activity in lymphocytes are changed after X-ray irradiation for breast examination and after exposure *in vitro* to chemical such as bisphenol A (Takahashi *et al.,* 2000). The activity in variant cells was found to be associated with heat shock protein (HSP) 27 expression, resulting in enhancement of error-free DNA repair function (Wano *et al.,* 2004). A HSP27-bound protein, annexin II, was also suggested to play some roles on the error-free function in human cell nuclei (Tong *et al.,* 2008 and Jin *et al.,* 2009). We recently developed a method to analyze base substitution mutation of the *K-ras* codon 12 and found that a decreased mutation frequency accompanied increased GRP78 expression in human RSa cells (Hirano *et al.,* 2008 and Zhai *et al.,* 2005). The repair function seems to suppress base substitution mutation of *K-ras* codon 12. The base substitution mutation was also found to be regulated by extracellular factors, human interferon and serum factors from cancer patients and stressors-exposed persons (Suzuki *et al.,* 2005, Hirano *et al.,* 2008 and Chi *et al.,* 2007). This regulation seems to be mediated by PA activation and the following chaperones expression (Suzuki *et al.,* 2005, Isogai *et al.,* 1994, Takahashi *et al.,* 2003 and Kita *et al.,* 2009). Thus, studies on molecular mechanisms to supervise cellular mutability, including frequency of Ras gene mutation, are important for discussion about relationships with a network of proteases and chaperones and/or cytokines.

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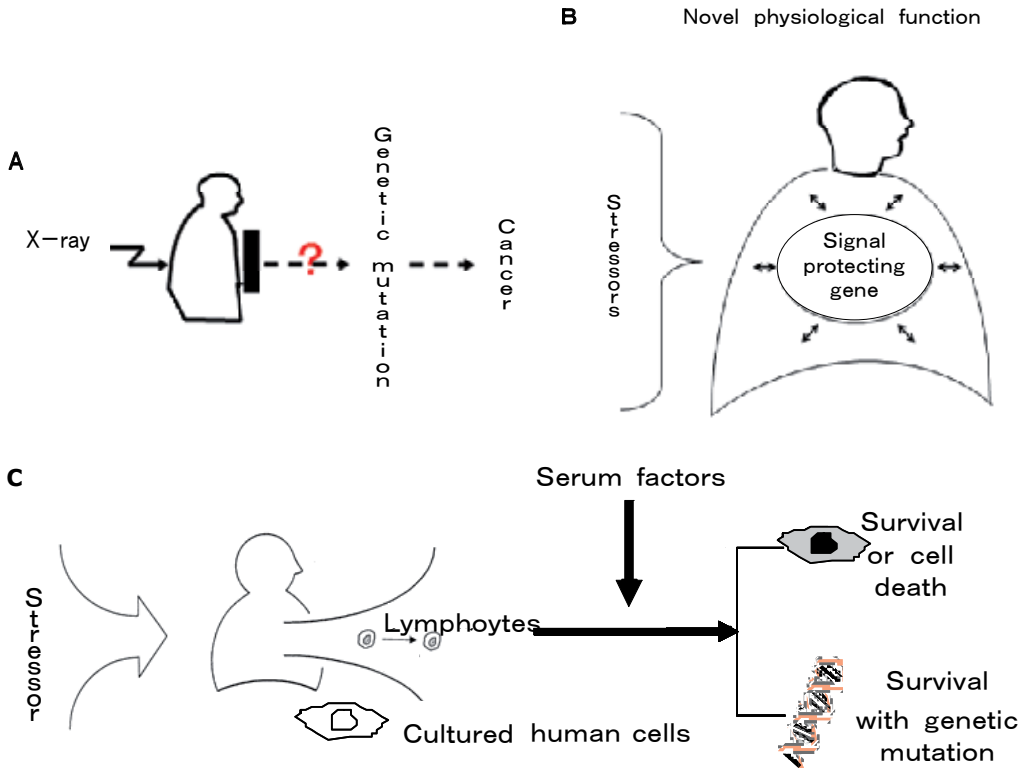

Fig. 1. Strategy of the method to search for human SOS response.
(A) A question concerning X-ray effects on genetic mutation and the following cancer. (B) A physiological function which supervises protection mechanisms for genetic information stability. (C) Modulation of cellular fate by serum factors and plasminogen activator-like protease (PA) activation, elucidated by the observation of peripheral blood lymphocytes and cultured human cells after stressors exposure.

Incidence of K-ras gene mutation is high in pancreatic carcinomas, and therefore suppression of the mutation could be beneficial for inhibiting pancreatic cancer occurrence (Hirano *et al.*, 2008). We were intrigued by the possibility that this carcinogen-induced mutation could be suppressed, via modulation of GRP78 expression, by agents such as foods.

In the present chapter, cellular levels of GRP78 in RSa cells that had been cultured with aqueous extracts of Japanese miso and the unfermented ingredients of miso are shown using immunoblotting analysis. The mutability of the treated cells are also evaluated after ultraviolet light C (UVC)(principally 254 nm in wave length) irradiation using the differential dot-blot hybridization test for *K-ras* codon 12 mutation. In previous reports, dietary supplementation with long-term fermented miso has been shown to act as a chemopreventive agent against gastric and colon carcinogenesis in rats (Ohara *et al.*, 2002a, 2002b, and Ohuchi *et al.*, 2005). Miso is a fermented food that has formed an important part of the Japanese diet for over 1300 years (Yoshikawa *et al.*, 1998). It is prepared by the microbial fermentation of a mixture of raw materials (soybean, wheat, barley and rice) over

a long period until the ripe miso is obtained (Hesseltine & Shibasaki,1961). Little is known about the ability of miso to modulate the mutability of human cells.

2. Human SOS response and suppression of *K-ras* mutation by Japanese miso possibly via GRP78 expression in human RSa cells

2.1 Effect of miso samples on RSa cell viability

2.1.1 Preparation of miso samples

The Japanese Enbunhikae miso (EM) was purchased from Marui Co., Ltd. (Chino, Japan). Rice-koji, a raw material used in the preparation of miso, was obtained from Hanamaruki Co., Ltd (Nagano, Japan). Aqueous extracts of miso and rice-koji were prepared as follows: each (10 g) was suspended in 20 ml of MilliQ water, and the suspension was heated at 90 °C for 5 minutes and then at 70 °C for 10 minutes. The suspension was centrifuged at 1,780 x g and 4 °C for 10 minutes and the supernatant was then filtered through a 0.22 μm membrane. The dose of sample used in each treatment is shown as a percentage of volume per volume (v/v); 1% is equivalent to 5 mg of miso or rice-koji per ml.

2.1.2 Culture conditions and UVC irradiation

RSa cells were established from human embryo-derived fibroblast cells by double infection with the Simian virus 40 and the Rous sarcoma virus. The cells were confirmed to have high UV sensitivity and low DNA-repair activity (Kuwata *et al.,* 1976, Suzuki & Fuse, 1981, Suzuki,1984). Cells were cultured in Eagle's minimal essential medium (EMEM) (Nissui, Tokyo, Japan) containing 10% calf serum (CS) (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. UVC was generated by a 6 W National germicidal lamp (Panasonic, Osaka, Japan). The intensity of UVC was 1 J/m^2/s, as measured by a UV radiometer, UVR-254 (Tokyo Kogaku Kikai, Tokyo, Japan). Irradiation of the cells with UVC was performed as described previously (Suzuki & Fuse, 1981) and mock irradiation of cells was carried out in the same manner but without UVC irradiation.

2.1.3 Colony survival assay

To determine the optimal concentration of miso sample for use in the culture medium in cell mutability tests, the colony survival capacity of RSa cells cultured with or without EM extracts was examined (Fig. 2). The survival capacity of cells treated with or without miso extracts was measured using a colony survival assay as reported previously (Suzuki *et al.,* 1984). Logarithmically growing cells were seeded in 100 mm dishes (800 cells/dish), incubated for 20 h to allow the cells to attach, and then treated with or without miso extracts. One h after treatment, the cells were grown in fresh medium containing 5% CS in 100 mm dishes for about 14 d and were then stained with 0.2% methylene blue in 30% methanol. The results of the colony survival assay are expressed as percentages of the colony numbers observed for miso extracts-treated cells relative to those of untreated cells. Colony survival rates were over 85% when a miso extract concentration of 1% was used, but at concentrations higher than 1% a decrease of more than 10% in survival rate was observed (Fig. 2). An MTT assay showed that miso extract concentrations of less than 1% were not cytotoxic after 48 h of culture (data not shown).

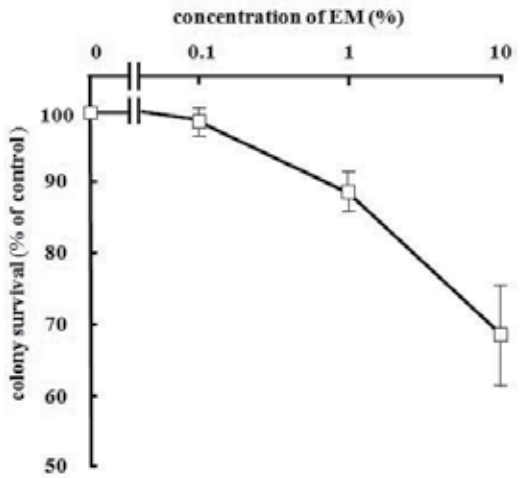

Fig. 2. Effect of EM extracts on cell survival.
RSa cells were treated with or without the indicated concentrations of EM extracts for 1 h. After treatment, the cells were cultured with fresh medium containing 5% CS in 100 mm dishes for 14 d and were then stained with 0.2% methylene blue in 30% methanol. Data are the means ±SD for three experiments.

2.2 Effect of miso extracts on GRP78 expression and the mutability of RSa cells

2.2.1 GRP78 expression

GRP78 expression was analyzed by immunoblotting as described previously (Wano *et al.*, 2004). Cells were lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% NP-40 and protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, 0.05 mM antipain and 0.05 mM pepstain A. The cell lysates were centrifuged at 15,000 × g and 4 °C for 20 min, and the supernatant was then treated with SDS sample buffer. Detection of the GRP78 protein was performed using mouse monoclonal anti-GRP78 (1:2000 dilution; SPA-827; StressGen, Victoria, Canada) antibodies. β-Actin was also analyzed using mouse monoclonal anti-β-actin antibodies (1:30000 dilution; ab40864; Abcam, Cambridge, UK) as a loading control. The antigen-antibody complexes were detected by horseradish peroxidase (HRP) conjugated anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK) following the ECL system (GE Healthcare, Buckinghamshire, UK). The GRP78 protein was quantified using Multi Gauge Ver2.2 image analysis software (Fuji Foto film, Tokyo, Japan) and expressed relative to the quantity of β-actin measured. When RSa cells were cultured with EM extracts at a concentration of 1%, expression of GRP78 was enhanced by over 1.5 fold compared with the expression observed in mock-treated cells (Fig 3A).

2.2.2 Mutability

Mutations in *K-ras* codon 12 were detected according to a method described previously (Suzuki N & Suzuki H., 1993). Logarithmically growing cells were inoculated at near confluency (5×10^5 cells/100 mm dish) to avoid cell selection by the lethal effects of UVC irradiation, as described elsewhere (Suzuki N & Suzuki H., 1995). Six d after UVC

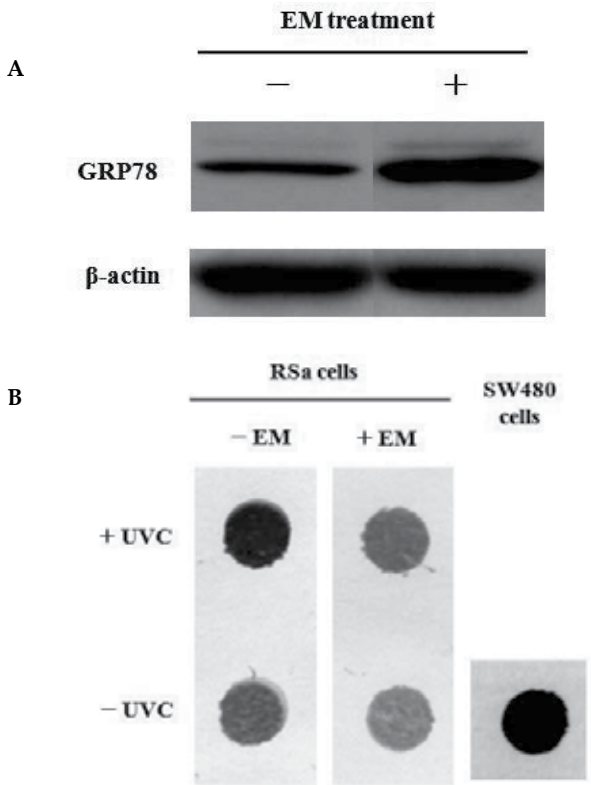

Fig. 3. Effect of EM extracts on GRP78 expression (A) and the mutability (B) of RSa cells. Cells were cultured with or without EM extracts at a concentration of 1% for 24 h. (A) Cell lysates were prepared after miso treatment and subjected to immunoblotting analysis of GRP78 and β-actin proteins. (B) After miso treatment, cells were irradiated with UVC or left unirradiated as controls (6 J/m^2). Six d after UVC irradiation, genomic DNA was extracted, and the *K-ras* codon 12 mutation was detected by PCR and differential dot-blot hybridization, as described in Materials and Methods.

or mock irradiation, genomic DNA was extracted using a standard proteinase K/SDS/phenol/chloroform procedure. DNA of SW480 cells carrying the *K-ras* mutation at codon 12 was used as a positive control for genomic DNA. Target sequences of sample DNA were amplified *in vitro* by PCR using the primers 5′-GACTGAATATAAACTTGTGG-3′ and 5′-CTATTGTTGGATCATATTCG-3′, and the amplified DNA (0.25 μg) was dot-blotted onto nylon membranes. After hybridization with digoxigenin-11-dUTP-3′ end-labeled *K-ras* codon 12 probes, the membranes were reacted with alkaline phosphatase conjugated polyclonal sheep anti-Dig Fab (Boehringer Ingelheim, Mannheim, Germany) and colored with the nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution (Boehringer Ingelheim, Mannheim, Germany). As a control probe, the oligonucleotide 5′-GTTGGAGCTGGTGGCGTAGG-3′ was used, and a mixed mutant probe, containing the following oligonucleotides mixed at the same concentration ratios, was used: 5′-GTTGGAGCTAGTGGCGTAGG-3′, 5′-GTTGGAGCTCGTGGCGTAGG-3′, 5′-GTTGGAGCTTGTGGCGTAGG-3′, 5′-GTTGGAGCTGATGGCGTAGG-3′, 5′-

GTTGGAGCTGCTGGCGTAGG-3′, 5′-GTTGGAGCTGTTGGCGTAGG-3′. Photographs were taken as permanent records of the results. To determine whether miso extracts suppress the mutability of RSa cells, the *K-ras* codon 12 mutation assay was performed. A black dot, indicating base substitution mutation, was detected after hybridization of the PCR products from genomic DNA of SW480 cells containing the *K-ras* codon 12 mutation, and this was used as a positive control (Fig. 3B). Under the assay conditions, the intensity of the black dot was clearly enhanced after UVC irradiation in mock-treated RSa cells (Fig. 3B). However, EM extracts-treated RSa cells did not show black dot signals either after UVC or mock irradiation (Fig. 3B).

2.3 Effect of GRP78 siRNA on the modulation of UVC cell mutability by miso extracts

To further examine whether GRP78 expression levels are causally related to the miso treatment modulation of RSa cells mutability, its expression was inhibited by GRP78 siRNA transfection. Duplex small interfering RNA (siRNA) with Stealth modification against human GRP78 (GRP78 siRNA) was synthesized based on the protein's nucleotide sequence (Invitrogen), as described previously (Suzuki T *et al.*, 2007). The sequence of the duplex was

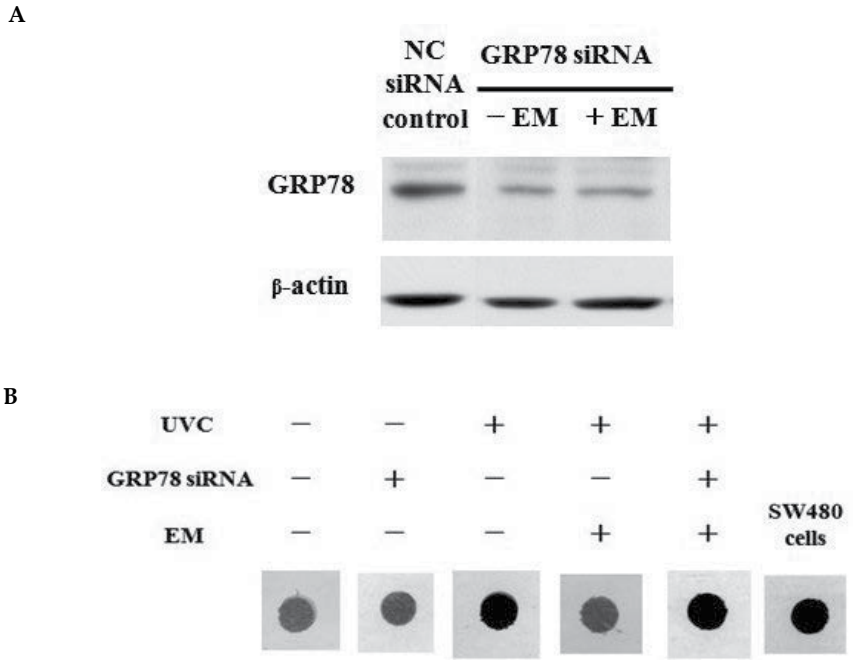

Fig. 4. Effect of GRP78 siRNA transfection on UVC-induced mutagenicity.
(A) After GRP78 or NC siRNA transfection, cell lysates were separated by SDS–PAGE and analyzed by immunoblotting analysis using anti-GRP78 and anti-β-actin (loading control) antibodies. (B) After siRNA transfection, cells were treated with or without EM extracts for 24 h and then irradiated with UVC (6 J/m^2). Mutability of RSa cells was determined by the *K-ras* codon 12 mutation assay using PCR and differential dot-blot hybridization, as described in Materials and Methods.

as follows: 5′-UAC CCU UGU CUU CAG CUG UCA CUC G-3′ / 3′-AUG GGA ACA GAA GUC GAC AGU GAG C-5′. Stealth RNAi negative control duplex (NC siRNA), with a GC content similar to that of the above Stealth RNAi, was used as a negative control. The NC siRNA was designed to minimize sequence homology to any known vertebrate transcript and for use in RNA interference (RNAi) experiments as a control for sequence independent effects following Stealth RNAi delivery in any vertebrate cell line. Treatment of cells with siRNA was carried out as described previously (Harborth *et al.*, 2001), with minor modifications. The siRNAs (128 nM) were transfected for 5 h into RSa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were detached from the test dish 48 h after transfection and used for each experiment.

In GRP78 siRNA-transfected cells, cellular levels of GRP78 protein were decreased in EM extracts-treated cells as well as in mock-treated cells, while no decrease was observed in NC siRNA-transfected cells (Fig. 4A). The decrease observed was up to 50% of the NC siRNA control (Fig. 4A). In RSa cells with GRP78 siRNA transfection that had been treated with miso extracts, the dot signal of *K-ras* codon 12 mutation was enhanced after UVC irradiation (Fig. 4B).

2.4 Effect of miso components on cell mutability

The effect of rice-koji, a component of miso, on the mutability of RSa cells was examined. The survival rate, measured by a colony survival assay (Fig. 5) and an MTT assay (data not shown), of cells treated with rice-koji at concentrations of less than 10% were more than 80%. On the basis of these results, we used 1% rice-koji in subsequent experiments to allow direct comparison with experiments using EM extracts.

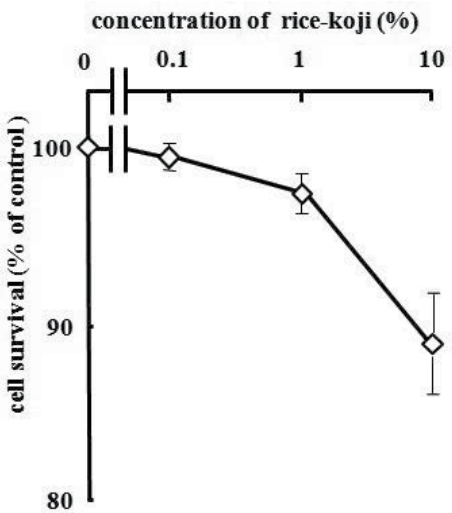

Fig. 5. Effect of rice-koji on cell survival.
RSa cells were treated at the indicated concentrations with rice-koji. After rice-koji treatment (1h) cells were grown in fresh medium containing 5% CS in 100 mm dishes for about 14 d, and then stained with 0.2% methylene blue in 30% methanol. Data are the means ±SD for three experiments.

Expression levels of GRP78 in rice-koji-treated RSa cells were slightly increased, at about 1.5 fold of the untreated cells (Fig. 6A). Rice-koji-treated cells showed no detectable black dot signal in the *K-ras* codon 12 mutation test with or without UVC irradiation treatment (Fig. 6B).

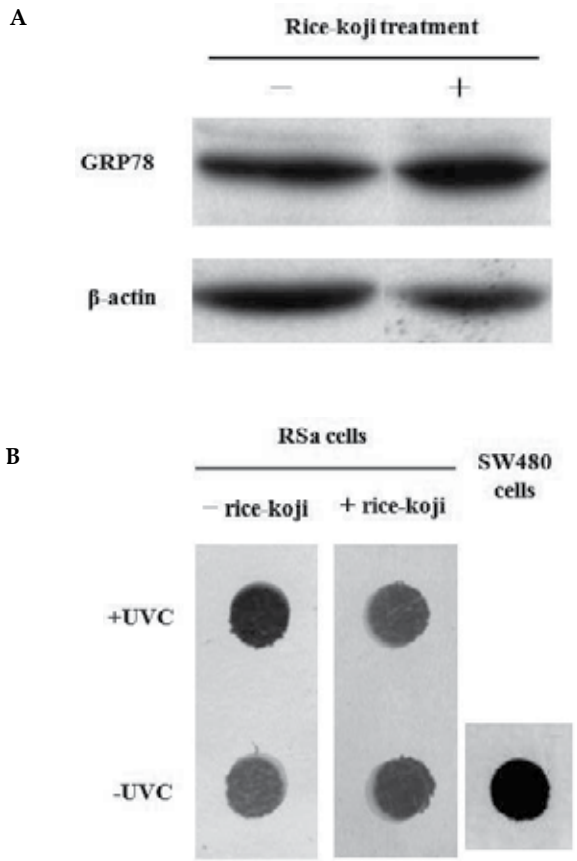

Fig. 6. Effect of rice-koji on GRP78 expression and mutability.
(A) Cells were treated with rice-koji (1%) for 24 h, and the cell lysates were subjected to immunoblotting analysis of GRP78 and β-actin proteins. (B) After rice-koji treatment, cells were irradiated with UVC (6 J/m^2). Six d after UVC irradiation genomic DNA was extracted, and the *K-ras* codon 12 mutation was detected using PCR and differential dot-blot hybridization.

We also examined whether GRP78 siRNA transfection affected UVC mutagenicity in rice-koji-treated RSa cells. In GRP78 siRNA-transfected cells, GRP78 expression levels decreased to about 40% of those in NC siRNA-transfected cells (Fig. 7A). Cells transfected with GRP78 siRNA and then treated with rice-koji, showed black dot signals indicating UVC-induced *K-ras* codon 12 mutation, similar to the signal observed in cells treated with UVC irradiation alone, and in SW480 cells (Fig. 7B).

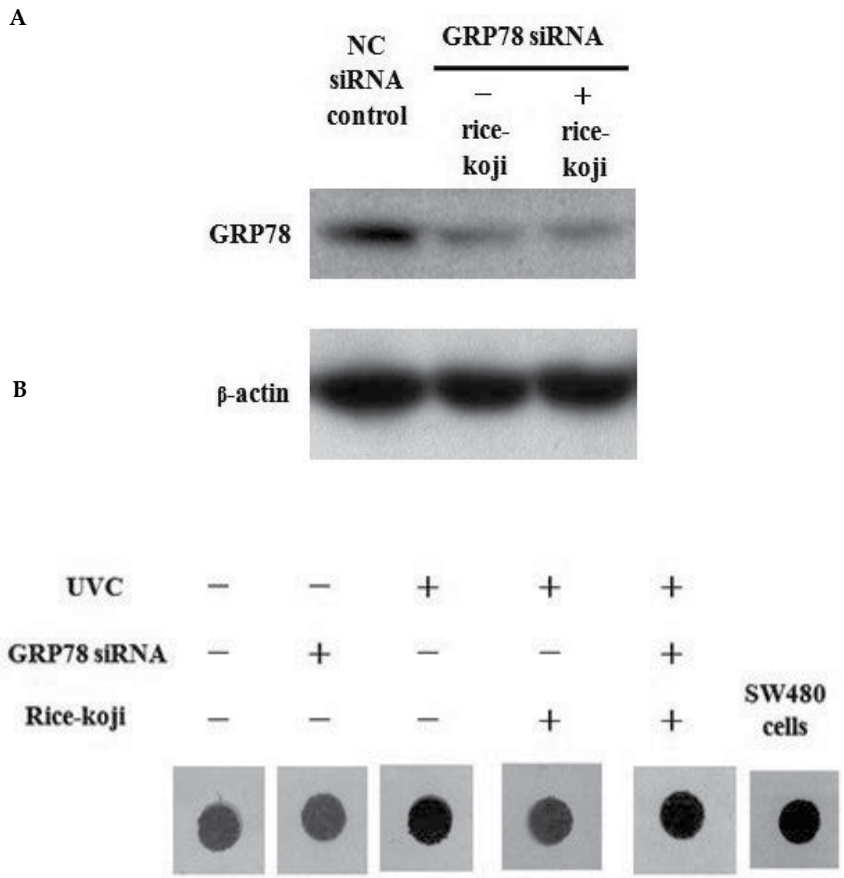

Fig. 7. Effect of GRP78 siRNA transfection on UVC-induced mutagenicity in rice-koji treated cells.
(A) After GRP78 or NC siRNA transfection, cell lysates were separated by SDS–PAGE and analyzed by immunoblotting analysis using anti-GRP78 and anti-β-actin (loading control) antibodies. (B) After siRNA transfection, cells were treated with or without rice-koji extracts for 24 h and then irradiated with UVC (6 J/m^2). Mutability of RSa cells was determined by the *K-ras* codon 12 mutation assay using PCR and differential dot-blot hybridization.

3. Conclusion

The *K-ras* base substitution mutation is thought to be a cancer-causing DNA sequence change in human cells, and its incidence is particularly high in pancreatic carcinomas. We previously found that proteases are activated in stress-exposed cells and/or human bodies, leading to modulation of chaperone expression and cellular mutability (so-called SOS response). Increased levels of glucose-regulated protein 78 (GRP78) expression are associated with the suppression of the *K-ras* mutation in human RSa cells irradiated with ultraviolet C UVC. RSa cells are hyper-mutable and are used to examine the modulation

of cell mutability using various agents. Here, we describe investigations into the effect of RSa cell treatment with Japanese miso on GRP78 expression and the suppression of UVC mutagenicity. Aqueous extracts of miso and its components were tested. Miso treatment was found to increase GRP78 levels, as estimated by immunoblotting analysis, and to decrease UVC-induced *K-ras* codon 12 base substitution mutation frequency. Increases in GRP78 expression and decreases in mutation frequency were not observed in cells whose GRP78 levels had been down-regulated using GRP78 siRNA transfection. This suggests that miso extracts suppress UVC mutagenicity by increasing GRP78 expression in human cells.

In the present study Japanese miso was tested concerning the modulation activity of genetic mutation, *K-ras* codon 12 base substitution mutation, in addition of human cytokines and serum factors. Several dietary factors have been postulated to act as risk factors for human carcinogenesis (Sugimura, 2000 and Mirvish, 1983). In Japan, intensive studies of the causal relationship between diet and cancer incidence have focused on stomach, lung and colorectal cancers (Masaki *et al.,* 2003, Ngoan *et al.,* 2002, Takezaki *et al.,* 2001, Ozasa *et al.,* 2001, Tajima & Tominaga, 1985). The association of dietary factors with pancreatic cancer has been significantly less well studied. In this study, the modulation of cell mutability via the GRP78 chaperone was examined by measuring GRP78 expression in Japanese miso-treated human RSa cells. It was found that levels of GRP78 expression increased upon treatment of RSa cells with EM and rice-koji extracts (Figs. 3A and 6A). The pretreatment of cells with these extracts was also found to suppress UVC mutagenicity (Figs. 3B and 6B). An intimate relationship between GRP78 up-regulation and hypo-mutability was also suggested by the results of experiments using GRP78 silencing (Figs. 4 and 7).

We reported that the down-regulation of GRP78 in RSa cells reduces DNA repair capacity in the nucleotide excision repair pathway. Nucleotide excision repair, a highly conserved DNA repair system in human cells, is essential for protection against UVC-induced DNA damage leading to, for example, (6-4)-photoproducts and cyclobutane thymine dimmers (Batty & Wood, 2000, de Laat *et al.,* 1999). Thus, one plausible mechanism for the observed hypo-mutable change in RSa cells pre-cultured with miso and rice-koji extracts may be the enhancement of cellular DNA repair function by the up-regulation of GRP78 expression.

The *K-ras* point mutation-enhancing activity of conditioned medium is detected from culture of human pancreatic cancer cells (Hirano *et al.,* 2008), suggesting involvement of extracellular factors from pancreatic cancer cells in tumor-worsening process. Extracellular materials released from cancer cells play crucial roles in development of cancers and resistance to anticancer treatment (Hidalgo *et al.,* 2010). Pancreas carcinoma shows resistance to chemotherapeutic agents (Gong *et al.,* 2010). Thus, we tried to search for the above materials in conditioned medium from pancreatic cancer cells, and identified one of HSP27-bound proteins, annexin II, by molecular mass analysis. Although the functions of extracellular annexin II are not fully understood, annexin II is known to act as a cell surface receptor for extracellular ligands and is suggested to play roles in regulation of proteolytic cascades including PA activities (Hajjar *et al.,* 1994), signal transduction (Singh, 2007), and tumor invasion and metastasis (Chung *et al.,* 1996, Esposito *et al.,* 2006, Singh *et al.,* 2007, Mai

et al., 2000). Studies on the mechanisms of PA-involved SOS functions are required for prevention of pancreatic cancer.

4. Acknowledgments

We thank Y. Sumiya for technical assistance. This work was supported in part by a grant from the Miso Central Institute in Japan, and in part by grants-in-aid from the following organizations: the Smoking Research Foundation, the Tokyu Foundation for a Better Environment, the Tsuchiya Foundation, the Goho Life Science International Foundation, the Ministry of Health, Labour and Welfare for the Treatment of Intractable Diseases Research Program and the Japan Society for the Promotion of Science (Japan).

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10

Vitamin D for the Prevention and Treatment of Pancreatic Cancer

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1. Introduction

Pancreatic adenocarcinoma (PCA), although infrequent, is one of the most lethal human malignancies. PCA ranks fourth in the Western countries and sixth worldwide among the most common cancer-related mortality based on GLOBOCAN 2008 (Jemal et al. 2011). Worldwide, an estimated 277,000 new cases of PCA were diagnosed in 2008 (Ferlay et al. 2008). In 2011, 44,000 new cases of PCA will be identified and 37,700 individuals will die from this disease in the US (Siegel et al. 2011). The nearly 1:1 ratio of incidence to mortality clearly implicates a poor prognosis and the lethal nature of PCA, which is the result of the difficulty of early diagnosis, early local spread, distant metastasis and resistance to traditional chemotherapy and radiotherapy. The overall five-year survival rate is estimated to be within the range of 1–4%, much lower than that of other types of cancers (Jemal et al. 2011). Up to the present time, the standard treatment for PCA is surgical extirpation, which may improve the overall five-year survival rate to 10-29% (Trede et al. 1990; Nitecki et al. 1995; Yeo et al. 1997). However, 40% of PCA patients already had distant metastasis at the time of diagnosis and another 40% were diagnosed with locally advanced cancer (Haller 2003; Jemal et al. 2011; Siegel et al. 2011), excluding them from being good candidates for resection.

Although the definite causes of pancreatic cancer are still poorly understood, several environmental risk factors have been implicated. Abundant epidemiological studies have indicated that the use of tobacco increases the risk of pancreatic cancer (Raimondi et al. 2009) and increased incidence of pancreatic cancer is positively associated with frequency and length of tobacco exposure (Hassan et al. 2007). A recent study based on a pooled analysis also concludes smoking is associated with an 18% increased risk of PCA (Lynch et al. 2009). On the other hand, the risk of PCA would dwindle after cessation of cigarette smoking for 10 years or longer (Iodice et al. 2008). Although alcohol has been related to increased risk of several types of cancers, the exact relationship between alcohol and PCA has not been established yet (Rohrmann et al. 2009; Jiao et al. 2009). A pooled analysis of 14 cohort studies reported a positive relationship between pancreatic cancer and women consuming more than 30 g of alcohol per day (Genkinger et al. 2009). A recent meta-analysis study also indicates that a 22% increased risk of PCA is observed in subjects with heavy

alcohol consumption (> 30g/day) (Tramacere 2010). High caloric intake and obesity are also identified to be risk factors for PCA (Reeves et al. 2007; Fryzek et al. 2005; Patel et al. 2005: Berrington de Gonzalez et al. 2003). While natural, plant-produced antioxidants, such as some flavonoids, are thought of traditional lyas protective factors for some cancers, their roles in PCA are still not established (Nothlings et al. 2007). Fruits and vegetables also failed to offer definitive protective benefit for PCA in a large-scaled cohort study (Vrieling et al. 2009). Other risk factors, such as intake of coffee, use of aspirin, previous cholecystectomy, and history of diabetes or chronic pancreatitis, although less conclusive, may contribute to pancreatic cancer as well (Batty et al. 2009: Landi 2009; Lowenfels & Maisonneuve 2006).

While investigating the incidence of PCA in different locations, a geographical variation has been observed; that is in the northern latitudes, the incidence of pancreatic cancer is three- to four-times higher than that in areas closer to equator (Curado et al. 2007). This finding has been attributed to sunlight or ultraviolet (UVB) exposure, which is directly related to vitamin D synthesis and the main determinant of vitamin D status in humans. In this regard, abundant epidemiologic studies have shown that vitamin D status is inversely associated with the incidence of some cancers such as prostate, colon and breast (Garland & Garland 1980; Gorham et al. 1990; Schwartz & Chen 2005).

Recently, due to the dismal outcome of PCA treatments and resistance of PCA to available chemotherapy and radiotherapy, some new regimens or strategies have been developed. In this chapter, we describe the recent findings on the relationship between sunlight, vitamin D and pancreatic cancer incidence, the potential role of vitamin D analogues for the prevention and treatment of pancreatic cancer, and the metabolism and functions of vitamin D as well as a brief history of vitamin D.

2. Current treatment of pancreatic cancer

Currently, the standard treatment for resectable pancreatic cancer remains surgery, including radically resection of the primary tumor, surrounding tissues, as well as neighboring lymph nodes. However, as described above, only 20% of PCA patients are suitable candidates for operation when diagnosed with PCA (Haller 2003; Jemal et al. 2011; Siegel et al. 2011). After operation, adjuvant chemotherapy with either gemcitabine or a combination of fluorouracil and leucovorin is able to improve progression-free period and overall survival (Neoptolemos et al. 2004; Oettle et al. 2007; Regine et al. 2008). Combination of adjuvant chemotherapy and radiation therapy seems to increase overall survival; however, the results are not impressive (Herman et al. 2008). For unresectable pancreatic cancer, the principle of treatment is mainly palliative. The standard chemotherapy for this group of patients is gemcitamine alone (Renouf & Moore 2010). Once gemcitamine fails to provide benefit in this group of patients, according to National Comprehensive Cancer Network guidelines, capecitabine, FOLFOX, or a combination of capecitabine and oxliplatin should be considered (National Comprehensive Network guidelines 2008). It has been reported that in general PCA patients who respond poorly to the first line therapy may have an unfavorable response to the second line therapy as well (Herrmann et al. 2007). Recently, target therapy has gained attention for the treatment of certain cancers. However, at the present time, no suitable target therapy is available against PCA. Under these bleak conditions, the development of new therapies to treat PCA should be one of the priorities in cancer research.

3. History of vitamin D

The discovery of vitamin D is closely associated with the disease rickets. Rickets was prevalent in the 17th century when two English physicians, Daniel Whistler and Francis Glisson described this deformality of bone in 1645 and 1650, respectively (Hess 1929). It was not until 1822, Sniadecki made an important observation relating the prevalence of rickets to locations of residence; lower incidence of rickets was found among children living on farms than children living in the city of Warsaw, Poland (Mozolowski, 1939). In 1889, Theodore Palm, a medical missionary and epidemiologist, reported that children living near the equator did not suffer from rickets and, thus, suggested sunbathing as a possible cure and strategy for rickets prevention (Palm 1890). Both of them attributed their finding of geographic differences in rickets incidence to varied exposures to sunlight. In 1919, Edward Mellanby successfully made dogs rachitic by keeping them indoors and feeding them with oats exclusively, followed by curing this disease with cod liver oil (Mellanby 1919). During that period, cod liver oil was used to treat night blindness and fracture. Mellanby did not know at that time whether the cure of rickets was attributed to the newly discovered vitamin A present in cod liver oil (McCollum et al., 1916) or another substance within. It was not until 1922 that McCollum clearly demonstrated that the anti-rachitic substance present in cod liver oil was a new substance and named it "vitamin D" (McCollum 1922). Around the same period, Huldshinsky in 1919 discovered that sunlight exposure could cure rachitic children (Huldshinsky 1919). Subsequently, there seemed to be a relationship between the cure of rickets by sunlight exposure and vitamin D in the cod liver oil. Steenbock and Black (1924) and Hess and Weinstock (1924) then noted independently that UV-irradiated food could cure rickets, which suggested that UV light was capable of transforming one substance stored in food to cure rickets. In other words, UV irradiation could produce vitamin D, which was responsible for the anti-rachitic activity found in food.

Vitamin D was believed as biologically active for decades until DeLuca's laboratory showed that injected radioactive vitamin D_3 disappeared instantly in the circulation of rats and the label appeared again later in the blood. The major radioactive compound in the blood was isolated and tested for its ability in stimulating intestinal calcium transport (Norman et al. 1964). His group reported that this unknown compound acted much quicker and had higher activity than the parent substance vitamin D_3 (Morii et al. 1967), suggesting that vitamin D_3 might be further metabolized to become active. Subsequently, the unknown compound was isolated in pure form and identified as 25-hydroxyvitamin D_3 [25(OH)D_3] in 1968 (Blunt et al. 1968). Later, when radioactive 25(OH)D_3 was synthesized and injected into rats, several more polar metabolites were found and isolated. One of them was shown to stimulate intestinal calcium transport much quicker and to a greater extent than 25(OH)D_3.The compound was identified in 1971, independently by three groups of researchers as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (Lawson et al. 1971; Norman et al. 1971; Holick et al. 1971).

Vitamin D_3 (cholecalciferol) can be obtained either from the diet, including supplements, or synthesized in the skin from the precursor 7-dehydrocholesterol (7-DHC) via sunlight exposure (wave length: 290-315 nm). Vitamin D_3 is then bound to vitamin D binding protein (DBP) and circulates in the blood. After entering the liver, vitamin D_3 is hydroxylated by vitamin D-25-hydroxylase (25-OHase, mainly CYP2R1) to generate the circulating prohormone 25(OH)D_3, which has the highest affinity for DBP and is bound to DBP

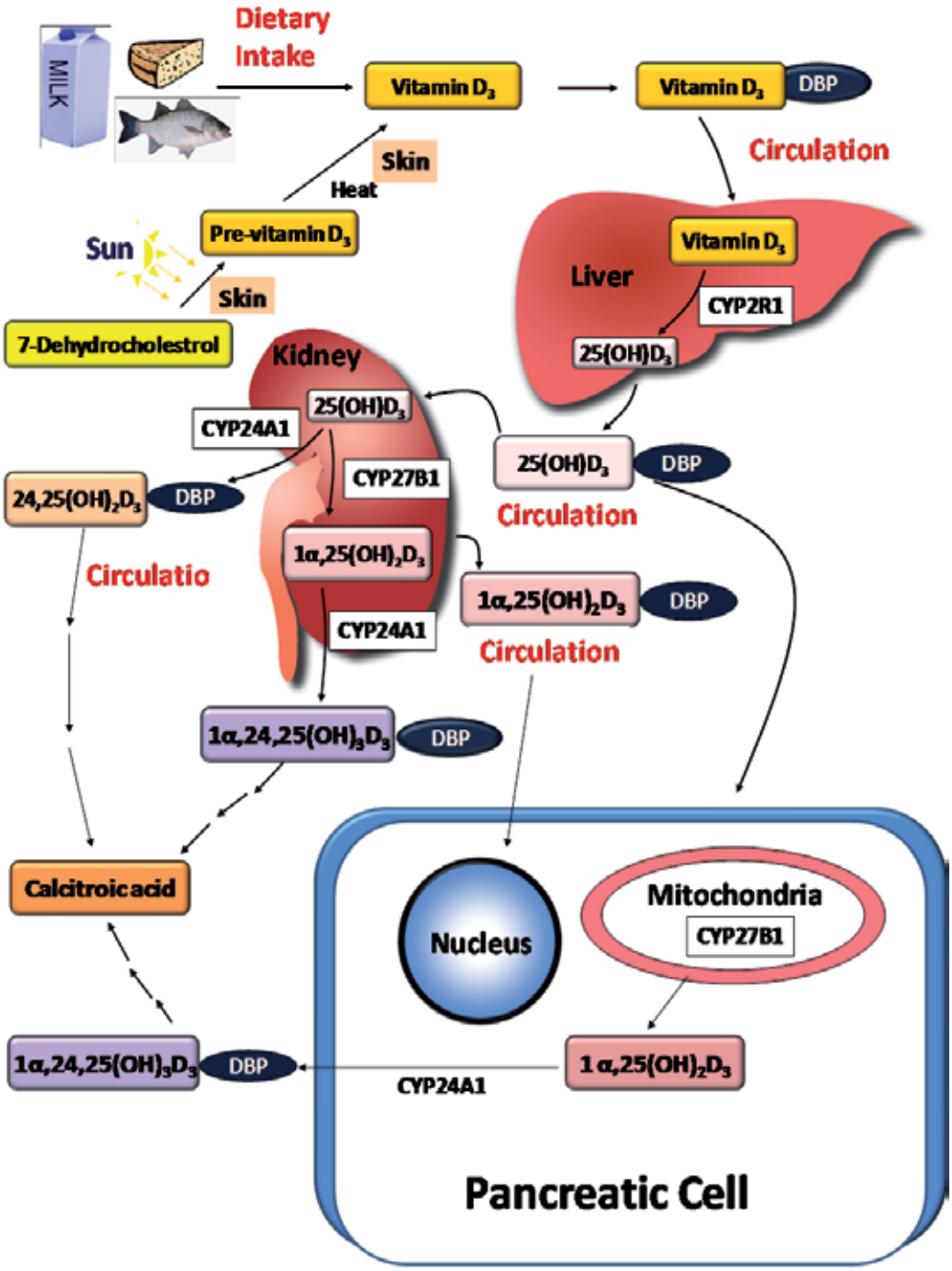

Fig. 1. Source and metabolism of vitamin D in pancreatic cells.

in the circulation. The subsequent conversion of $25(OH)D_3$ to the active form, 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], occurs in the kidneys and is catalyzed by a tightly regulated enzyme 25(OH)D-1α-hydroxylase (1α-OHase or CYP27B1). The active form then will be bound to DBP in the circulation and transported to its target organs, tissues and cells to induce gene transcription, including the up-regulation of *CYP24A1* as shown in Figure 2. The activation of $25(OH)D_3$ may also take place in many extra-renal tissues, including pancreas, bone, breast, colon, prostate. The extra-renal synthesis of $1\alpha,25(OH)_2D$ may explain why serum 25(OH)D level, instead of the circulating level of the active form, $1\alpha,25(OH)_2D$, is the index of vitamin D nutritional status. $1\alpha,25(OH)_2D_3$, either obtained from the circulation or within the cells in an autocrine fashion, will be hydroxylated by CYP24A1 (or 24-OHase) to form 1α,24,25-trihydroxyvitamin D_3 [$1\alpha,24,25(OH)_3D_3$], the first step to inactivate $1\alpha,25(OH)_2D_3$, leading to the formation of calcitroic acid, which is water-soluble and is secreted in the urine.

4. Sources and metabolism of vitamin D

Two major forms of vitamin D exist in nature: vitamin D_2 and vitamin D_3. Commonly, vitamin D_2 (ergocalciferol) is produced from ergosterol of yeast and vitamin D_3 (cholecalciferol) is synthesized from 7-dehydrocholesterol (7-DHC) of lanolin. Vitamin D (representing D_2 and D_3) is rare in foods, only few foods contain sufficient vitamin D naturally (Chen et al. 2010). Therefore, fortification of vitamin D in foods, like orange juice, cheese, cereal and milk, is becoming popular in some countries. For most humans, exposure to sunlight remains the major source, accounting for about 90% of vitamin D requirement (Chen et al. 2010) (Figure 1). The basal and suprabasal layers of human skin contain 7-DHC, which can be converted to pre-vitamin D_3 as the skin receives UV irradiation (wavelength 290–315 nm). Pre-vitamin D_3 is further thermoisomerized to vitamin D_3 in the skin. Vitamin D, obtained from food (may contain vitamin D_2 and/or vitamin D_3) or synthesized from skin after exposure to sunlight, enters the blood circulation carried by vitamin D binding protein (DBP). Upon entering the liver, vitamin D is hydroxylated at the C-25, catalyzed by vitamin D-25-hydroxylase (25-OHase) (Schuster 2011), to produce $25(OH)D_3$. 25(OH)D is further hydroxylated by the enzyme 1α-OHase or CYP27B1 mainly in the renal proximal tubules at the C-1 position to form the active metabolite, $1\alpha,25(OH)_2D_3$. While $1\alpha,25(OH)_2D_3$ is the active form and is responsible for the various biological activities exerted by vitamin D_3, $25(OH)D_3$ is the major circulating form of vitamin D_3 and is considered as the most reliable index of vitamin D nutritional status. $25(OH)D_3$ has the highest affinity for DBP and circulates as a DBP-bound form in the blood stream. Another renal enzyme, which also plays a crucial role in vitamin D metabolism, is 25(OH)D-24-hydroxylase (24-OHase or CYP24A1). CYP24A1 is responsible for the degradation of $1\alpha,25(OH)_2D_3$, forming $1\alpha,24,25(OH)_3D_3$, and thus terminating the actions of $1\alpha,25(OH)_2D_3$. In addition, when there is an excess of $25(OH)D_3$, 24-OHase in the kidneys is capable of converting it into $24,25(OH)_2D_3$ to prevent the over-production of $1\alpha,25(OH)_2D_3$ (Schuster 2011). Of note, originally it was believed that CYP27B1 and CYP24A1 exist exclusively in the kidneys, the two enzymes have been found to express in many extra-renal tissues (Zehnder et al. 2001; Chen & Holick 2003; Schwartz et al. 2004; Kemmis et al. 2006; Chiang & Chen 2009), including the pancreas. Given that anephric individuals have no detectable $1\alpha,25(OH)_2D_3$ in their circulation, it is believed that extrarenal-generated $1\alpha,25(OH)_2D_3$ acts and is degraded only locally in an autocrine and paracrine manner. This autocrine/paracrine pathway seems

to be regulated in a tissue-specific manner and is not associated with systemic calcium homeostasis. Based on this theory, once 25(OH)D_3 is internalized into the cells, the fate of 25(OH)D_3 may depend on the relative expression levels of CYP27B1 to CYP24A1. In the cells with dominant expression of CYP27B1, 25(OH)D_3 will be converted to 1α,25(OH)$_2D_3$ to exert its cellular functions. Meanwhile, the locally generated 1α,25(OH)$_2$D will up-regulate the expression of CYP24A1 within the cells to hydroxylate 1α,25(OH)$_2D_3$ and excess 25(OH)D_3 to form their respective 24-hydroxylated metabolites leading to their catabolism. On the other hand, in cells dominated with the expression of CYP24A1, the generated 1α,25(OH)$_2D_3$ will be degraded very quickly with little or no chance to exert biological actions (Ly et al. 1999; Schuster 2011).

5. Functions of vitamin D

The genomic action of 1α,25(OH)$_2$D is mediated through its binding to vitamin D receptor (VDR) to modulate various gene expressions in a cell- and tissue- specific manner (Norman 2006) (Figure 2). VDR is a member of the nuclear receptor superfamily and is expressed in almost all tissues (Hauslller et al. 1997). To date, 1α,25(OH)$_2D_3$ has been well described to exert anti-proliferation, anti-inflammation, pro-differentiation, pro-apoptosis and immune regulation in a tissue- and cell-specific manner (Chiang & Chen 2009; Bikle 2009; Adams & Hewison 2010). So far, more than 2770 VDR binding sites have been identified within 229 vitamin D-regulated genes as shown by a Chip-sequencing method (Ramagopalan et al. 2010). Many cancer cell lines, including prostate, lung, liver, breast, pancreas and liver cancers, have been shown to express VDR, and 1α,25(OH)$_2D_3$ has been found to have growth inhibitory effect on these cells (Colston et al., 1980; Skowronski et al., 1993; Hulla et al., 1995; Chen & Holick 2003; Flanagan et al., 2009; Chiang et al., 2009).

The active form of vitamin D_3, 1α,25(OH)$_2D_3$, either synthesized in an autocrine fashion or obtained from the kidneys, exerts its genomic effects by binding to the VDR/retinoid X receptor (RXR) complex on vitamin D response element (VDRE) in the promoter region of vitamin D-regulated genes. The transcriptional effects include cell cycle arrest, pro-differentiation, pro-apoptosis, anti-inflammation, regulation of immune response and *etc*. After 1α,25(OH)$_2D_3$ elicits its function, it is then inactivated by CYP24A1. Since many tissues possess CYP27B1 and CYP24A1 simultaneously, the internalized 25(OH)D_3 can be activated or inactivated to form 1α,25(OH)$_2D_3$ or 24-25(OH)D_3 based on the expression rates of 1α-OHase to 24-OHase.

Once 1α,25(OH)$_2D_3$ is internalized into cells, it binds to VDR. The liganded VDR then form a heterodimer with RXR and binds to VDRE (Tsai & Omalley 1994) located in the promoter regions of vitamin D responsive genes to modulate the gene expression. In cancer cells, the action mainly leads to the inhibition of cancer growth and the prevention of cancer cells from invading to surrounding normal tissues. Mechanistically, the genomic pathways are regulated by multiple co-factors (Haussler et al. 1998). The VDR conformational change occurs upon 1α,25(OH)$_2D_3$ binding to VDR, leading to subsequent phosphorylation, and gives rise to the release of co-repressors and the recruitment of co-activators (Tagami et al. 1998; Li et al. 2007). In addition to the genomic pathways, 1α,25(OH)$_2D_3$ has been shown to be able to induce instant biologic reaction at the plasma membrane or in the cytoplasm by changing transmembrane signals quickly (Norman 2006).

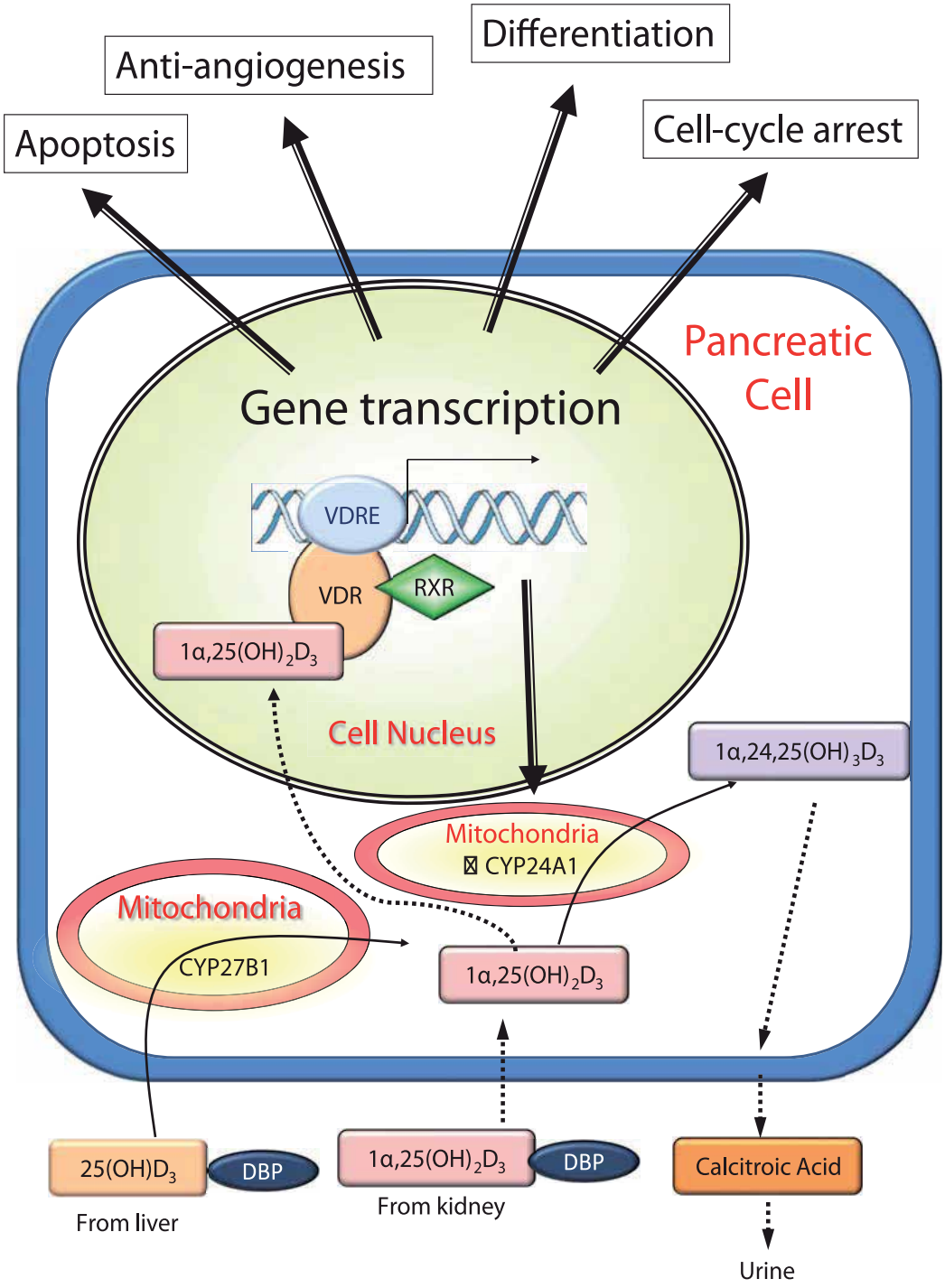

Fig. 2. Functions of vitamin D in pancreatic cells

This kind of action does not influence gene expression directly, though, its cross-talk with varied signaling pathways still can modulate gene transcripton (Losel & Wehling 2003). To date, the exact mechanisms for non-genomic actions of $1\alpha,25(OH)_2D_3$ are not well understood. Nevertheless, the existence of non-classical membrane VDR has been found to be related to the rapid actions (Huhtakangas et al. 2004), including activation of protein kinase C and protein phosphatase PP1c. The actions have been shown to result in subsequent ion channel activity modulation (Bettoun et al. 2002; Shah et al. 2006), which is also implicated in the growth inhibition of cancer cells.

6. Vitamin D and pancreatic cancer- biological studies

To date, $1\alpha,25(OH)_2D_3$ has been shown to possess anti-tumor activity in many cancer cells expressing VDR through its anti-proliferative, pro-apoptotic, and pro-differentiation actions in a cell- and tissue-specific manner. In terms of pancreatic cancer, $1\alpha,25(OH)_2D_3$ has been demonstrated to up-regulate the expression of p21 and p27 and down-regulate the expression of cyclins A, D_1, and E and cyclin dependent kinases 2 and 4, leading to cell cycle arrest at G_0/G_1 phase (Kawa et al. 1997). However, $1\alpha,25(OH)_2D_3$ is known to cause hypercalcemia and hypercalciuria side effects when administered systemically. To overcome these lethal side effects caused by systemic administration of $1\alpha,25(OH)_2D_3$, thousands of $1\alpha,25(OH)_2D_3$ analogues have been synthesized in an effort to potentiate its anti-tumor effect while decreasing its hypercalcemic activity. Some of them have been found to induce greater cell-cycle arrest, differentiation, and/or apoptosis on pancreatic cancer cells *in vitro* and to inhibit tumor growth in the xenograft animal model. For example, 22-oxa-$1\alpha,25(OH)_2D_3$ has been reported to cause growth inhibition on three pancreatic cancer cell lines and to inhibit xenografted BxPC-3 cell growth *in vivo* (Kawa et al. 1996). Similarly, EB-1089, a well-studied$1\alpha,25(OH)_2D_3$ analogue, has been shown to inhibit pancreatic cancer growth *in vitro* and *in vivo* (Colston et al. 1997; Pettersson 2000), and has been investigated in a phase II clinical trial to treat advanced pancreatic cancer. While EB-1089 failed to prolong the survival of patients significantly in this trial (Evans et al. 2002), $1\alpha,25(OH)_2D_3$ (0.5 μg/kg) in a combination with docetaxel successfully increased the period of time-to-progress of pancreatic cancer in a recently published phase II study enrolling 25 advanced pancreatic cancer patients as compared to treatment with docetaxel alone (Blanke et al. 2009). Several new analogues have been shown to possess promising results in *in vitro* studies. For example, a VDR-alkylating derivative of $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3-3-bromoacetate ($1\alpha,25(OH)_2D_3$-3-BE), was able to inhibit pancreatic cancer cell grow at a lower concentration and to a greater extent than $1\alpha,25(OH)_2D_3$, especially in combination with 5-amino-imidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) (Persons et al. 2010). In another study, 19-nor-$1\alpha,25(OH)_2D_2$ (Paricalcitol), which has been approved by the Food and Drug Administration for treating secondary hyperparathorodism, has been demonstrated to have comparable growth inhibition as $1\alpha,25(OH)_2D_3$ in pancreatic cancer *in vitro* and *in vivo* (Schwartz et al. 2008). Given that 19-nor-$1\alpha,25(OH)_2D_2$ and 19-nor-$1\alpha,25(OH)_2D_3$, are less calcemic analogues of $1\alpha,25(OH)_2D_3$, we have studied a carbon-2 modified analogue of 19-nor-$1\alpha,25(OH)_2D_3$, 19-nor-2α-(3-hydroxypropyl)-$1\alpha,25(OH)_2D_3$ or MART-10, in pancreatic cancer cells *in vitro* and found to be 100-1000 times as potent as $1\alpha,25(OH)_2D_3$ to inhibit tumor cell growth. Most importantly, MART-10 does not increase serum calcium in rats (Iglesias-Gato, D. *et al*, 2011). Furthermore, MART-10 has been shown

to be a poor substrate of CYP24A1 and has a lower binding affinity for DBP compared to $1\alpha,25(OH)_2D_3$, suggesting that this analogue is likely more bio-available than $1\alpha,25(OH)_2D_3$ in circulation (Flanagan et al. 2009). Thus, MART-10 is a promising compound to treat pancreatic cancer.

7. Epidemiological evidence associating vitamin D and pancreatic cancer

Circulating vitamin D level, primarily determined by solar UVB exposure and partially influenced by food uptake and oral vitamin D supplementation, has been shown to be inversely associated with the incidence of many cancers, including prostate, colon and breast cancers in a number of epidemiological studies (Garland & Garland 1980; Gorham et al., 1990; Schwartz & Chen 2005). Garland et al. (2009) further reported that 58,000 new cases of breast cancer and 49000 new cases of colon cancer could be prevented annually through vitamin D supplement. In addition, recent studies applying Hill's criteria for causality also clearly showed that UVB exposure and vitamin D status are negatively associated with cancer risk (Grant 2009; Grant & Boucher 2009). For pancreatic cancer, its exact relationship to vitamin D status has not been well understood. Although two earlier epidemiologic studies published in 2006 showed inconsistent findings about the relationship between pancreatic cancer incidence and serum 25(OH)D level (Skinner et al. 2006; Stolzenberg-Solomon et al. 2006), the death rate of pancreatic cancer has been shown to be inversely related to sun exposure (Mizoue 2004; Boscoe & Schymura 2006; Grant 2007; Tuohimaa et al. 2007). More recently, Stoleznberg-Solomon *et al*. (2010) conducted two pooled nested case control studies to investigate the potential association of vitamin D status and pancreatic cancer, and reported that the circulating 25(OH)D concentration was not related to the risk of pancreatic cancer. Furthermore, Stoleznberg-Solomon et al. showed that a high 25(OH)D level, exceeding 100 nmol/L (40 ng/mL), increased pancreatic cancer incidence two folds (odds ratio = 2.12, 95% confidence interval: 1.23, 3.64) (Stoleznberg-Solomon et al. 2010). However, they did find subjects with lower estimated annual residential solar UVB exposure would have higher risk of pancreatic cancer (Stoleznberg-Solomon et al. 2009). The reason behind the lack of association between serum levels and pancreatic cancer and other cancers maybe that serum 25(OH)D levels were only measured at one time point years prior to diagnosis of pancreatic cancer and, in fact, 25(OH)D levels change from season to season. For this reason, Yin *et al*. (2010) conducted case-control studies with zero lag time between diagnosis and serum 25(OH)D measurement, not nested studies, and found an inverse correlation between serum 25(OH)D level and breast cancer. Mohr SB *et al.* (2010) also demonstrated an inverse association between UVB irradiation and incidence rates of pancreatic cancer worldwide. They found that the incidence rate of pancreatic cancer was only half in countries with estimated serum 25(OH)D> 30 ng/ml as compared to those with serum 25(OH)D ≤ 30 ng/mL. There are other studies also showing inverse relationship between UVB and pancreatic cancer (Kato et al. 1985; Giovannucci et al. 2006; Neale et al. 2009). Interestingly, high insulin and glucose levels have been found to be related to pancreatic cancer positively (Hennig et al. 2004; Stolzenberg-Solomon et al. 2005; Huxley et al. 2005; Michaud et al. 2007). Since vitamin D is able to regulate the synthesis, binding and actions of insulin (Maestro et al. 2000; Maestro et. 2003; Mathieu et al. 2005), there seems to be an inverse relationship between pancreatic cancer incidence and vitamin D status. Due to these contradictory findings, more careful studies should be conducted to investigate the

potential impacts of gene polymorphisms, including VDR, DBP, *CYP27B1*, and *CYP24A1*, on vitamin D status in order to determine whether adequate vitamin D nutrition has a survival and/or a preventive benefit against the pancreatic cancer.

8. Conclusion

Pancreatic cancer is often diagnosed at a late stage with a 5-year survival of merely 1-4%. Its characteristics of early spread and distant metastasis at the time of diagnosis make it a poor candidate for surgical treatment. Moreover, traditional chemotherapy and radiotherapy fail to show significant benefit on survival of PCA patients, and no effective target therapy against PCA is available at the present time. Since clinicians are faced with the dilemma of dealing with advanced PCA, developing new regimens against PCA deserve more attention. Vitamin D, originally discovered for treating rickets a century ago, has been found to go through a series of hydroxylation steps, leading to the synthesis of the active metabolite, $1\alpha,25(OH)_2D$. The active metabolite exerts an array of actions through its binding to VDR, which is found to exist in almost all tissues in humans. Although $1\alpha,25(OH)_2D_3$ possesses antitumor effects on many cancer cells *in vitro* and *in vivo*, its clinical application is impeded by the lethal side effect of hypercalcemia when administered systemically. To overcome this drawback, thousands of $1\alpha,25(OH)_2D_3$ analogues have been synthesized, and some of them have much less calcemic activity and/or a more potent antitumor effect. Regarding pancreatic cancer, although several analogues have shown promising antiproliferative effect on cells in culture and animal experiments, they fail to offer any benefits in clinical trials. However, in combination with docetaxel, $1\alpha,25(OH)_2D_3$ was able to prolong the period of time-to-progression of patients with advanced pancreatic cancer. Recently, two analogues of $1\alpha,25(OH)_2D_3$, $1,25(OH)_2D_3$-3-BE and MART-10, have been shown to exert much greater antiproliferative effect on pancreatic cancer cells *in vitro*. Under the current situation without an effective treatment for the advanced PCA, further investigation of these two analogues in animal models and clinical trials is warranted.

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11

Molecular Targets of Benzyl Isothiocyanates in Pancreatic Cancer

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1. Introduction

Addiction of cancer cells to survival pathways has been well documented in most of the cancer models including the pancreatic cancer. Pancreatic cancer is one of the most aggressive tumors with an average five year survival rate of less than 5% (Jemal A et al., 2010). It is associated with high expression levels of various survival pathways, such as KRAS, STAT3, AKT, NFkB, HDAC etc. Furthermore, pancreatic cancer acquires resistance to various apoptosis signals such as FasL, TRAIL. In addition, pancreatic cancer gets resistance to various chemo-drugs including gemcitibine by altering survival pathways.

Currently, there is no effective treatment for pancreatic cancer because conventional chemotherapy including the gemcitabine and 5-FU, and radiation treatment has shown very limited success in improving the patient survival. Therefore, the development of novel approaches to prevent and treat pancreatic cancer is an important mission.

Evidence from epidemiological, pharmacological, and case-control studies continue to support the notion that isothiocyanates (ITCs) present in cruciferous vegetables may have substantial chemopreventive activity against various human malignancies including pancreatic cancer (Zhang Y et al., 1992); Stoner GD & Morse MA, 1997). Benzyl isothiocyanate (BITC), an agent that is present in cruciferous vegetables such as, watercress, cabbage, cauliflower, mustard, and horseradish, is widely consumed as part of a routine diet. BITC has been reported to inhibit initiation, growth, and metastasis of human cancers in rodents (Batra S et al., 2010; Boreddy SR et al., 2011a; Boreddy SR etal., 2011b; Kim EJ et al., 2011; Sahu RP & Srivastava SK, 2009; Zhang Y et al., 1992). The structure of BITC is shown in Fig.1.

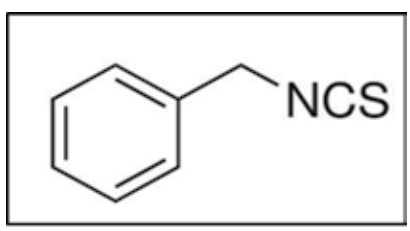

Fig. 1. Chemical structure of BITC

Our laboratory has shown that BITC potentially suppresses the growth and induces apoptosis in pancreatic cancer cells by targeting various key survival molecules (Batra S et al., 2010; Boreddy SR et al., 2011a; Sahu RP & Srivastava SK, 2009). In the present chapter, we discuss the current understanding of BITC, its targets and mechanism of action in pancreatic cancer.

2. Effect of BITC on STAT3 signalling pathway

Signal Transducer Activator Transcription (STAT3) transcription factors are latent proteins that bind to the genome on activation to either induce or to repress gene expression (Bromberg et al., 1999). STAT3 is aberrantly activated in majority of the cancers including pancreatic cancer (Wei et al., 2003). Clinical specimens have revealed that more than 50% of the breast and lung cancer, and over 95% of head and neck cancers have hyperactive STAT3 signaling (Darnell, 2005). Interestingly, STAT3 deficient mice in a chemical carcinogenesis model have shown the reduced proliferation of epithelial cells due to inability to pass through G1-S-G2 cell cycle progression (Chan et al., 2004). Furthermore, Chiarle et al. have demonstrated that disruption of STAT3 signaling by anti-sense oligoneclosides was sufficient to impair the growth of solid tumors (Chiarle, 2005), highlighting the potential of anti-STAT3 therapy in clinical medicine. Recently, numerous natural and synthetic compounds have been discovered to target STAT3 signaling. Results from our laboratory showed that benzyl isothiocyanate (BITC) targets STAT3 signaling to induce apoptosis in pancreatic cancer (Sahu & Srivastava, 2009).

Our laboratory showed that BITC significantly suppress the phosphorylation of STAT3 at both Tyr-705 and Ser-727 to induce apoptosis in BxPC-3 (Fig. 2), MIA PaCa-2, Capan-1 and PanC-1 pancreatic cancer cell lines, in a dose and time dependent manner (Sahu & Srivastava, 2009). Interestingly, BITC also down regulated the protein levels of STAT3 in these cell lines, although its functional implications are yet to be explored. Furthermore, down regulation of STAT3 protein expression by BITC was transcriptional, as evidenced by RT-PCR analysis of BITC treated BxPC-3 cells (Fig. 2).

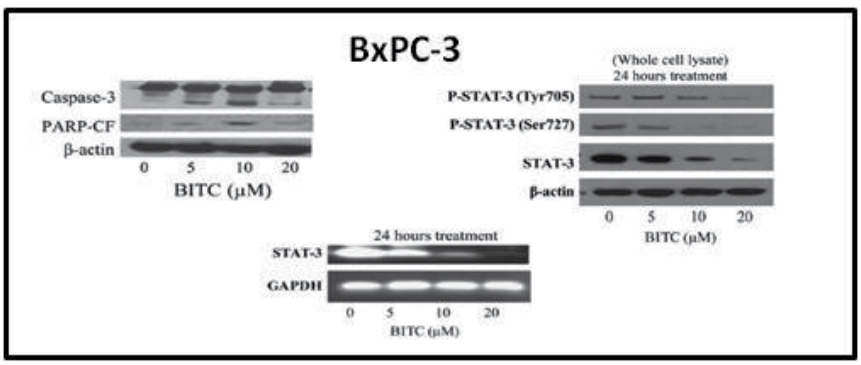

Fig. 2. Benzyl isothiocyanate induces apoptosis in pancreatic cancer cells by inhibiting the phosphorylation of STAT3. (J Natl Cancer Inst 2009;101: 176 – 193).

BITC-induced apoptosis was further substantiated by IL-6 treatment, which specifically phosphorylates STAT3 at Tyr-705 (Berishaj, 2007) and STAT3α overexpression. IL-6 pre-

treated BxPC-3 cells showed significant resistance to BITC-induced apoptosis (Fig. 3). Similarly, when STAT3α was over expressed in BxPC-3 cells, BITC-induced apoptosis was severely abrogated, indicating that BITC targets STAT3 to induce apoptosis in pancreatic cancer cells.

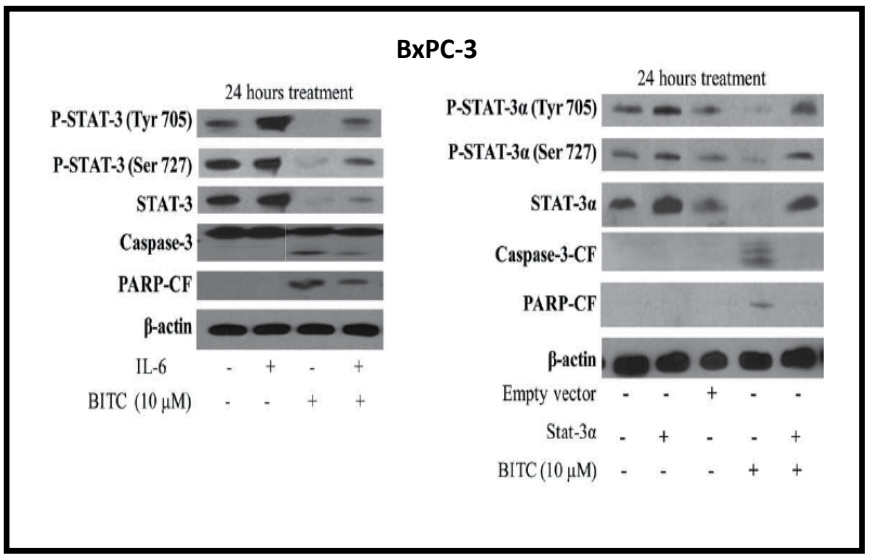

Fig. 3. IL-6 pre-treatment or STAT3α overexpression abrogates BITC-induced apoptosis in pancreatic cancer cells. (J Natl Cancer Inst 2009:101; 176 – 193).

3. Effect of BITC on AKT/FOXO/Bim signaling pathway

Phosphotidyl inositol 3phosphate (PI3K)/AKT signaling plays a critical role in cell survival and growth during embryonic development as well as during normal cell survival (Finkielsztein & Kelly, 2009). However, cancer cells exploit the same pathway to overcome apoptosis induced by either therapeutic drugs or internal stimuli such as oxidative stress. Upon binding of growth factors to Tyrosine Kinase Receptor (TKR), PI3K is directly or indirectly activated by TKRs by inhibiting or removing the P85 regulatory unit of PI3K (Vivanco & Sawyers, 2002). Activated PI3K phosphorylates phosphatidylinositol and converts inositol 4,5biphosphate (PIP2) into PIP3. Consequently, AKT and PDK translocate to membrane and interact with PIP3 through PH domain leading to conformational changes in AKT to expose phosphorylation sites. AKT is phosphorylated by PDK1 at Ser-308 leading to stabilization of AKT. Yet another phosphorylation takes place at Tyr-473, which is required for full activation of AKT. In addition, another protein complex mTOR has been shown to be required for the phosphorylation of AKT (Sarbassov et al., 2005). This pathway is negatively regulated by phosphatases, such as PTEN, which dephosphorylates PIP3 thus limiting its availability (Osaki et al., 2004).

Recently, FOXO transcription factors received ample of attention in cancer because of direct involvement in apoptosis and drug resistance (Salih & Brunet, 2008). FOXO1 and FOXO3a are the members of FOXO transcription factors, which operate right under the AKT signaling. Upon growth signal stimulation, AKT is activated by phosphorylation at Ser-473,

which further phosphorylates FOXO1 or FOXO3a transcription factors. Phosphorylated FOXOs bind to 14-3-3 chaperons and transported out of nucleus and subjected to proteosomal degradation (Tzivion et al., 2011). But during oxidative stress or growth factor withdrawal, AKT is dephosphorylated leading to nuclear import of FOXOs and induction of pro-apoptotic proteins such as Bim and PUMA (Obexer, 2011).

A recent report has shown that 59% of the pancreatic tumors harbor aberrantly activated AKT signaling (Schlieman et al., 2003). One of the possible reasons behind hyperactive AKT signaling in pancreatic cancer is due to mutation or deletion of PTEN gene (Sawai et al., 2008). Indeed, strategies aimed at blocking AKT activation could be a promising treatment for pancreatic cancer. Interestingly BITC significantly inhibited AKT signaling *in vitro* and *in vivo.*

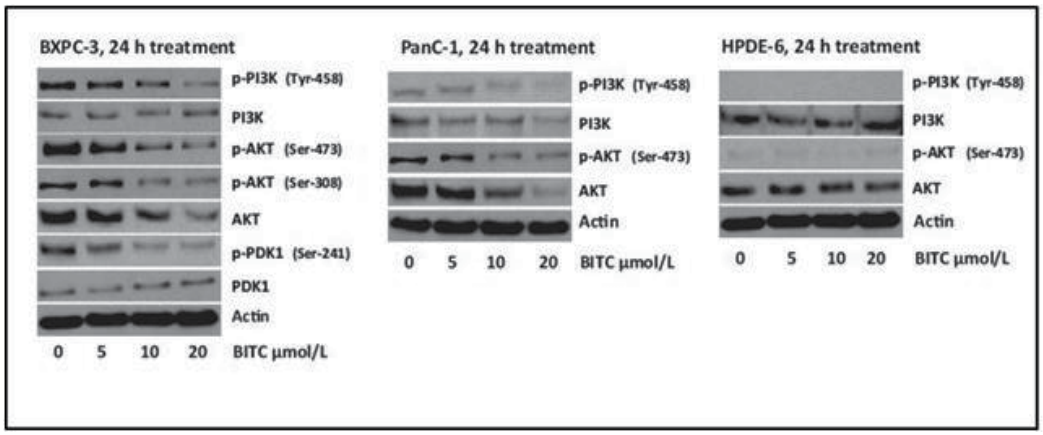

Fig. 4. BITC down regulates the phosphorylation of key molecules of PI3K/AKT pathway. (Clin Cancer Res; 17(7); 1784–1795).

BITC suppressed the phosphorylation of AKT at both Ser-308 and Ser-473 in BxPC-3 and PanC-1 cells, in dose dependent manner (Boreddy et al., 2011a). Furthermore, BITC also suppressed the phosphorylation of various other key molecules of PI3K/AKT pathway such as PI3K (Tyr-458), PDK1 (Ser-241), mTOR (Ser-2448) etc. (Fig. 4), indicating that BITC targets PI3K/AKT signaling to induce apoptosis in pancreatic cancer cells. Interestingly, BITC was almost ineffective in human pancreatic ductal epithelial (HPDE-6) cells (Fig. 4). Over expression of AKT blocked the apoptosis inducing effects of BITC in pancreatic cancer cells.

Recently, FOXO transcription factor received plenty of attention as a potential target for cancer therapy, as they are directly involved in apoptosis induction. Interestingly, BITC significantly suppressed the phosphorylation of FOXO1 (Ser-256) and FOXO3a (Ser-253), without effecting the protein levels in both BxPC-3 and PanC-1 cells (Fig. 5). Moreover, immunoprecipitation studies showed that BITC treatment significantly masked 14-3-3 binding motif on FOXO proteins indicating that more of FOXO proteins were retained in the nucleus (Fig. 5B). Furthermore, BITC significantly increased the expression FOXO1 transactive genes such as P21, P27 and Bim in both the cell lines, BxPC-3 and PanC-1 (Fig. 5C).

Apart from phosphorylation, another tier of FOXO transcription factor regulation is acetylation. Interestingly, BITC also reduced the acetylation of FOXO proteins. Probably, inhibition of acetylation by BITC was due to down regulation of CBP protein expression, since SIRTs were not altered by BITC treatment.

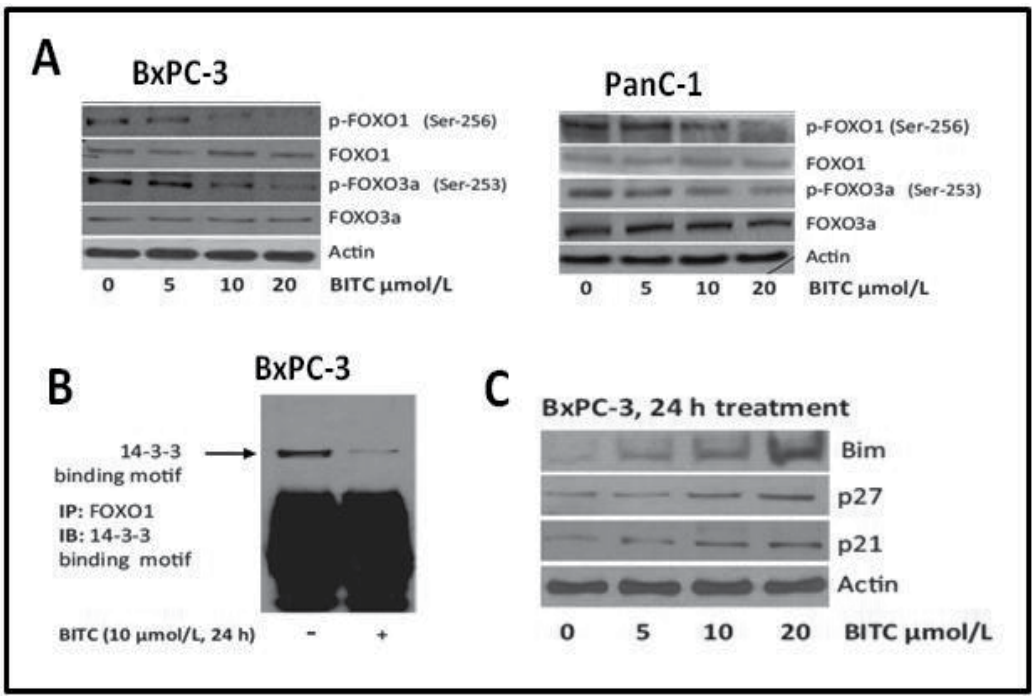

Fig. 5. BITC activates FOXO transcription factors and pro-apoptotic proteins in pancreatic cancer cells. (Clin Cancer Res; 17(7); 1784–1795).

Role of AKT in BITC-induced apoptosis was further confirmed by using PI3K inhibitor LY-294002 and overexpression of wild type AKT in BxPC-3 cells. Interestingly, when BxPC-3 cells were pre-treated with LY-294002 followed BITC (10µM) for 24h, apoptosis induction was potentiated, as compared to BITC alone treated cells (Fig. 6), whereas AKT overexpression severely abrogated BITC-induced apoptosis in BxPC-3 cells (Fig. 6). In line with apoptosis results, phosphorylation of FOXO proteins were increased with AKT overexpression, whereas Bim, P27, P21 expression was reduced. However, BITC partially blocked these effects, indicating that BITC targets AKT pathway to induce apoptosis in pancreatic cancer cells lines (Fig. 6).

4. BITC Regulates NFkB Activity by Inhibiting HDACs

NFkB transcription factors are mainly involved in the regulation of immune and inflammatory response, apart from cell proliferation and apoptosis Ghosh et al., 1998; Hart et al., 1998). NFkB is normally located in the cytoplasm sequestered by its endogenous inhibitor IkB. Upon cellular stimulation, IkB proteins are phosphorylated at Ser-32/36 liberating NFkB, which translocates to the nucleus and gets involved in the transcription of responsive genes such as Cyclin D1 (Sun & Andersson, 2002).

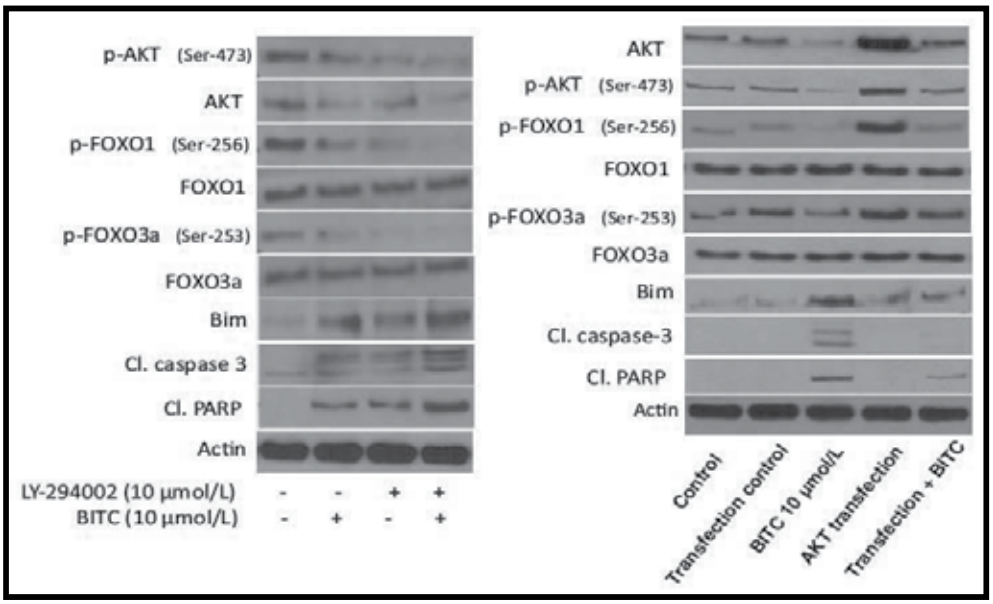

Fig. 6. AKT inhibitor potentiates BITC-induced apoptosis, whereas AKT overexpression abrogates BITC-induced apoptosis in BxPC-3 pancreatic cancer cells. (Clin Cancer Res2011: 17(7); 1784–1795).

NFkB may activate various survival signals to promote cell survival. NFkB is known to interfere with inducers of extrinsic apoptosis pathway by up regulating the FLIP-like inhibitory protein (Kreuz et al., 2001). NFkB also induces the expression of inhibitors of apoptosis proteins such as IAP (Deveraux et al., 1998) and some members of the Bcl2 (Shou et al., 2002) family proteins, thereby protecting the cells from various apoptosis stimuli. NFkB is also known to play critical role in drug resistance is various cancer models (Arlt et al., 2003). Hence, inhibiting of NFkB activation may potentiate the clinical efficacy of the drugs.

BITC significantly inhibits the phosphorylation of NFkB at both Ser-276 and Ser-536 in both BxPC-3 and Capan-2 pancreatic cancer cells, in a dose and time dependent manner (Fig. 7A&B). Interestingly, BITC down regulated the expression of NFkB in BxPC-3 cells but not in Capan-2 cells, indicating that BITC differentially act on different cells (Batra et al., 2010). Furthermore, BITC drastically inhibited the nuclear localization of NFkB in BxPC-3 cells (Fig. 7C). BxPC-3 cells that were transfected with a luciferase gene containing NFkB-promoter and treated with BITC demonstrated around 90% decrease in luciferase activity, as compared to control cells (Fig. 7D). Furthermore, BITC also decreased Cyclin D1 expression and transcriptional activity, as it is one of the target genes of NFkB (Fig. 7E & Fig. 7F). Interestingly expression of IKK was decreased with BITC treatment, but neither phosphorylation (Ser32/36) nor protein levels of IkB were altered in BITC treated BxPC-3 cells (Fig 7A), indicating that down regulation of IKK by BITC treatment could be the reason for inhibition of NFkB phosphorylation (Ser-536).

Apart from the phosphorylation, NFkB is known to be regulated by acetylation. Interestingly, BITC also inhibited the acetylation of NFkB on lysine residue in BxPC-3 cells. BITC suppressed the acetylation of NFkB by altering the expression of HDAC1 and HDAC3

(Fig. 8A&B), as these molecules play critical role in NFkB acetylation. In agreement with other HDAC inhibitors' data, such as veronistat (SAHA) and tricostatin A (TSA), BITC also up regulated the expression of p21 in BxPC-3 and Capan-2 cells, in a dose dependent manner (Fig. 8C).

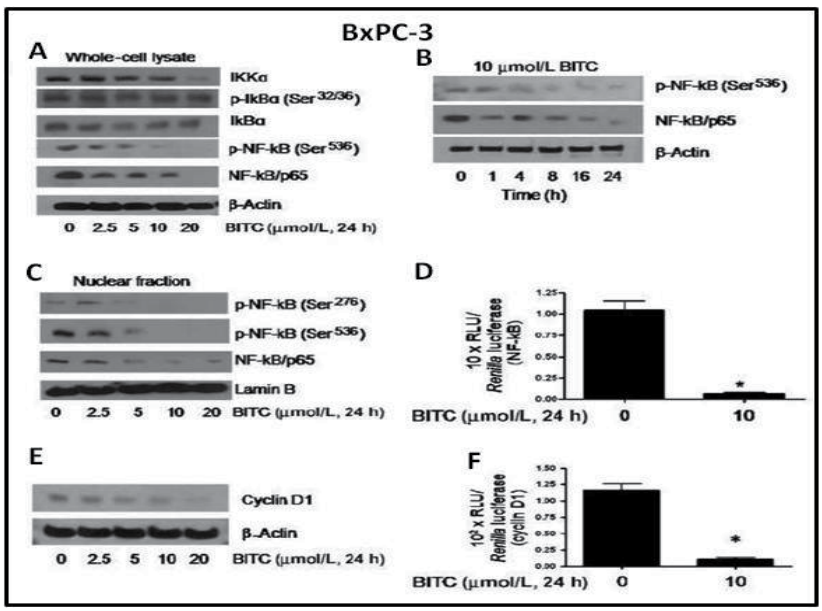

Fig. 7. BITC treatment causes inhibition of NF-κB and cyclin D1 in BxPC-3 pancreatic cancer cells. (Mol Cancer Ther 2010: 9(6):1596-608).

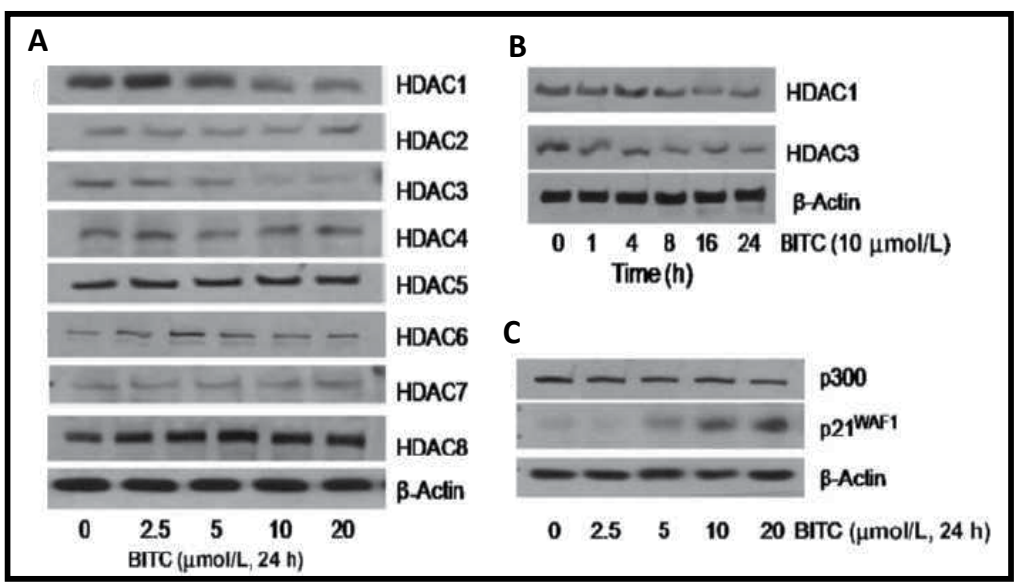

Fig. 8. BITC down regulates the expression of HDACs and p21 in BxPC-3 cells. (Mol Cancer Ther. 2010: 9(6);1596-608).

Role of HDACs in BITC-induced NFkB deacetylation was further substantiated by HDAC overexpression in BxPC-3 cells. HDAC1/3 overexpression significantly outweighed the effects of BITC in BxPC-3 cells. Furthermore, overexpression of HDACs protected BxPC-3 cells from BITC-induced apoptosis, as indicated by the reduced cleavage of caspase-3, PARP and increased survival in HDACs overexpressing BxPC-3 cells, as compared to BITC alone treated cells (Fig. 9).

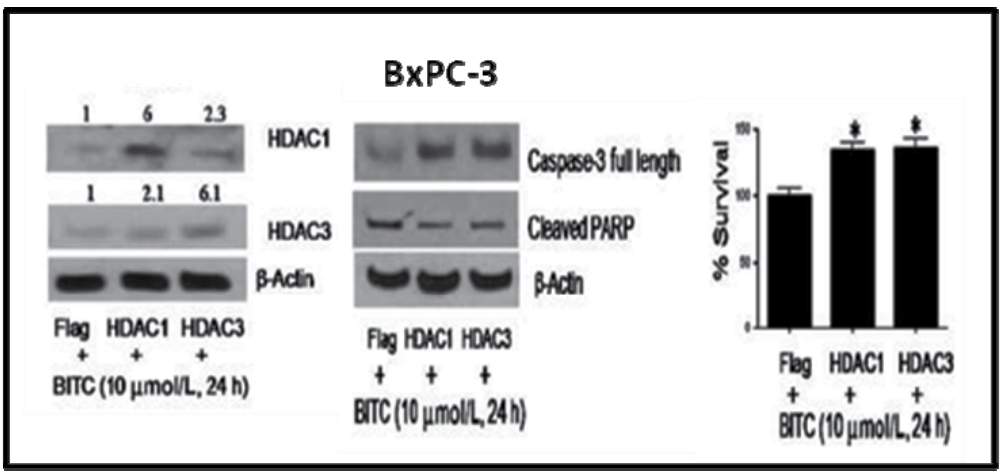

Fig. 9. Over expression of HDAC1/HDAC2 rescue BxPC-3 cells from BITC-induced apoptosis. (Mol Cancer Ther. 2010: 9(6);1596-608).

5. BITC induces ROS generation, DNA damage and cell cycle arrest in pancreatic cancer cells

As many drugs induce cell death in cancer cells by triggering ROS generation, it was quite obvious to see whether BITC could induce ROS generation in pancreatic cancer cells. In agreement with other drugs, BITC caused significant generation of H2O2 in Capan-2 cells in a dose and time dependent manner (Fig. 10). On the contrary, BITC induced a modest increase in the generation of hROS, such as singlet oxygen, superoxide, nitric oxide, hydroxyl and alkyl peroxide radicals in response to BITC treatment.

Eventually, BITC-induced ROS production substantially increased the phosphorylation of stress sensors, such as ERK (Thr202/Thy204), JNK (Thr183/Tyr185) and P38 (Thr180/Tyr182), (Fig. 11). The activation of ERK and JNK was as early as1 h after BITC treatment and was sustained until 12h. On the other hand, activation of P38 was observed around 24 h of BITC treatment (Sahu et al., 2009b).

BITC–induced ROS generation also resulted in DNA damage as evidenced by the phosphorylation of H2A.X at Ser-139, which is considered to be the hall mark of DNA double strand breaks (Sedelnikova et al., 2003). Interestingly, when BITC-treated cells were cultured in fresh medium without BITC for additional 48h cells showed persistent H2A.X phosphorylation (Fig. 12), indicating that BITC induce permanent DNA damage in Capan-2 cells (Zhang et al., 2006). As protective mechanism, DNA damage lead to cell cycle arrest to obtain brief window of time to compensate/repair the damage that occurred due to ROS

production. Accordingly, treatment of Capan-2 cells with BITC (10μM) for 24h resulted in the increased accumulation of cells in G2/M phase (42%) (Srivastava, 2004). The increased expression and phosphorylation of Chk2 (Thr-68) by BITC treatment caused G2/M arrest. Furthermore, BITC also decreased the phosphorylation and expression of Cdc25C (ser-216), Cdc2 (Tyr-15) and Cyclin B1in apan-2 cells, as compared to control cells (Fig.12).

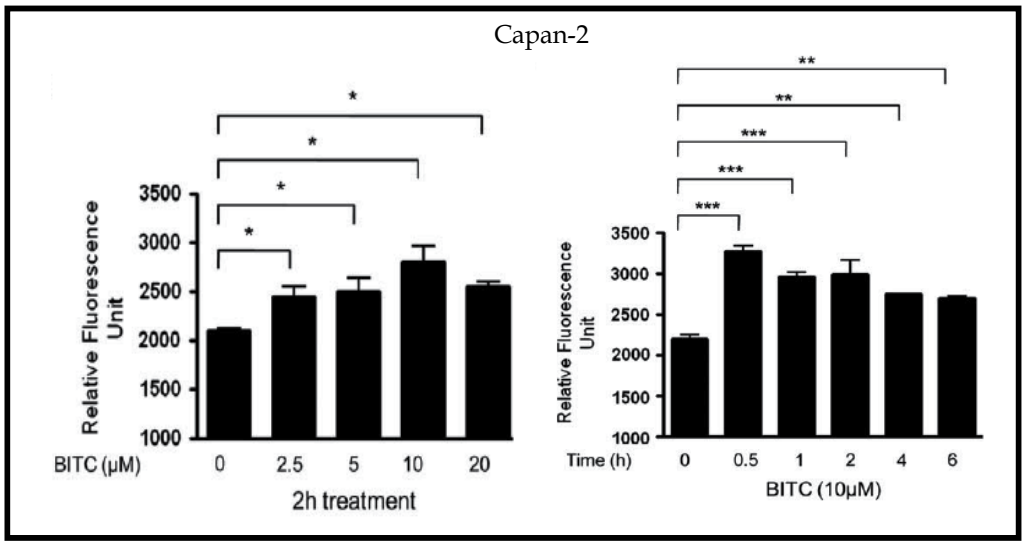

Fig. 10. BITC induces ROS generation in Capan-2 cells. (Carcinogenesis 2009: 30;1744–1753).

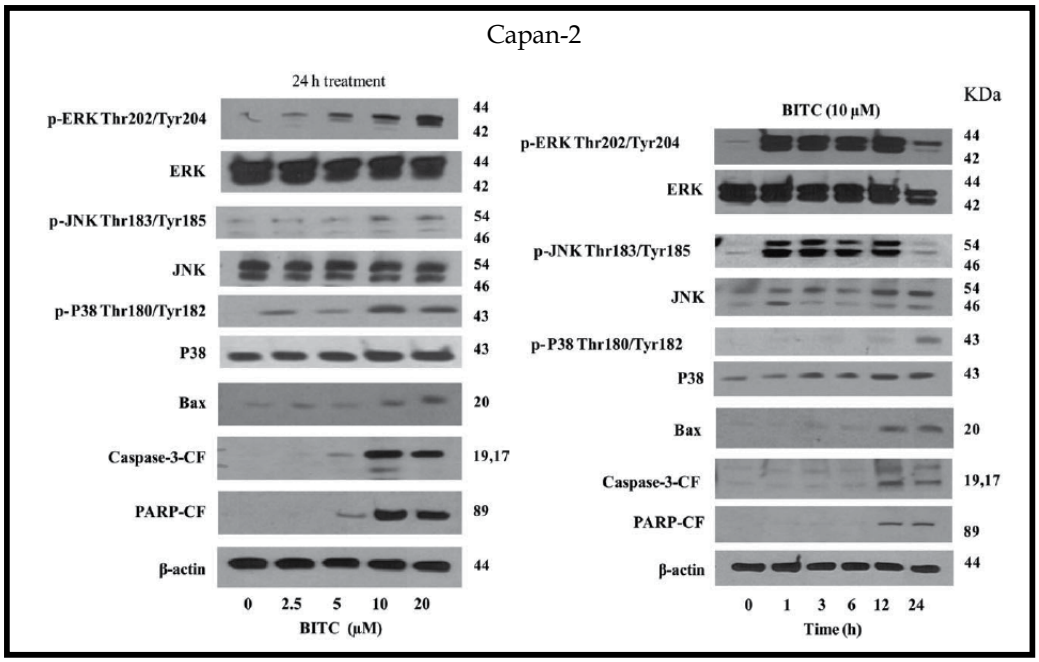

Fig. 11. BITC induces phosphorylation of MAP kinases. (Carcinogenesis 2009: 30;1744–1753).

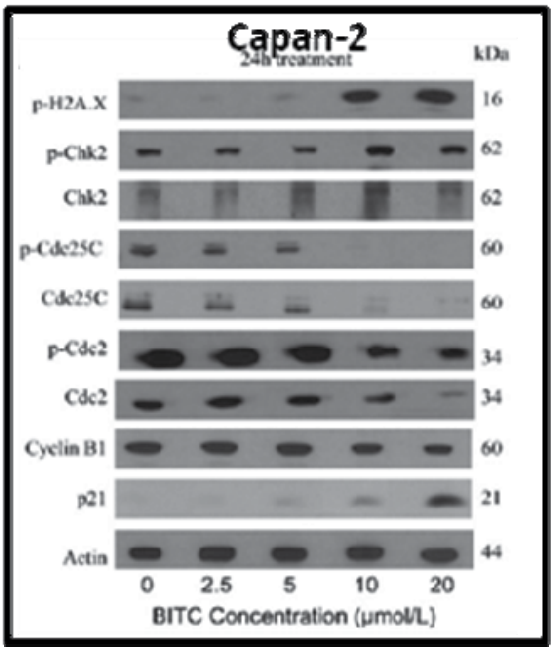

Fig. 12. Effect of BITC on cell cycle proteins. (J. Nutr. 2006: 136; 2728–2734).

Interestingly, although ERK, JNK and P38 were activated in response to BITC treatment, they had different roles in BITC-induced cell cycle arrest and apoptosis. MEK-1 inhibitor PD98059 significantly abrogated BITC induced G2/M cell cycle arrest and apoptosis (Fig. 13A, B&D). Whereas, both JNK (SP600125) and P38 (SB202190) inhibitors failed to protect the cells from BITC-mediated G2/M cell cycle arrest. Further, MEK-1 inhibitor blocked BITC-mediated activation of ERK as well as down-regulation of G2/M regulatory proteins such as cyclin-dependent kinase-1 (Cdk1), cyclin B1, Cdc25C and cleavage of caspase-3 and PARP, suggesting the involvement of ERK in BITC-induced G2/M cell cycle arrest and apoptosis (Fig. 13C). BITC-mediated apoptosis was almost completely blocked in the cells pre-treated with ERK, JNK or P38 inhibitors as evaluated by cell death apoptosis ELISA assay (Fig.13D). Similar results were obtained with MAPK8-shRNA in Capan-2 cells, indicating that all the MAPK were involved in BITC-induced apoptosis but only ERK was involved in BITC-induced cell cycle arrest.

Involvement of BITC-induced ROS generation in cell cycle arrest and apoptosis was further confirmed by treatment with antioxidants such as NAC, tiron, GSH and SOD. BITC-induced phosphorylation of MAPK and down regulation of cell cycle proteins such as GSH, Cdk1, Cdc25C, Cyclin B1 were significantly blocked by the treatment also with NAC (Fig.14). Furthermore, BITC-induced apoptosis was inhibited when cells were pre-treated with antioxidants, such as tiron, GSH and SOD. These results indicate that BITC induces ROS in pancreatic cancer cells which leads to DNA damage, cell cycle arrest and apoptosis.

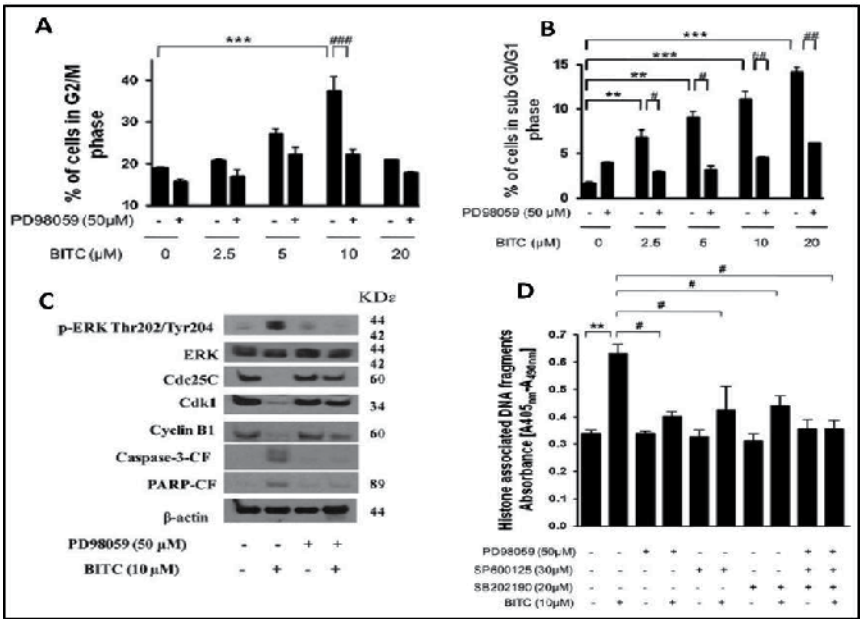

Fig. 13. MAPK inhibitors rescue pancreatic cancer cells from BITC induced apoptosis and cell cycle arrest. (Carcinogenesis 2009: 30;1744–1753).

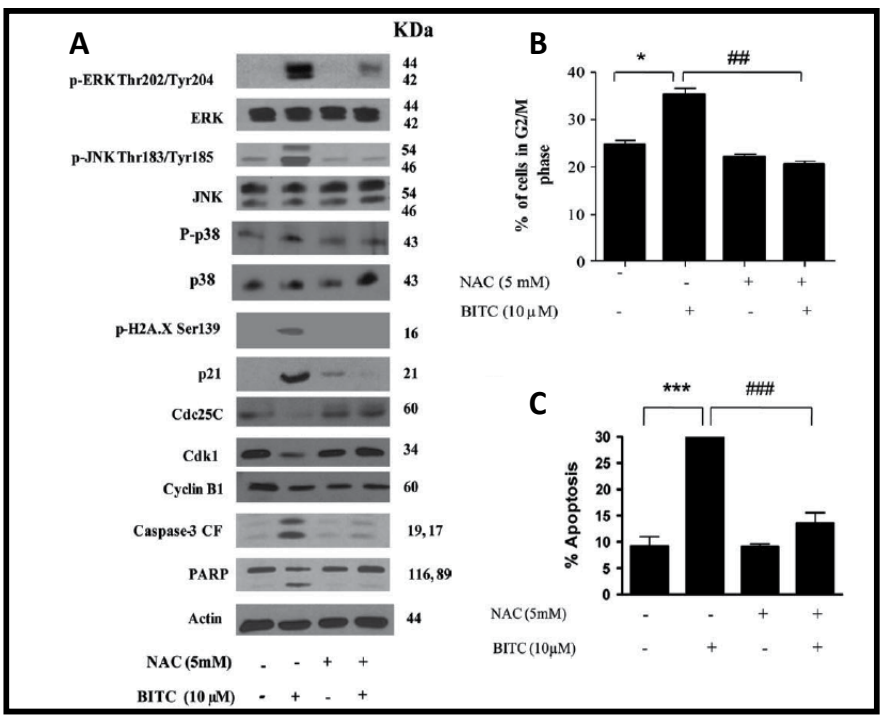

Fig. 14. Antioxidants protects Capan-2 cells from BITC-induced cell cycle arrest and apoptosis. (Carcinogenesis 2009: 30;1744–1753).

6. BITC sensitizes human pancreatic cancer cells to radiation and TRAIL treatment

Increased systemic toxicity and resistance are the major drawbacks of radiation therapy in pancreatic cancer treatment. Interestingly, BITC potentiated therapeutic effect of γ-irradiation in BxPC-3 cells. BxPC-3 cells were pre-treated with 2.5 or 5 μM BITC for 24h, followed by treatment with different doses of γ-irradiation (2.5, 5, 10 and 20Gy) at a dose of 4Gy/minute. The cells were allowed for 24 or 48h before being analyzed for survival assay. BxPC-3 cells pre-treated with BITC and treated with 5Gy γ-irradiation show intense cell death, as compared to either treatment alone, indicating that BITC sensitizes the cells to γ-irradiation (Sahu et al., 2009c). Furthermore, as shown in Table 1, more cells were accumulated in G2/M arrest in response to combination treatment, as compared to either treatment alone. In addition, expression of cell cycle proteins Chk2 and Cdc25 was increased in combination treated cells, as compared to control cells. Interestingly, DNA damage markers H2A.X (Ser-139) and ATR (Ser-428) also increased in combination treatment, indicating that BITC sensitizes cells to γ-irradiation. In agreement with cell cycle data, apoptosis induction was more in combination treated cells.

Similarly, BITC also potentiated the apoptosis inducing activity of TRAIL in pancreatic cancer cells. BxPC3 cells had a 3.84 fold increase in apoptosis upon treatment with BITC alone, an 8.65 fold increase was observed with TRAIL alone, and a 12.39 fold increase was seen when cells were treated with BITC combined with TRAIL. Similarly, Panc-1 cells underwent a 1.49 fold increase in apoptosis upon treatment with BITC, a 1.82 fold increase with TRAIL alone, and a 3.45 fold increase with BITC combined with TRAIL compared to vehicle. Interestingly, sensitization of pancreatic cancer cells to TRAIL by BITC was more in Kras wild type cells (BxPC-3) as compared to Kras mutated cells (PanC-1 and MIA PaCa-2). Further studies are needed to elucidate the role of Kras mutation in TRAIL or BITC-induced apoptosis.

7. BITC inhibits pancreatic cancer angiogenesis

Pancreatic tumors can acquire substantial development of new blood vessels in a process called angiogenesis (Philip, 2008). This vascular development is a necessary component of solid tumor growth and progression. Numerous reports have shown that disrupting tumor angiogenesis could effectively inhibit tumor growth and metastasis. BITC has shown promising potentials as anti-angiogenesis agent for pancreatic cancer *vitro* and *in vivo.*

In a rat aorta ring assay model, treatment with 5 μM BITC reduced sprouting of new blood vessels by 67% as compared to control aortic rings (Fig. 15A). Furthermore, 5 μmol BITC treatment drastically (70%) suppressed new embryonic blood vessel growth in each egg as compared to control eggs in a CAM assay model (Fig. 15B), indicating that BITC has potential to inhibit tumor angiogenesis (Boreddy et al., 2011b).

BITC was also effective in suppressing the secretion of pro-angiogenic factors from pancreatic cancer cells under both, normoxia and hypoxia conditions. Hypoxia alone induced the secretion of both MMP-2 and VEGF around 2-4 folds in both BxPC-3 and PanC-1 cells; however, BITC significantly inhibited the secretion of both VEGF and MMP-2 from the both BxPC-3 and PanC-1 cells under normoxia and hypoxia conditions (Fig. 16A-D). Interestingly, BITC significantly inhibited the migration and invasion of both, BxPC-3 and PanC-1 cells in a dose dependent manner. These steps are critical for the migration of the tumor cells *in vivo*.

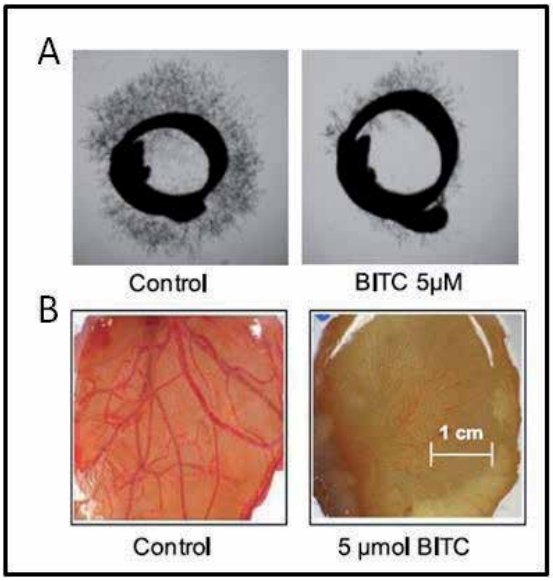

Fig. 15. BITC inhibits ex vivo angiogenesis. (PLoS ONE 2011: 6(10); e25799).

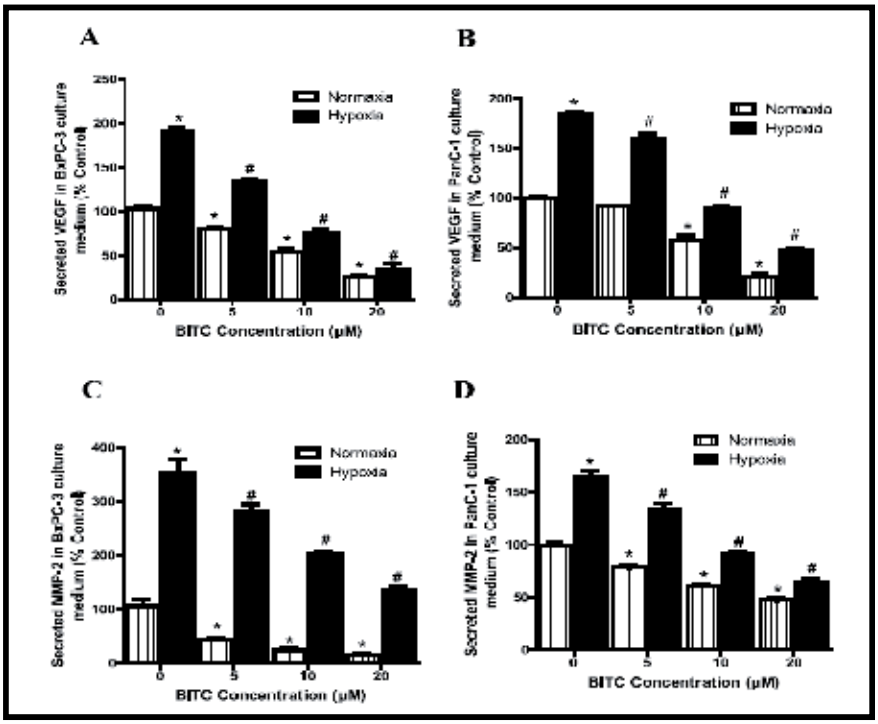

Fig. 16. BITC inhibits the secreastion of VEGF and MMP-2 in pancreatic cancer celss under both normoxia and hypoxia conditions. (PLoS ONE 2011: 6(10); e25799, 1-12).

Furthermore, BITC was quite effective in down regulating various angiogenic factors such as, HIF1-α, VEGFR-2, MMP-2, Rho A, Rho C and RAC1,2,3 in dose dependent manner in BxPC-3 and PanC-1 cells (Fig. 17A). Similarly, BITC inhibited the expression of angiogenic proteins in human endothelial cells (HUVEC) (Fig. 17B), in a dose dependent manner. Interestingly, BITC was ineffective in STAT3-overexpressing BxPC-3 cells. Furthermore, when STAT-3 was silenced in BxPC-3 cells the molecular changes were similar to that of BITC treatment changes indicating that BITC inhibits tumor angiogenesis by targeting STAT-3 (Fig. 17C&D).

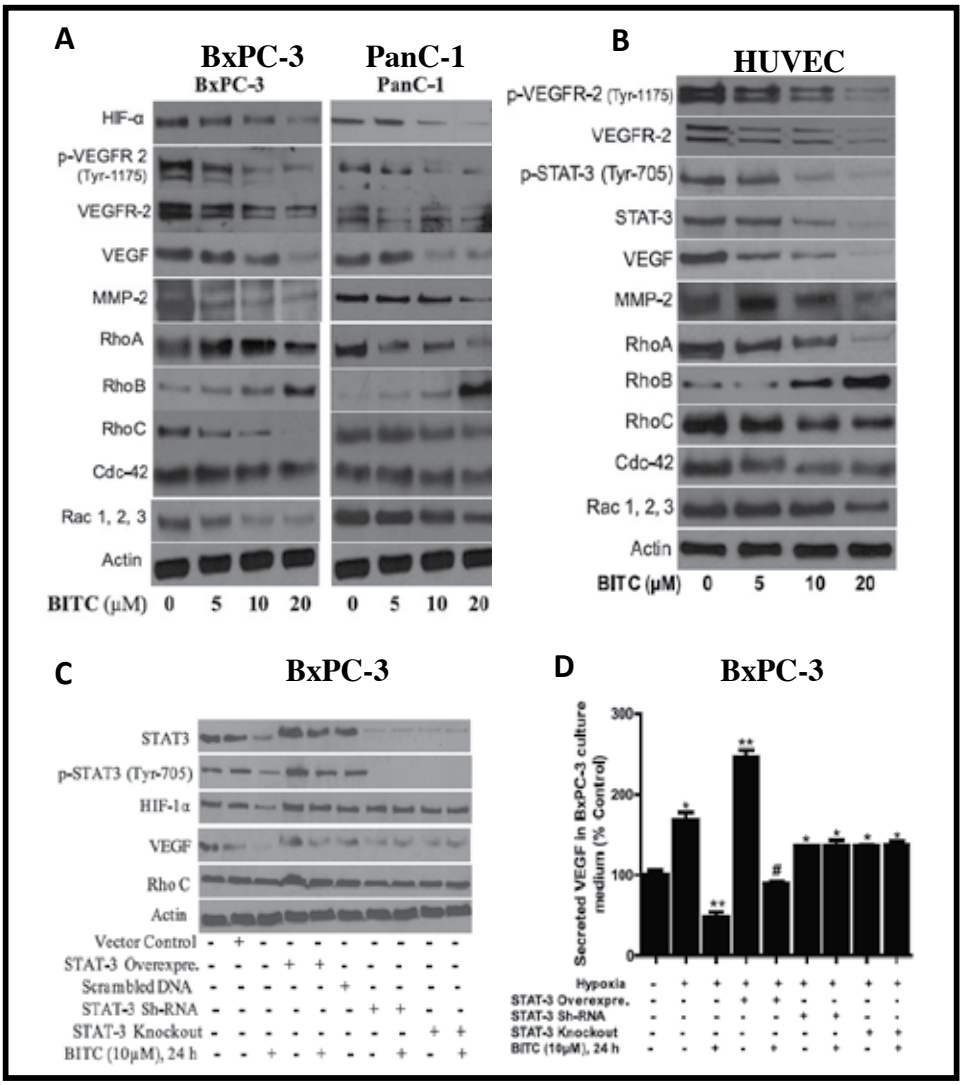

Fig. 17. BITC down regulates the critical molecules of angiogenesis in BxPC-3, PanC-1 and HUVECs by targeting the STAT3. (PLoS ONE 2011: 6(10); e25799, 1-12).

8. BITC suppresses pancreatic tumor growth and angiogenesis in vivo

BITC exhibited similar results *in vivo* as observed in culture. Tumor growth in BITC-fed mice was substantially retarded, as compared to control mice. Tumors appeared to grow more slowly in BITC-fed mice as compared with control mice. For example, 6 weeks after treatment with12 µmol BITC, the average tumor volume in control mice was about 1.92-fold higher than that in BITC-treated mice (mean tumor volume, control *vs* BITC treated: 334 vs172 mm^3, difference = 162 mm^3, 95% CI = 118 to 204 mm^3 ;P = .008; Fig.18A). Furthermore, average tumor weight in BITC-treated mice was 225mg, whereas in control mice tumor weight was 425mg (Fig. 18B), indicating that BITC potentially suppress the growth of pancreatic tumors *in vivo*. Interestingly, BITC-treated mice did not show any toxicity symptoms such as weight loss (Fig. 18C).

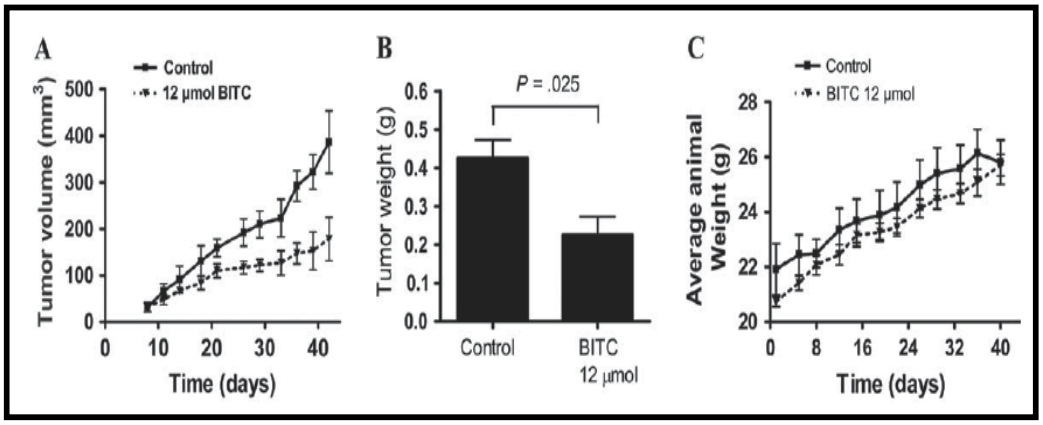

Fig. 18. BITC suppresses the growth of pancreatic cancer xenografts in vivo. (J Natl Cancer Inst 2009;101: 176-193).

It is noteworthy that when animals were orally gavaged with 12µmol/day BITC for 46 days, mean BITC concentration in plasma after1 hour of BITC administration was 6.5 ± 0.1 mmol/ L (n=10), whereas accumulated BITC concentration in the tumors after 46 days was 7.5 ± 0.3 µmol/g (n=10). These results indicate that the therapeutic concentration of BITC could be achieved *in vivo* by oral feeding.

A 76% reduction in hemoglobin content was observed in BITC-treated matrigel plugs that were implanted in Nu-Nu athymic nude mice as compared to untreated plugs (Fig. 19A). Similarly, BITC-treated tumor xenografts showed 61% reduced hemoglobin content as compared to untreated xenografts (Fig. 19B).

Tumors excised from BITC-treated mice showed reduced phosphorylation of STAT3 (Tyr-705 and Ser-727) (Fig. 20A), AKT (Ser-473 and Ser-308), FOXO1 (Ser-256) and FOXO3a (Ser-253) (Fig. 20B). Furthermore, protein expression of STAT3 and angiogenic proteins (Fig. 20C) was down regulated, whereas expression of AKT, FOXO1, FOXO3a remained unaltered. Nonetheless, Bim expression was significantly increased in BITC-treated tumor as compared to vehicle alone treated tumors indicating that the *in vivo effect* of BITC was similar to *in vitro* effects.

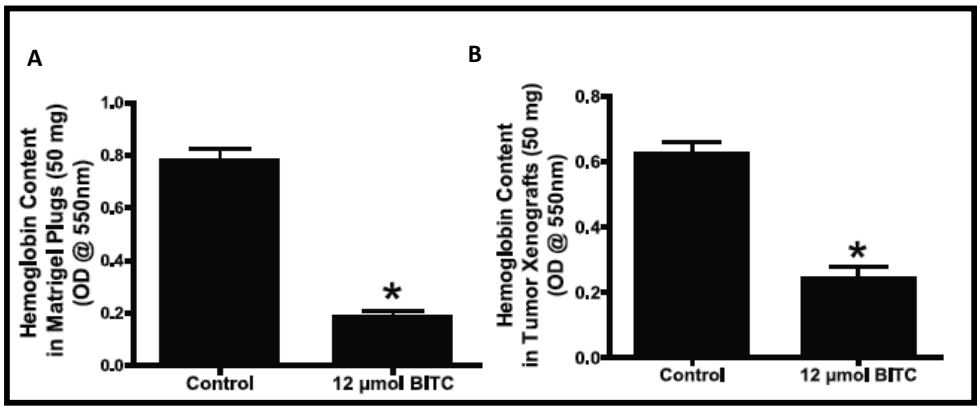

Fig. 19. BITC inhibits angiogenesis in vivo. (Clin Cancer Res 2011: 17(7); 1784–1795).

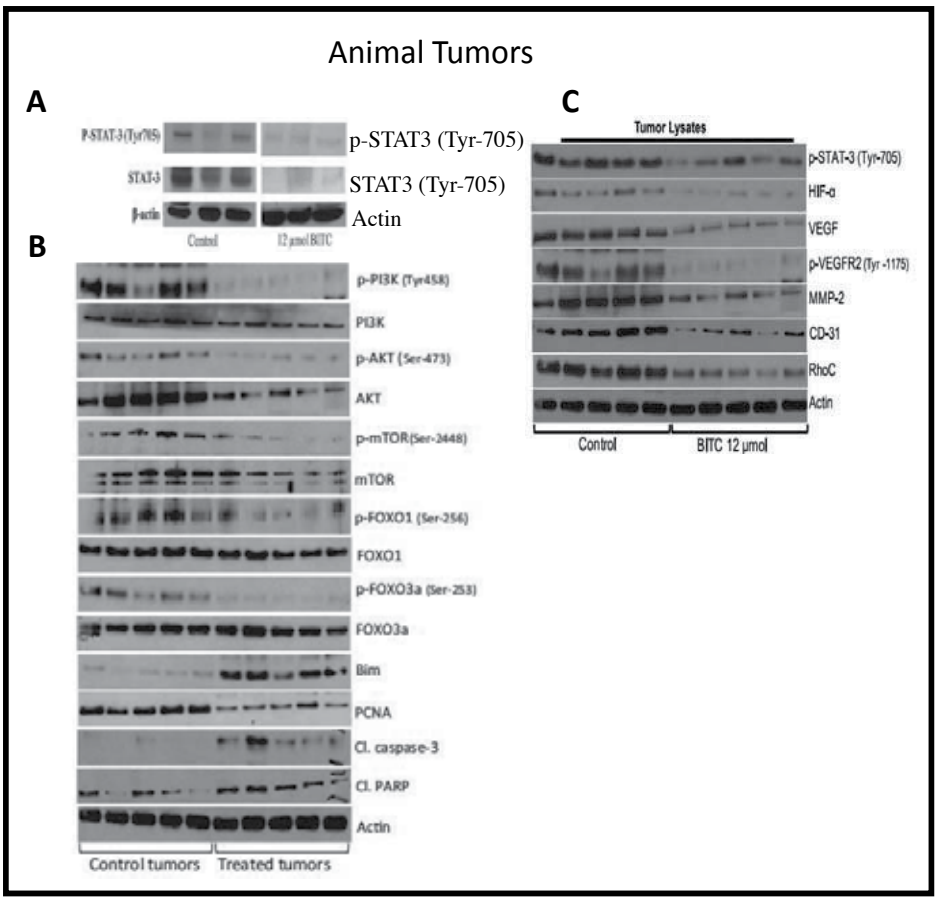

Fig. 20. BITC down regulates key molecules of survival and angiogenesis pathways. (Natl Cancer Inst 2009;101: 176-193. Clin Cancer Res 2011: 17(7); 1784–1795. PLoS ONE 2011: 6(10); e25799, 1-12).

9. Conclusion

9.1 Does BITC have multiple targets in pancreatic cancer?

Since, BITC inhibits the phosphorylation and protein levels of various key survival molecules such as STAT3, AKT and NFkB, indicating that BITC has multiple targets in pancreatic cancer. However, at this time, it is not clear whether BITC is targeting various survival pathways individually or it is the tandem effect upstream regulators. Since previous reports showed that STAT3 is being regulated by AKT through FOXO1 (Kortylewski et al., 2003) and NFkB is a direct target of AKT (Dan, 2008), presently we assume that AKT is the main target of BITC and other targets are obligated events but further studies are needed to conclude interaction of these pathways (Fig. 21).

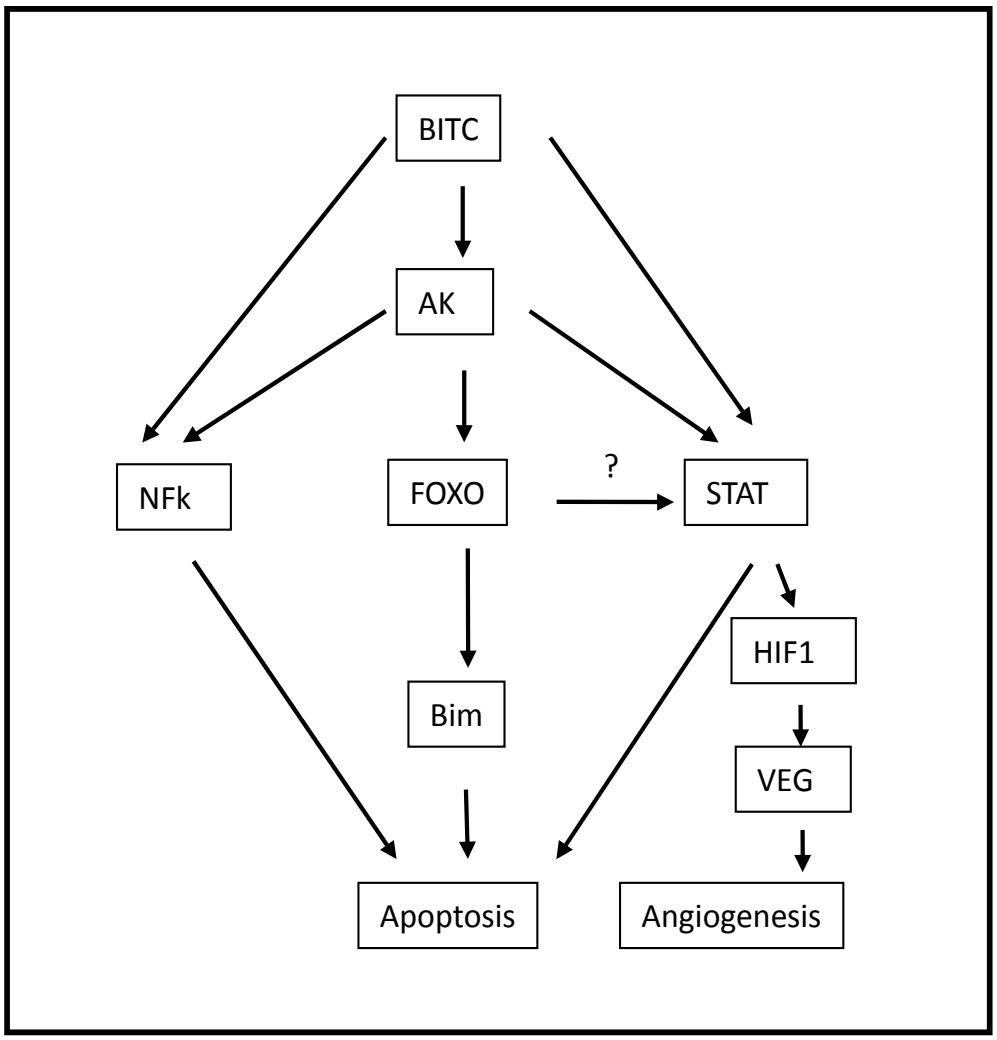

Fig. 21. BITC mechanism of action in pancreatic cancer

10. References

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12

The Potential Role of Curcumin for Treatment of Pancreatic Cancer

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1. Introduction

Gemcitabine has been the standard chemotherapy for advanced pancreatic cancer since 1997, when a randomized phase III study demonstrated that gemcitabine significantly improved cancer-related symptoms in comparison with 5-fluorouracil (5-FU) (Burris *et al.*, 1997). However, the survival benefit of gemcitabine is modest and the median survival time was 5.7 and 4.4 months for gemcitabine and 5-FU arm, respectively. Thus the prognosis of this disease still remains dismal and the development of a more effective therapy is urgently needed in daily clinical practice. For the past decade, many efforts have been made to improve the overall survival of patients with this disease by adding a second cytotoxic agent to gemcitabine. Several large phase III trials have compared gemcitabine alone with gemcitabine combination therapy (e.g. capecitabine, 5-fluorouracil, irinotecan, oxaliplatin, pemetrexed). However, none of them could demonstrate a significant survival advantage for the gemcitabine combination therapy over the gemcitabine monotherapy, despite a significant improvement in response rates (Berlin *et al.*, 2002; Herrmann *et al.*, 2007; Louvet *et al.*, 2005; Oettle *et al.*, 2005; Rocha Lima *et al.*, 2004). It is likely that the benefit of adding a second cytotoxic agent to gemcitabine is countered by increased toxicity and the decreased dose intensity of gemcitabine. Therefore, a new approach other than adding cytotoxic agents to gemcitabine is warranted. Since pancreatic cancer patients often suffer from cancer-related symptoms (e.g. fatigue, appetite loss, pain), it is very important to maintain a balance between efficacy and quality of life in palliative chemotherapy.

Curcumin is derived from turmeric (Curcuma longa) and is a natural polyphenol (Figures 1 and 2). Curcumin has long been used as a food (e.g. the popular Indian curry), coloring agent and traditional medicine (Aggarwal *et al.*, 2007; Strimpakos & Sharma, 2008).

Fig. 1. Turmeric (left panel) and curcumin (right panel)

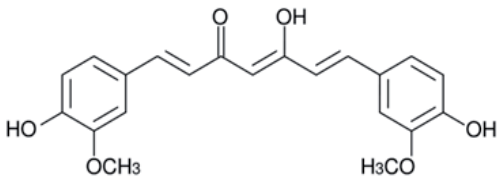

Fig. 2. Chemical structure of curcumin

A number of preclinical studies have demonstrated the anticancer effects of curcumin in a variety of tumors including pancreatic cancer, both *in vitro* and *in vivo,* and these promising data are now attracting the interest of many researchers in developing this agent as a chemopreventive as well as a chemotherapeutic drug (Corson & Crews, 2007). In contrast to conventional cytotoxic drugs, curcumin causes little toxicity, which is a great advantage of developing this agent for the treatment of pancreatic cancer patients, who are often intolerant to cytotoxic combination therapy due to their poor clinical condition. Safety is another advantage of this agent. The safety of curcumin has been approved by the Food and Drug Administration (FDA) and World Health Organization (WHO); however, its safety is most strongly supported by the fact that this agent has been used as a traditional Hindu or Chinese medicine for thousands of years. In this chapter, we highlight the potential role of curcumin for the treatment of pancreatic cancer by reviewing the published preclinical and clinical data.

2. Anticancer effects of curcumin

A Pubmed search using the key words 'curcumin' and 'cancer' demonstrated that more than 1500 articles have been published since 1983 and that this number has rapidly increased over the past 5 years (Figure 3). The potential anticancer effects of curcumin have been reported in a variety of preclinical models including breast, colon, gastric, head and neck, hepatic, ovarian, pancreatic and prostate cancer, leukemia and multiple myeloma, and well described in several review articles (Aggarwal *et al.*, 2007; Shishodia *et al.*, 2007; Strimpakos & Sharma, 2008). Curcumin can modulate a variety of molecules which play an important role in cancer progression. Among these molecules, nuclear transcription factor-κB (NF-κB) is one of the major targets of curcumin. Diverse upstream signals (e.g. growth factors, cytokines, hypoxia) can induce constitutive NF-κB activation in patients with cancer, including those with pancreatic cancer, and its activity is positively correlated with cancer

progression (Fujioka *et al.*, 2003; Wang *et al.*, 1999). For example, NF-κB activation can up-regulate the expression of a number of genes involved in anti-apoptosis (e.g. Bcl-2, Bcl-xL), proliferation (e.g. cyclin D1, *c*-myc), angiogenesis (e.g. vascular endothelial growth factor (VEGF), interleukin-6), and invasion (e.g. matrix metalloproteinases (MMP)), all of which play a pivotal role in cancer progression (Aggarwal, 2004). Therefore, inhibition of NF-κB activity by curcumin can effectively suppress tumor growth (Figure 4).

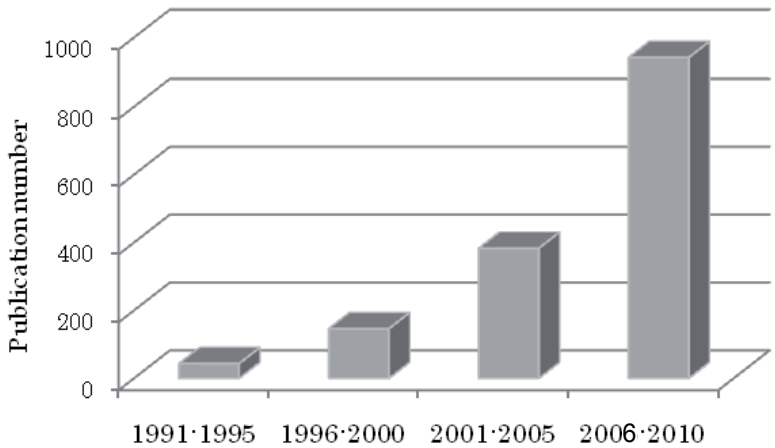

Fig. 3. Pubmed search results using the key words 'curcumin' and 'cancer'

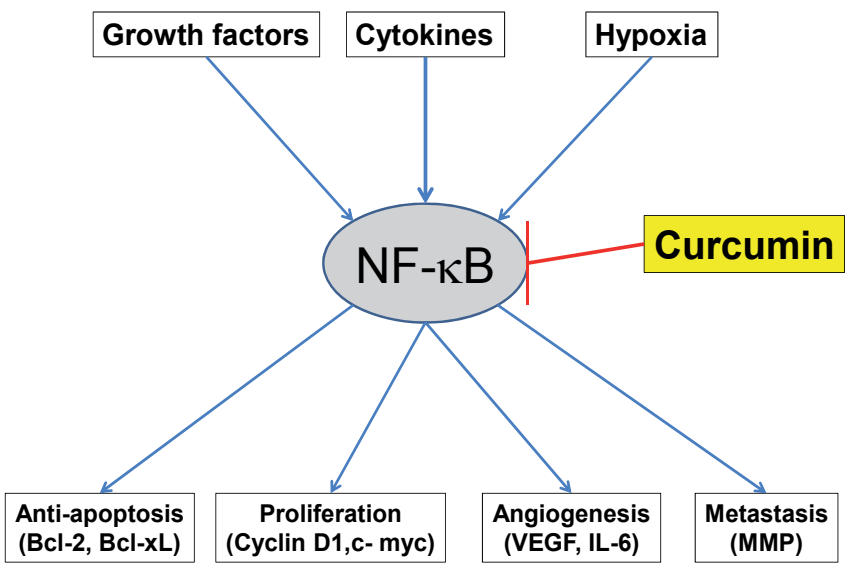

Fig. 4. NF-κB is one of the major molecular targets of curcumin

Other mechanisms involved in the anticancer effects of curcumin include the down-regulation of Akt, cyclooxygenase-2 (COX2), prostaglandin E2 or signal transducers and activators of transcription 3 (STAT3) (Aggarwal & Shishodia, 2006), which prevents cancer cells from escaping through alternative signaling pathways. Curcumin can also potentiate the anticancer effects of cytotoxic agents such as cisplatin, 5-fluorouracil and gemcitabine (Du *et al.*, 2006; Kunnumakkara *et al.*, 2007; Tsai *et al.*, 2011).

3. Preclinical data on anticancer effects of curcumin

Li et al. first reported the anticancer effects of curcumin on pancreatic cancer cells *in vitro* (Li *et al.*, 2004). They demonstrated that curcumin can suppress tumor growth of pancreatic cancer cell lines in a time and dose dependent manner through NF-κB inhibition**.** The efficacy of curcumin *in vivo* has also been demonstrated using an orthotopic mouse model of pancreatic cancer (Kunnumakkara *et al.*, 2007). While treatment with either curcumin (1 g/kg orally) or gemcitabine (25 mg/kg through intraperitoneal injection) demonstrated modest antitumor effects, the combination of curcumin with gemcitabine suppressed tumor growth more effectively than curcumin or gemcitabine alone. As expected, inhibition of NF-κB activity as well as the down-regulation of a variety of NF-κB-dependent gene products (cyclin D1, c-myc, Bcl-2, Bcl-xL, cellular inhibitor of apoptosis protein-1, cyclooxygenase-2, matrix metalloproteinase and VEGF) was observed in orthotopic tumor tissue after administration of curcumin. Other preclinical studies have also demonstrated the anticancer effects of curcumin either alone or in combination with gemcitabine in pancreatic cancer (Ali *et al.*, 2010; Strimpakos & Sharma, 2008; Wang *et al.*, 2006).

Based on these promising preclinical data, the main focus of research has now moved on to demonstrating the anticancer effects of curcumin in clinical trials.

4. Clinical trials using curcumin in patients with pancreatic cancer

Although the number of clinical trials is still limited compared to the numerous preclinical studies, several phase I or pharmacokinetic studies have been conducted with curcumin and found no dose limiting toxicity (DLT) up to at least 12 g/day when administered orally in both healthy volunteers (Lao *et al.*, 2006; Vareed *et al.*, 2008) and cancer patients (Cheng *et al.*, 2001; Garcea *et al.*, 2005; Sharma *et al.*, 2004). Minor toxicities of Grade 1-2 diarrhea and nausea have been reported, probably due to the oral intake of a bulky volume of curcumin at one time. Higher doses than 8 g/day of oral curcumin do not cause any DLT; however, these bulky volumes are unacceptable for daily oral intake. Moreover, doses above 8 g/day did not lead to a further increase in plasma curcumin levels due to poor bioavailability (Cheng *et al.*, 2001; Lao *et al.*, 2006; Vareed *et al.*, 2008). For these reasons, 8 g of daily oral curcumin is accepted to be the optimal dose for clinical trials in cancer patients.

Dhillon et al. were the first to report a phase II clinical trial, using 8 g of daily oral curcumin in patients with pancreatic cancer (Dhillon *et al.*, 2008). Twenty-five patients were enrolled in this study and 22 patients (88%) had a history of prior chemotherapy. Out of the 22 patients evaluable for response, 2 patients demonstrated some clinical benefit. One patient had stable disease for more than 18 months and the other patient achieved a partial response in a liver metastasis (73% decrease in the size), although it lasted for only 1 month. Curcumin was safe in patients with pancreatic cancer and no toxicity associated with

curcumin intake was reported. Furthermore, inhibition of NF-κB activity after curcumin intake was demonstrated using peripheral mononuclear cells from patients.

We also conducted a phase I/II clinical trial using curcumin for patients with pancreatic cancer who had become resistant to gemcitabine-based chemotherapy (Kanai *et al.*, 2010). In contrast to the study by Dhillon et al. which tested the safety and efficacy of curcumin monotherapy, our study evaluated the safety and feasibility of adding curcumin to gemcitabine-based chemotherapy, because no previous studies had demonstrated the safety and feasibility of this combination. In the phase I study, the safety of 8 g of daily curcumin in combination with gemcitabine-based chemotherapy was evaluated. In line with previous reports evaluating curcumin monotherapy, the first 3 assessable patients enrolled for the phase I study completed their first cycle without a predefined DLT (Grade 4 leucopenia; Grade 4 neutropenia; Grade 3 or more thrombocytopenia; non-hematological of Grade 3 or more; patient refusal due to the intolerability of curcumin intake). Therefore, we selected this dose as the recommended dose for the following phase II study. In total, 21 patients who showed disease progression during gemcitabine-based chemotherapy (gemcitabine/S-1 combination therapy for 19 patients and gemcitabine monotherapy for 2 patients) were enrolled. Adding curcumin did not increase the risk of clinically relevant toxicity, and the toxicity profile was comparable with that observed in pancreatic cancer patients treated with gemcitabine-based chemotherapy. No patients showed intolerance to 8 g of daily oral curcumin, and the median compliance rate was as high as 100% (range 79-100%), indicating that there was little toxicity due to curcumin even if administered concurrently with cytotoxic agents. Cumulative toxicity due to curcumin was not observed and 4 patients were able to continue this intake for more than 6 months, which indicates the safety of this agent for long-term use. Albeit the preliminary results from a small sample size, the median survival time (MST) of 161 days (95% CI 109-223 days) and a 1-year survival rate of 19% (95% CI 4.4-41.4%) were encouraging considering the poor prognosis of pancreatic cancer patients for whom gemcitabine-based chemotherapy has failed (Figure 5).

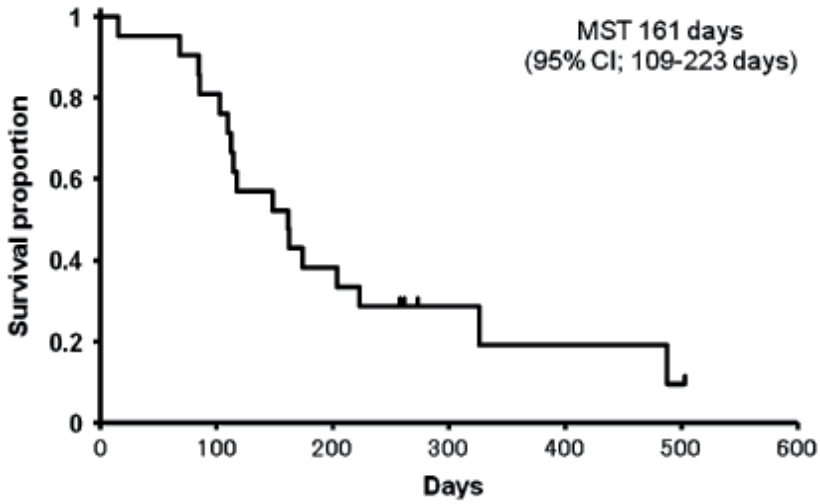

Fig. 5. Overall survival of patients with advanced pancreatic cancer treated with gemcitabine-based chemotherapy plus curcumin (n = 21) (adapted from Kanai et al. 2011)

Interestingly, several patients reported an improvement in cancer- or chemotherapy-related symptoms (e.g. fatigue, pain, constipation). We cannot rule out the placebo effect; however, several preclinical studies demonstrating that curcumin can improve fatigue, depression or neuropathic pain support our current observation (Gupta *et al.*, 2011; Sharma *et al.*, 2006; Xu *et al.*, 2005). Therefore, curcumin could improve the quality of life of patients with pancreatic cancer by alleviating cancer-related symptoms, and this could indirectly contribute to the improved overall survival.

Recently, another clinical trial has been reported by Epelbaum et al., who investigated the efficacy and feasibility of curcumin in combination with gemcitabine monotherapy in patients with advanced pancreatic cancer (Epelbaum *et al.*, 2010). Seventeen chemo-naïve patients were enrolled and received a standard dose and schedule of gemcitabine in combination with 8 g of daily oral curcumin. In contrast to our results showing a good compliance rate and low toxicity using 8 g of daily oral curcumin, this study reported that 5 patients (29%) discontinued curcumin after a few days to 2 weeks due to intractable abdominal fullness or pain. Furthermore, the dose of curcumin was reduced to 4 g/day because of abdominal complaints in 2 other patients. They discussed the possibility that increased gastrointestinal toxicity could be caused by the combination of curcumin and gemcitabine and concluded that 8 g of oral curcumin is not feasible when combined with gemcitabine in patients with pancreatic cancer. The reasons for the discrepancy between our study and that of Epelbaum et al. are unclear at this moment. Ethnic differences may exist in compliance to the combination therapy of curcumin and gemcitabine. Another possible explanation is that the patients' clinical condition at base line was poorer in Epelbaum's study than in ours, and abdominal fullness or pain could therefore be mainly attributable to cancer-related symptoms.

Table 1 summarizes the published clinical trials using curcumin in patients with pancreatic cancer.

	Dhillon et al.	Epelbaum et al.	Our study
Sample size	25	17	21
Study design	Phase II	Phase II	Phase I/II
Study period	2008*	2004-2006	2008-2009
Dose of curcumin	8 g/day	8 g/day	8 g/day
Prior history of chemotherapy	22	none	21
Combination with gemcitabine	no	yes	yes
Toxicity attributable to curcumin	none	7 (Abdominal discomfort)	none

* Publication year

Table 1. Comparison of the published clinical trials using curcumin in patients with pancreatic cancer

5. Development of a new form of curcumin with improved bioavailability

Several investigators, including ourselves, have tested plasma curcumin levels in clinical trials, and most studies report that plasma curcumin levels remained at low ng/ml levels in spite of taking gram doses of curcumin (Cheng *et al.*, 2001; Garcea *et al.*, 2005; Kanai *et al.*, 2010; Sharma *et al.*, 2004; Shoba *et al.*, 1998) (Table 2). As described in the previous section, the intake of more than 8 g of oral curcumin did not lead to a further increase in plasma curcumin levels in human subjects (Cheng *et al.*, 2001; Lao *et al.*, 2006; Vareed *et al.*, 2008). Thus, poor bioavailability is the major weak point of curcumin and has been the main challenge for physicians seeking to verify the therapeutic efficacy of this promising agent in clinical trials. Therefore, many efforts have been made to improve its bioavailability through several approaches including innovative drug delivery systems (liposomes, nanoparticles and phospholipids) (Anand *et al.*, 2010; Antony *et al.*, 2008; Bisht *et al.*, 2007; Das *et al.*, 2010; Gupta *et al.*, 2009; Koppolu *et al.*, 2010; Li *et al.*, 2005; Liu *et al.*, 2006; Marczylo *et al.*, 2007; Mukerjee & Vishwanatha, 2009; Sahu *et al.*, 2008; Shaikh *et al.*, 2009; Sou *et al.*, 2008; Takahashi *et al.*, 2009), or the development of new curcumin analogues (Lin *et al.*, 2011; Mosley *et al.*, 2007; Ohori *et al.*, 2006; Sato *et al.*, 2011). A nanoparticle-based drug delivery system is effective in improving the water solubility of hydrophobic agents like curcumin, and the development of at least 8 different types of nanoparticle-based curcumin have been published up to this point (Anand *et al.*, 2010; Bisht *et al.*, 2007;

Subjects	Dose of curcumin	Sample size	Plasma curcumin level (mean ± SE)	Reference
Healthy volunteers	2 g/day	8	6 ± 5 ng/ml	(Shoba *et al.*, 1998)
Patients with precancerous lesions	8 g/day	2	651 ± 688 ng/ml	(Cheng *et al.*, 2001)
Patients with colorectal ca.	3.6 g/day	3	4 ± 0.2 ng/ml	(Sharma *et al.*, 2004)
Healthy volunteers	12 g/day	3 (1)	57 ng/ml*1	(Lao *et al.*, 2006)
Patients with colorectal ca.	3.6 g/day	3	below 1 ng/ml	(Garcea *et al.*, 2005)
Healthy volunteers	8 g/day	6	2300 ± 260 ng/ml	(Vareed *et al.*, 2008)
Patients with pancreatic ca.	8 g/day	5	134 ± 70 ng/ml	(Kanai *et al.*, 2010)
Healthy volunteers	0.03 g/day*2	7	29.5± 13 ng/ml	(Sasaki, 2011)
Healthy volunteers	0.21 g/day*2	6	275 ± 67 ng/ml	(Kanai *et al.*, 2011)

*1 Plasma curcumin was detected in only one subject.

*2 THERACURMIN® was used in these studies

Table 2. Comparison of the published plasma curcumin levels in human subjects (adapted from Kanai et al. 2011)

Das *et al.*, 2010; Gupta *et al.*, 2009; Mukerjee & Vishwanatha, 2009; Sasaki, 2011; Shaikh *et al.*, 2009; Sou *et al.*, 2008).

Out of these new forms of nanoparticle-based curcumin, we chose to focus on THERACURMIN®, which demonstrated a more than 30-fold higher bioavailability compared to that of conventional curcumin in rat models (Sasaki, 2011). We conducted a dose-escalation and pharmacokinetic study using this newly developed nanoparticle curcumin to verify its improved bioavailability in human subjects. Six healthy human volunteers were recruited and received THERACURMIN® at a single oral dose of 150 mg. After an interval of 2 weeks, the same subjects then received THERACURMIN® at a single oral dose of 210 mg. C_{max} for THERACURMIN® at 150 mg and 210 mg was 189 ± 48 and 275 ± 67 ng/ml (mean ± S.E.M.), respectively and the area under the curve for 24 h was estimated to be 2649 ± 350 and 3649 ± 430 ng/ml × h (mean ± S.E.M.), respectively (Figure 6. Kanai, 2011).

These results indicate that an intake of 150 mg of THERACURMIN® could lead to similar or even higher plasma curcumin levels in comparison with those observed after the intake of 8 g of conventional curcumin (Table 2). As for the safety, only one subject reported grade 1 diarrhea lasting from day 1 to day 4 after 150 mg of THERACURMIN® intake. However, diarrhea did not recur after the second, 210 mg dose of THERACURMIN® intake in this subject. No other adverse events were observed. These results suggest that THERACURMIN® can safely increase plasma curcumin levels in a dose dependent manner up to at least 210 mg without saturating the absorption system. If we can achieve higher plasma curcumin levels, there is a greater chance that patients will benefit from this agent. Therefore, we consider that this new form of curcumin could be a promising tool when testing the potential anticancer effects of curcumin in clinical trials, and we are now conducting clinical trials to test this new agent in patients with pancreatic cancer.

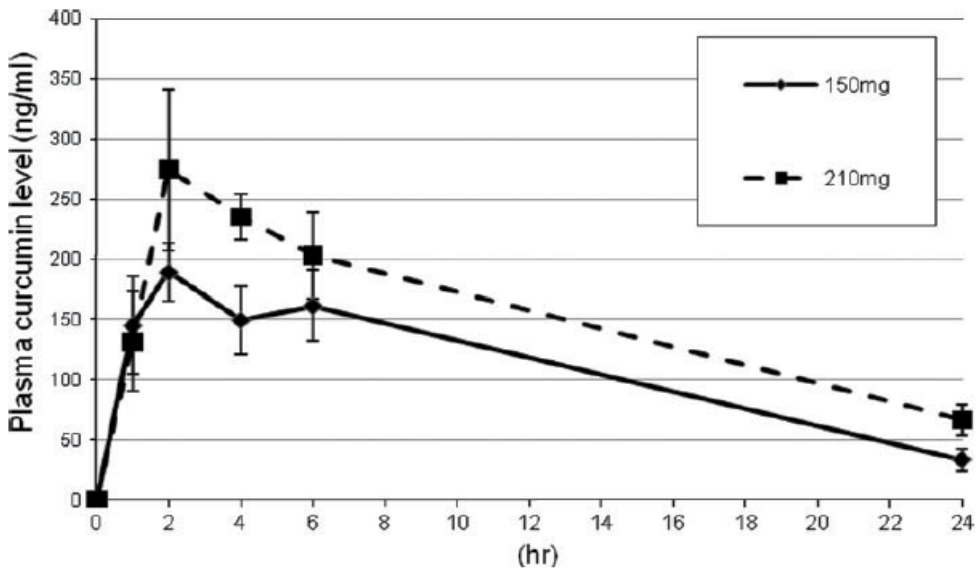

Fig. 6. Time course of plasma curcumin levels after intake of 150mg (solid line) and 210 mg (dash line) of THERACURMIN® (n = 6). Error bar represents S.E.M. (adapted from Kanai et al. 2011)

6. Conclusion

More and more data support the idea that curcumin could be a promising anticancer drug. Curcumin can exhibit anticancer effects through inhibiting diverse signaling pathways with minimal toxicity. On the other hand, poor bioavailability has been the main challenge in demonstrating the benefits of this promising agent in clinical trials. This problem has now been overcome by the development of nanoparticle curcumin and we can achieve higher plasma curcumin levels without saturating the absorption system. We are now conducting clinical trials to test the safety and efficacy of this new form of curcumin in patients with pancreatic cancer.

7. Acknowledgements

We thank Yasuko Nakagawa, Atsushi Imaizumi, Yoshitaka Ohtsuka, Hiroki Sasaki for their contribution to this work. This work was supported by the Grant-in-Aid from the Japanese Research Foundation for Clinical Pharmacology.

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Immunotherapy for Pancreatic Cancer

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1. Introduction

Patients with pancreatic cancer have an especially poor prognosis, with a 5-year survival rate of <1% and a median survival of 4-6 months (Jemal, Siegel et al., 2010). The management of patients with pancreatic cancer depends on the extent of the disease at diagnosis. However, approximately 80% of patients present with advanced-stage disease that precludes surgical resection (pancreaticoduodenectomy) and long-term survival is poor (Sener, Fremgen et al., 1999). Even after resection, the majority of patients relapse, leading to a median survival of about 18 months after resection (Neoptolemos, Stocken et al., 2004). In this time, gemcitabine-based chemotherapy is typically offered as standard of care. However, most patients treated with gemcitabine alone do not survive longer than 6 months, as the tumor cells are naturally resistant to current chemotherapy (Neoptolemos, Stocken et al., 2004). Importantly, the tumors that develop gemcitabine resistance would still be a suitable target for immunotherapy. Therefore, cancer immunotherapy for pancreatic cancer may be one attractive approach to treatment. This chapter summarizes the effect of immunotherapy for inducing cytotoxic T lymphocytes (CTLs) in patients with pancreatic cancer and discusses recent advances in concept of combination therapy of immunotherapy and chemotherapy.

2. Chemotherapy

Gemcitabine (2′2′-difluorodeoxycytidine) is a synthetic pyrimidine nucleoside analog that has become the standard first-line treatment for patients with advanced pancreatic cancer

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based on clinical benefit and survival improvement compared with 5-fluorouracil (5-FU)-based chemotherapy (Burris, Moore et al., 1997). Gemcitabine is phosphorylated intracellularly to difluorodeoxycytidine triphosphate, which terminates DNA-chain elongation and competitively inhibits DNA polymerase and ribonucleotide reductase, leading the cells into the apoptotic pathway (Storniolo, Allerheiligen et al., 1997). However, most patients treated with gemcitabine alone do not survive longer than 6 months. Moreover, the addition of the cytotoxic agents (platinums, fluoropyrimidines, or topoisomerase inhibitors) or radiation therapy to gemcitabine did not lead to a statistically significant improvement in overall survival (OS) in patients with metastatic pancreatic cancer (Moore, Goldstein et al., 2007; Van Cutsem, Verslype et al., 2007; Philip 2008b; Cascinu, Berardi et al., 2008). Recently, Thierry Conroy and colleagues randomly assigned 342 patients to receive combination chemotherapy regimen of FOLFIRINOX (consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin) (n=171) or gemcitabine (n=171) (Conroy, Desseigne et al., 2011). In selected patients with good performance status ECOG 0-1, the FOLFIRINOX regimen, when compared with gemcitabine, was associated with significantly increased median survival from 6.8 to 11.1 months. However, as compared with gemcitabine, FOLFIRINOX had increased toxicity. Gemcitabine is still the reference treatment in patients with ECOG performance status 2. Therefore, there is still great need for a novel therapeutic approach with low toxicity for advanced pancreatic cancer. Cancer immunotherapy for pancreatic cancer may be one attractive approach to cancer treatment.

3. Targeted therapy

The era of targeted therapies has generated a lot of interest in discovering better approaches for patients with pancreatic cancer. While traditional cytotoxic drugs also target specific cellular process, the newer generation of agents is set apart by their targeting of a pathway or molecule that derives the growth, speed, survival, or maintenance of tumor cells specially. Overexpression of human epidermal growth factor receptor type 1 (HER1/EGFR) has been suggested to be associated with the malignant transformation of pancreatic cancer (Tobita, Kijima et al., 2003). Therefore, there is a sound rationale for combining HER1/EGFR inhibitor and gemcitabine in pancreatic cancer. Erlotinib (Taraceva, Genentech, South San Francisco) is a small molecule HER1/EGFR tyrosine kinase inhibitor. Pancreatic cancer patients given the combination of erlotinib with gemcitabine showed a statistically significant improved survival compared with those given gemcitabine alone (Moore, Goldstein et al., 2007). The median and 1-year survival rates were better for the combination treatment: 6.24 months versus 5.91 months and 23% versus 17%, respectively. Therefore, the US Food and Drug Administration (FDA) recently approved erlotinib for use in the first-line setting of advanced pancreatic cancer in combination with gemcitabine. However, this survival benefit was small and, therefore, erlotinib has not yet been widely incorporated into standard treatment protocols. On the other hand, cetuximab, a monoclonal antibody, has been shown to significantly suppress the growth of implanted pancreatic cancer cells, and this effect was enhanced by the addition of gemcitabine in mice study (Bruns, Harbison et al., 2000). The study evaluating cetuximab in pancreatic cancer has been completed. In patients with advanced pancreas cancer, cetuximab did not improve the outcome compared with patients treated with gemcitabine alone (Philip, Benedetti et al., 2010). Moreover, the

addition of cetuximab to gemcitabine did not contribute to improvement in the patient-reported health-related quality of life (HRQL) outcomes (Moinpour, Vaught et al., 2010). The next generation of single-target trials is moving toward a focus on antiangiogenic agents, including anti-VEGF and anti-VEGFR strategies combined with gemcitabine. However, the addition of Axitinib that is a potent, selective inhibitor of vascular endothelial growth factor (VEGF) receptors 1, 2, and 3 tyrosine kinase also did not improve overall survival in advanced pancreatic cancer (Kindler, Ioka et al., 2011). These results add to increasing evidence that targeting of EGFR or VEGF signaling is an ineffective strategy in pancreatic cancer. Other chemotherapy, including S-1, ixabepilone, nanoparticle albumin-bound (nab) paclitaxel, FOLFOX (5-FU, leucovorin, oxaliplatin), and XELOX (capecitabine, oxaliplatin) may be better partners with targeted agents (Philip 2008a).

4. Immunotherapy

T cells with the αβ T-cell receptor (TCR) generally express CD4+ or CD8+ lineage markers and mostly fall into helper or cytotoxic subsets, respectively (Boon, Coulie et al., 1997). On the other hand, T cells expressing the alternate γδ TCR generally do not express lineage markers. Although CD8+ naive T cells recognize peptides (usually 8-10 amino acids) derived from tumor-associated antigens (TAAs) bound by major histocompatibility complex (MHC) class I molecules on tumor cells, it is not sufficient to initiate a productive generation of antigen-specific CTLs. Induction of CD8+ CTLs need peptides derived from TAAs to be presented on the surface of antigen presenting cells (APCs) in the context of MHC molecules. Moreover, CD4+ T cells recognize peptides (usually 10-30 amino acids) in association with MHC class II molecules on APCs and mediate their helper functions by enhancing the persistence of antigen-specific CD8+ CTLs or through secretion of cytokines such as interleukin (IL)-2 and interferon (IFN)-γ (Steinman and Swanson 1995; Banchereau & Steinman 1998). Therefore, the αβ TCR interaction with complex of peptides and MHC class I and class II molecules on APCs is a central event in T-cell-mediated antitumor immune responses. Antigen-specific CD8+ CTLs can respond to TAAs derived peptides presented in the context of MHC class I molecules on tumor cells. Therefore, efforts have focused on generating TAAs-specific αβ CD8+ CTLs (Waldmann 2003).

Dendritic cells (DCs) are powerful APCs that play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses (Steinman 1991). DCs can process endogenously synthesized antigens into antigenic peptides, presented to the cell surface as MHC class I-peptide complexes, and recognized by the αβ TCR in CD8+ naive T cells (Steinman 1991). DCs are also capable of capturing and processing of exogenous antigens, and presenting antigenic peptide on MHC class I molecules through an endogenous pathway, a process known as antigen cross-presentation (Berard, Blanco et al., 2000). In the case of cancer, cross-presentation after uptake and processing of soluble or particulate matter from apoptotic, necrotic cancer or even live cancer cells is the only important natural mode of presentation (Melief 2003). On the other hand, exogenous antigens from the extracellular environment are captured and delivered to the compartments of the endosome/lysosome, where they are degraded to antigenic peptides by proteases and peptidases, which are complexed with MHC class II and recognized by the αβ TCR in CD4+ naive T cells (Steinman 1991). Although both immature and mature DCs

are capable of processing and presenting MHC/peptide complexes to TCR, mature DCs are significantly better at CTL induction due to higher expression of MHC class I and class II and costimulatory molecules (Banchereau & Steinman 1998). On the other hand, presentation of antigens by immature DCs, in the absence of proper costimulation, may lead to tolerance induction (Banchereau & Palucka 2005). After antigens uptake and inflammatory stimulation, immature DCs in peripheral tissues undergo a maturation process characterized by the up-regulation of MHC class I and class II and costimulatory molecules, chemokine receptors such as CCR7, and the secretion of cytokines such as IL-12 (Banchereau & Steinman 1998; Forster, Schubel et al., 1999; Steinman 1991). During the process, mature DCs migrate to T-cell areas of secondary lymphoid organs, where they present antigens to CD4+ and CD8+ T cells through MHC class I and class II pathways, respectively (Steinman 1991; Banchereau & Steinman 1998; Banchereau & Palucka 2005). The αβ TCR in CD8+ CTL can recognize MHC class I-peptide complexes on cancer cells and destroy cancer cells through effector molecules such as granzyme B and perforin (Finn 2008). On the other hand, γδ T cells generally do not require MHC for antigen presentation, and recognize nonpeptidic antigens. As effective antitumor responses depend on the presence and function of immune cells that are able to recognize and eliminate cancer cells, the aim of immunotherapy is to activate both CD8+ CTLs that recognize TAAs-specific antigens and CD4+ T helper (Th) cells that mediate helper function.

4.1 Immune homeostasis

Now, it is becoming clear that CD4+ Th cells are critical in combating cancer cells and maintaining immune homeostasis. Upon TCR-mediated cell activation, naive CD4+ T cells can differentiate into at least four major polarization patterns including Th1, Th2, regulatory T (Treg), and Th17 cells, all of which participate in different types of immune responses (Zhu & Paul 2010) (Fig. 1). Mainly, immune homeostasis is controlled by two distinct helper T cell subsets, Th1 and Th2 cells. The Th1 cells secrete type I cytokines such as IFN-γ, tumor necrosis factor (TNF)-α, and TNF-β, to activate DCs, which can regulate the survival and persistence of CD8+ CTLs as memory cells (Bachelet, Mariethoz et al., 1998). IL-12 secreted from DCs is a potent inducer of Th1 differentiation. Both CD8+ CTLs and Th1 cells secrete IFN-γ, which can further sensitize tumor cells to CTLs by upregulation of MHC class I molecules on tumor cells and antigen-processing machinery of DCs (Steinman 1991). On the other hand, Th2 cells secrete type II cytokines, such as IL-4 and IL-10 resulted in enhanced generation of a humoral immunity, antibody-based antitumor response (Steinman 1991; Bradley, Yoshimoto et al., 1995; Banchereau & Steinman 1998; Wiethe, Debus et al., 2008). The newly identified Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity (Dong 2008). Importantly, cancer cells-derived soluble factors promote the induction of tolerance through the generation of CD4+α chain of IL-2R (CD25)+ forkhead box P3 (Foxp3)+ natural (n) Treg cell subset (Koido, Homma et al., 2008). Induced (i) Treg cells (CD4+CD25+Foxp3-) secrete transforming growth factor-β (TGF-β) and IL-10 and suppress effector T cells of either Th1 or Th2 phenotype in a cell contact and antigen-specific manner (Shevach 2009; Mougiakakos, Choudhury et al., 2010). Treg cells play a pivotal role in the tumor progression and the suppression of antitumor immunity.

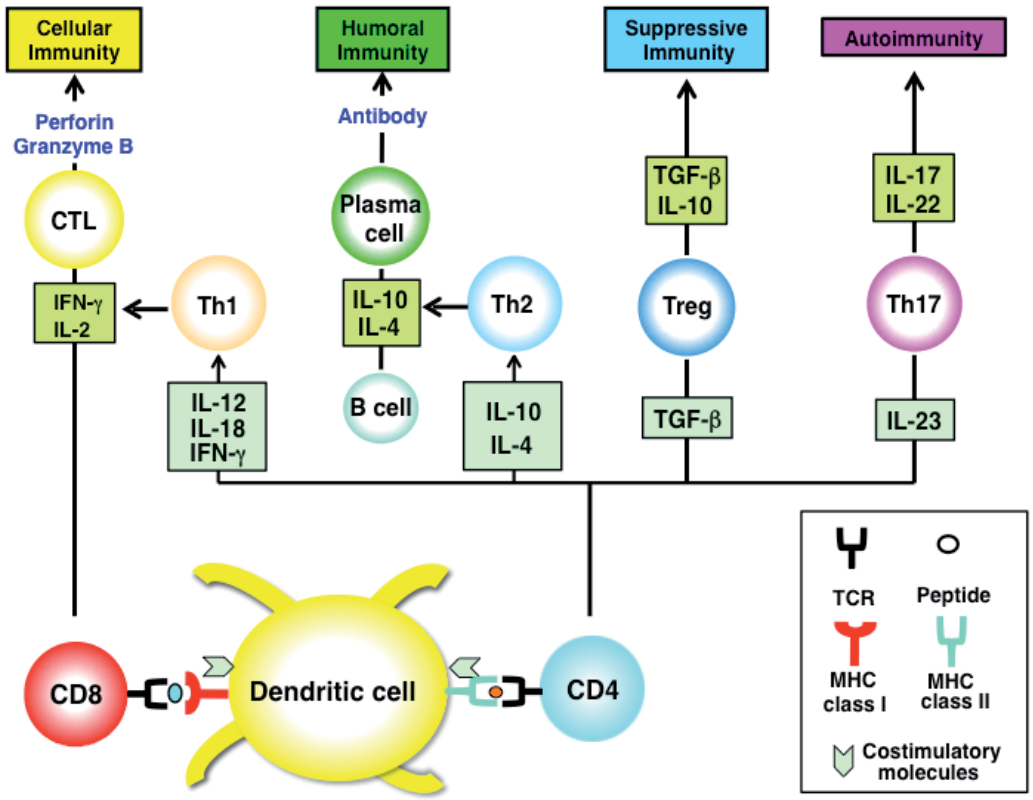

Fig. 1. Immune homeostasis. Upon TCR-mediated cell activation, naive CD4 T cells can differentiate into at least four major lineages, Th1, Th2, Treg, and Th17 cells, all of which participate in different types of immune responses. The Th1 cells produce signature type I cytokines, such as IFN-γ and IL-2 resulting in induction of CD8+ CTLs. Th2 cells secrete type II cytokines, such as IL-4 and IL-10. The Th2 response is associated with the humoral, antibody-based antitumor response. Treg cells that secrete TGF-β and IL-10 suppress Th1 or Th2 cells. Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity.

4.2 Immunosuppression in tumor microenvironment

Pancreatic cancer cells express TAAs such as Wilms' Tumor gene 1 (WT1) (Sugiyama 2005), mucin 1(MUC1) (Mukherjee, Ginardi et al., 2000), human telomerase reverse transcriptase (hTERT) (Seki, Suda et al., 2001), mutated K-RAS (Gjertsen, Bakka et al., 1995), survivin (Wobser, Keikavoussi et al., 2006), carcinoembryonic antigen (CEA) (Nair, Hull et al., 1999), HER-2/neu (Larbouret, Robert et al., 2007), or p53 (Hoffmann, Nakano et al., 2000) as potential targets for immunotherapy. Therefore, immunotherapy targeted such a TAA may be an approach in patients with advanced pancreatic cancer. However, the microenvironment in pancreatic cancer is consisted not only cancer cells but also stroma cells such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and

Treg cells (Fig. 2). These immune suppressive cells secrete vascular endothelial growth factor (VEGF), IL-6, IL-10, TGF-β, soluble Fas ligand (Fas-L), and indolamine-2,3-dioxygenase (IDO) (Koido, Homma et al., 2010c). As a result, immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO). The tumor microenvironment also promotes the accumulation of Treg cells that suppress CD8+ CTL function through secretion of IL-10 or TGF-β from Treg cells and tumors. Therefore, immunotherapies that struggle against pancreatic cancer cells with CTLs as well as inhibition of Treg cells may tip the balance in favor of immunostimulation. Currently, the field of cancer immunotherapy using peptide- or cell (DC or whole tumor cell)-based approaches is in an active state of preclinical and clinical investigations.

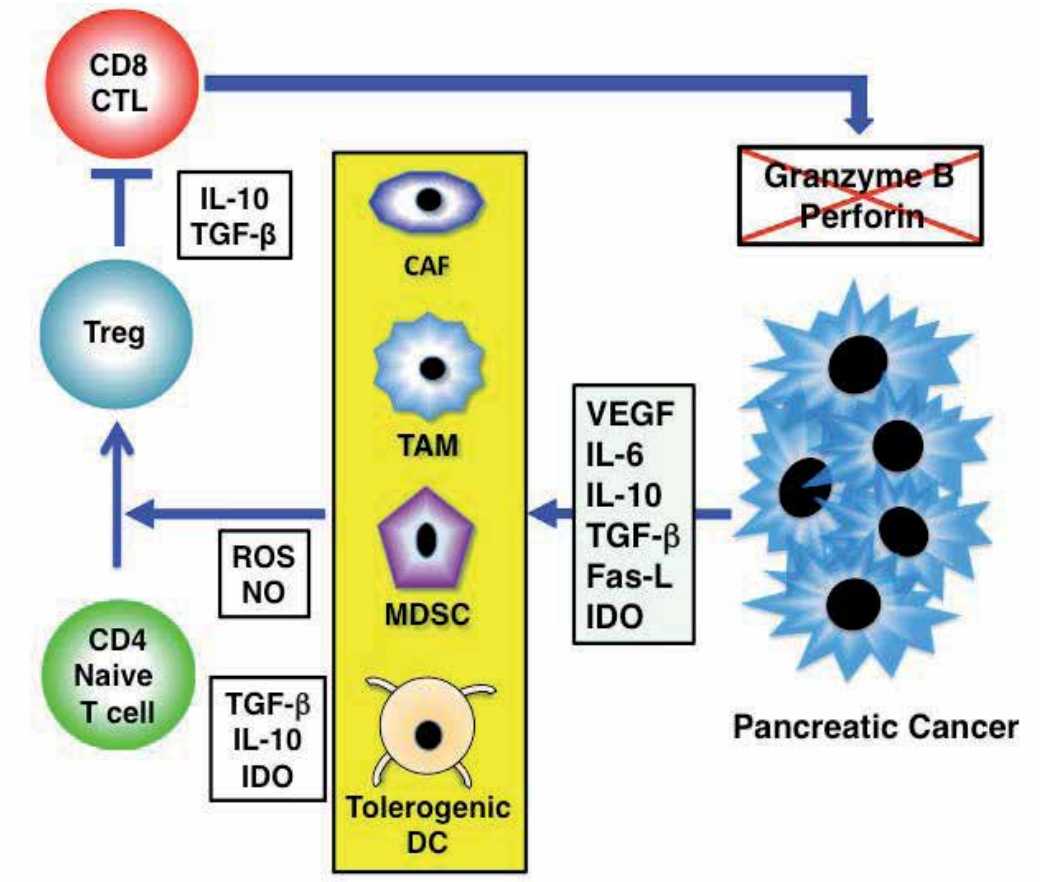

Fig. 2. Immunosuppression in tumor microenvironment. Pancreatic cancer cells secrete various factors such as VEGF, IL-6, IL-10, TGF-β, Fas-L, and IDO, all of which promote the accumulation of heterogeneous populations of CAFs, TAMs, MDSCs, or tolerogenic DCs. These immunosuppressive cells in tumor microenvironment inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of ROS and NO. The tumor microenvironment also promotes the accumulation of Treg cells that suppress CD8+ CTL function.

5. Peptide vaccines

Peptide-based cancer vaccines are preparations made from antigenic protein fragments that represent the minimal immunogenic region of TAA (Purcell and McCluskey 2007; Bijker, Melief et al., 2007). As peptide vaccines are simple, safe, stable, and economical, multiple MHC class I-binding peptides have been identified and vaccination with synthetic peptides has been examined for their immunogenicity in clinical trials for pancreatic cancer (Dummer 2001; Jaffee, Hruban et al., 2001; Yanagimoto, Mine et al., 2007; Miyazawa, Ohsawa et al., 2010). In early phase clinical trials, vaccination of mutant K-ras (Gjertsen, Bakka et al., 1995; Gjertsen, Buanes et al., 2001; Abou-Alfa, Chapman et al., 2011), MUC1 (Yamamoto, Ueno et al., 2005b; Ramanathan, Lee et al., 2005), or telomerase (Bernhardt, Gjertsen et al., 2006) peptide to patients with advanced pancreatic cancer are significantly associated with antitumor responses. As almost all pancreatic cancers involve mutations in the K-ras oncogene, it is believed that activating K-ras mutations are critical for initiation of pancreatic cancer (Gjertsen, Bakka et al., 1995; Gjertsen, Buanes et al., 2001; Abou-Alfa, Chapman et al., 2011). In a clinical phase I/II trial involving 48 patients with pancreatic cancer (10 surgically resected and 38 with advanced disease), vaccination of synthetic mutant K-ras peptides in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) produced immune responses to mutant K-ras and showed prolonged survival from the start of treatment compared to non-responders (Gjertsen, Buanes et al., 2001). Abou-Alfa et al. (Abou-Alfa, Chapman et al., 2011) also vaccinated 24 patients with resected pancreatic cancer with mutant K-ras peptide in combination of GM-CSF and found that the vaccination proved to be safe and tolerable with no elicitable immunogenicity and unproven efficacy. On the other hand, almost all pancreatic cancer cells also express MUC1 that is high molecular weight glycoproteins (Chhieng, Benson et al., 2003). The MUC1 peptide derived from tandem repeat core was also recognized by CD8+ T cells in an MHC-restricted and -unrestricted manner. Therefore, MUC1 peptide vaccine was subsequently used in the immunization of patients with pancreatic cancer (Finn, Jerome et al., 1995). Ramanathan et al. (Ramanathan, Lee et al., 2005) used 100 mer MUC1 peptide with SB-AS2 adjuvant in 16 patients with resected or locally advanced pancreatic cancer. They found that 100 mer MUC1 peptide with SB-AS2 adjuvant induced low but detectable MUC1-specific humoral and T-cell responses in some patients. Yamamoto et al. (Yamamoto, Ueno et al., 2005b) also, in a clinical phase I trial involving 6 patients with advanced pancreatic cancer, reported that vaccination with 100 mer MUC1 peptide and incomplete Freund's adjuvant resulted in increased circulating anti MUC1 IgG antibody in some patients. Moreover, human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase and a prototype for a novel class of universal tumor antigens due to its expression in the vast majority of human tumors (Beatty & Vonderheide, 2008). Therefore, it is one of widely applicable target antigen recognized by CTLs in pancreatic cancer. Bernhardt et al. (Bernhardt, Gjertsen et al., 2006) reported the results of a phase I trial of telomerase peptide in combination with GM-CSF for non-resectable pancreatic cancer patients (n=48). The immunotherapy was safe and induction of an immune response was correlated with prolonged survival. Recently, Itoh et al. (Itoh, Yamada et al., 2009) have developed personalized peptide vaccines. In this regimen, pre-vaccination peripheral blood mononuclear cells (PBMCs) were screened for their reactivity in vitro to each peptide in

patients, and only the reactive peptides were used as vaccines to 11 patients with advanced pancreatic cancer. In the personalized peptide vaccine, increased cellular and humoral immune responses to at least one of peptides used for vaccination were observed in the post-vaccination PBMCs (Yamamoto, Mine et al., 2005a). In all of these peptide vaccines, only a limited success has occurred in clinical trials. Generally, the drawback of this strategy comes from numerous factors: (i) only a limited number of known synthesized antigenic peptides can be available (Mocellin, Pilati et al., 2009), (ii) CD8+ CTLs may be ineffective in reacting with pancreatic cancer cells due to down regulation of certain antigens and MHC class I molecules, (iii) impaired function of DCs in patients with advanced pancreatic cancer (Yanagimoto, Takai et al., 2005; Koido, Hara et al., 2010a), and (iv) tumor microenvironment where immune suppressive cells such as Treg cells, CAFs, MDSCs, or TAMs exist (Finn 2008). The more attractive peptide-based vaccines may be synthetic long peptides. As synthetic long peptides are not able to bind directly on MHC class I and class II molecules on DCs, they need to be taken up, processed and presented by DCs. Therefore, the long peptide vaccines can be presented on MHC class I and class II molecules long time resulted in induction of antigen-specific polyclonal CD4+ and CD8+ T cells (Melief & van der Burg 2008; Bijker, van den Eeden et al., 2008). Peptide vaccines for the treatment of established pancreatic cancer may require long-lived presentation of epitopes by MHC class I and class II molecules on appropriately activated DCs. Such presentation is essential for induction of robust therapeutic CD4+ and CD8+ T-cell responses. Recently, Weden et al. (Weden, Klemp et al., 2011) treated 23 patients who were vaccinated after surgical resection for pancreatic cancer with long synthetic mutant K-ras peptides designed mainly to elicit T-helper responses. Surprisingly, 10-year survival was 20% (four patients out of 20 evaluable) versus zero (0/87) in a cohort of nonvaccinated patient treated in the same period. The key elements for the development of therapeutic peptide vaccines for pancreatic cancer may be the combination with chemotherapy to overcome robust cancers. Indeed, Wobser et al. (Wobser, Keikavoussi et al., 2006) reported a case of complete remission (CR) of liver metastasis of pancreatic cancer refractory to gemcitabine chemotherapy under vaccination with a survivin peptide. Peptide vaccines alone should be tested in cancer patients in remission to prevent recurrence and metastasis after surgical resection.

6. DC-based vaccines

For T-cell activation, three signals are required: (i) effective presentation of multiple TAAs in MHC class I and class II molecules; (ii) costimulation by membrane-bound receptor-ligand pairs; and (iii) soluble factors to direct polarization of the ensuing efficient antitumor immune responses. DCs derive their potency from constitutive and inducible expression of essential costimulatory ligands on the cell surface including B7, ICAM-1, LFA-1, LFA-3, and CD40 (Inaba, Witmer-Pack et al., 1994). These proteins function in concert to generate a network of secondary signals essential for reinforcing the primary antigen-specific signal in T-cell activation (Inaba, Pack et al., 1997). Therefore, now it is clear that DCs have the ability to provide all three signals essential for induction of antitumor immunity (Banchereau & Palucka 2005). These findings have provided the rationale for ex vivo antigen loading of DC as vaccines. More than 200 clinical trials have been performed using DC as cellular adjuvants in cancer. Several strategies to deliver TAAs into DCs have been developed to generate potent antitumor immune responses (Fig. 3).

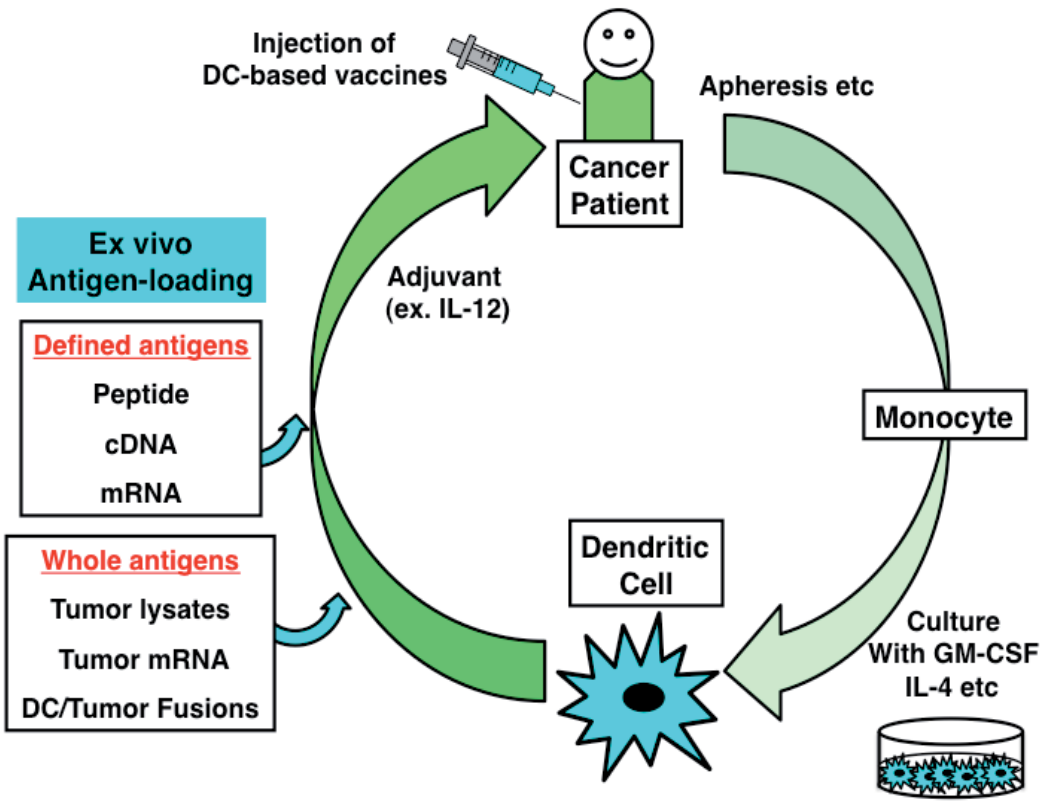

Fig. 3. Strategies to deliver defined or whole antigens to DCs. DCs used for cancer vaccines have been generated from the peripheral blood monocytes of the patients using cytokines including GM-CSF and IL-4 etc. To generate antigen-specific CTL responses against tumor cells, DCs loaded with synthetic peptide, antigenic cDNA, or mRNA have been used. Moreover, whole tumor associated antigens including defined and unidentified have been also loaded to DCs.

DCs have been loaded with tumor antigens in the form of peptides (Nestle, Alijagic et al., 1998), tumor lysates (Mackensen, Herbst et al., 2000), apoptotic tumor cells (Palucka, Ueno et al., 2006), or mRNA (Nair, Boczkowski et al., 1998; Koido, Kashiwaba et al., 2000). Alternatively, whole tumor cells have been fused with DCs to facilitate the entry of TAAs, including both known and unidentified, into the endogenous antigen-processing pathway in the DCs (Fig.4). The strategy for DC/tumor fusion vaccine is based on the fact that DCs are the most potent antigen-presenting cells in the body, whereas tumor cells express abundant tumor antigens. In animal studies, DC/tumor fusion vaccines have been shown to possess the elements essential for processing and presenting tumor antigens to host immune cells, for inducing effective immune response, and for breaking T-cell tolerance to TAAs (Gong, Chen et al., 1997; Koido, Hara et al., 2007; Gong, Koido et al., 2008; Koido, Hara et al., 2009; Koido, Hara et al., 2010a; Koido, Homma et al., 2010b). Recently, we have reported that fusions of human pancreatic cancer cells and DCs induce CTL responses against pancreatic cancer cells in vitro (Koido, Hara et al., 2010a). Although DC/tumor fusion

vaccines have proven clinically safe and efficient to induce tumor-specific immune responses, only a limited number of objective clinical responses have been reported in cancer patients (Avigan, Vasir et al., 2004; Kikuchi, Akasaki et al., 2004; Homma, Kikuchi et al., 2005; Homma, Sagawa et al., 2006).

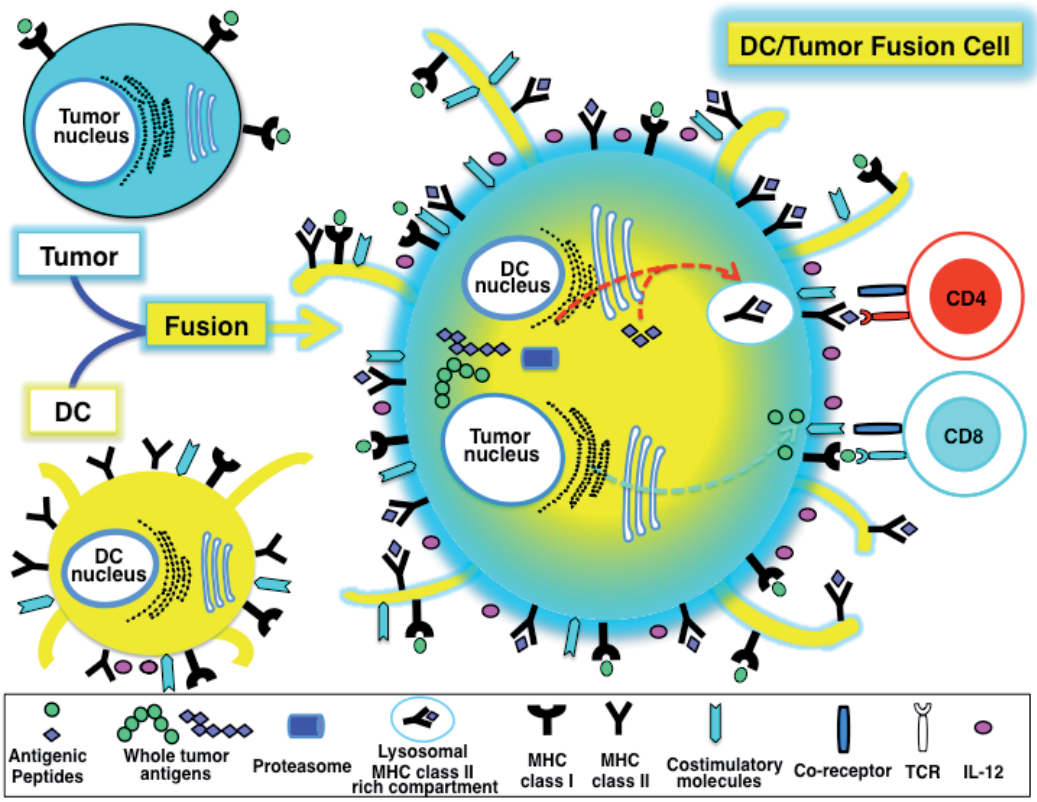

Fig. 4. Fusions of DC and tumor cell. DC/tumor fusions express MHC class I and class II, costimulatory molecules (CD80 and CD86), and multiple tumor-associated antigens. The DC/tumor fusions are able to process multiple TAAs derived from tumor. They form MHC class I-peptide complexes, in the endoplasmic reticulum, which are transported to the cell surface of DC/tumor fusions and presented to CD8+ T cells. The DC/tumor fusions can also synthesize antigenic peptides in the endoplasmic reticulum, which are transported to the cytoplasm, where MHC class II-peptide complexes are assembled with multiple tumor-derived peptides. These complexes are presented to CD4+ T cells, which are essential for induction of efficient antigen-specific polyclonal CTLs.

Clinical trials of antigen-pulsed DCs have been conducted in patients with various types of tumors including pancreatic cancer. In a phase I/II clinical trial of a MUC1 peptide-loaded DC vaccines in pancreatic and biliary cancer patients following resection of their primary tumors, 4 of the 12 patients followed for over four years were alive, all without evidence of recurrence (Lepisto, Moser et al., 2008). Moreover, MUC1 specific immune responses were observed even in patients with pretreated and advanced disease, following immunization with DC transfected with MUC1 cDNA (Pecher, Haring et al., 2002). Findings from initial

clinical trials demonstrate that hTERT-specific immune responses can be safely induced in cancer patients. Suso et al. (Suso, Dueland et al., 2011) recently reported that vaccination with DC transfected with hTERT mRNA (DC/hTERT mRNA) had the potential to induce strong immune responses to multiple hTERT epitopes. In this therapy, a patient who could not continue chemotherapy due to sever neutropenia had been treated with DC/hTERT mRNA alone for 3 years and resulted in no evidence of active disease. Moreover the CR was associated with induction of hTERT-specific immune responses against several hTERT-derived Th and CTL epitopes. Therefore, DC/hTERT mRNA may be an attractive approach to induce potent antitumor immunity. On the other hand, combined injection of unloaded DCs and activated lymphocytes resulted in prolonged survival of refractory pancreatic cancer patients (Nakamura, Wada et al., 2009). To improve the clinical efficacy of DC-based cancer vaccines, we need to design novel and improved strategies that can boost adaptive antitumor immunity to break overcome the immunosuppressive tumor microenvironment.

7. Whole tumor cell-based vaccines

Although cancer vaccines with defined TAAs are commonly used, the advantage of using autologous whole tumor cells is that tumor cells express a whole array of TAAs that are both characterized and uncharacterized. Moreover, this rich source of antigens contains epitopes of both CD8+CTLs and CD4+ T helper cells (Koido, Hara et al., 2005; Koido, Hara et al., 2009; Koido, Homma et al., 2010c). Thus, whole tumor cells could greatly diminish the chance of tumor escape compared to using single epitope peptide vaccines. In clinical trials, autologous tumor cells have been used as cancer vaccines to induce polyclonal CTL induction against colorectal (Harris, Ryan et al., 2000), renal cell cancer (Jocham, Richter et al., 2004), or melanoma (Berd, Sato et al., 2004), and several trials have shown clinical responses in the initial clinical studies. However, in many cases, even though a tumor-specific immune response has been observed, none has shown significant efficacy in the randomized phase III trials. To improve immunogenicity of vaccines, autologous whole tumor cells have been genetically modified to secrete GM-CSF and have shown promising results in patients with prostate (Simons, Jaffee et al., 1997) renal cell (Simons, Mikhak et al., 1999), metastatic non-small-cell lung carcinoma (Salgia, Lynch et al., 2003), and melanoma (Soiffer, Hodi et al., 2003). This approach is based on the concept that GM-CSF is required at the site of the tumor to prime TAAs-specific immunity effectively (Nemunaitis 2005).

Autologous cancer cells would be the best source of immunizing proteins, however, only 10-15% of pancreatic cancer patients diagnosed are eligible for surgical treatment. Therefore, autologous tumor cells may not be provided in almost of the patients with pancreatic cancer. Moreover, even if the patients are treated by surgical resection, it is difficult to prepare sufficient amounts of autologous tumor cells due to the length of culture time and potential contamination of bacteria and fungus (Koido, Hara et al., 2005a). To circumvent this problem, allogeneic pancreatic tumor cell lines with shared TAAs have been used instead of autologous tumor cells to deliver shared TAAs into autologous DCs (Jaffee, Hruban et al., 2001; Lutz, Yeo et al., 2011). The whole allogeneic tumor cell line-based vaccines have numerous advantages. (i) Allogeneic tumor cell lines that share one or even several of the TAAs as autologous tumor cells. (ii) Allogeneic tumor cell lines can be propagated in large quantities in cell factories. (iii) It is not necessary to determine HLA typing of patients and allogeneic tumor cells, because autologous DCs can process and

present multiple TAAs from allogeneic tumor cells owing to cross-presentation in the context of autologous MHC class I and class II alleles. (iv) Both antigens-specific polyclonal CD4+ and CD8+ T cells can be induced simultaneously. While currently explored allogeneic approaches as whole tumor cell-based vaccines represent an improvement in terms of standardization over their autologous counterparts, they nevertheless entail the culture of large batches of cells under good manufacturing practice (GMP) grade conditions. Further optimization of these in vitro culture methodologies may be required. Moreover, the quality must be easily assessed and monitored in GMP facilities. One of the challenges that face the generation of whole allogeneic tumor-based vaccines for clinical use may be to overcome the potential hazards of fetal calf serum (FCS) (Koido, Hara et al., 2010a).

In a phase I trial, cancer vaccines using irradiated allogeneic pancreatic cancer cells secreting GM-CSF were safe and induced systemic antitumor immunity in patients with surgically resected pancreatic cancer (Jaffee, Hruban et al. 2001). From the same group, GM-CSF secreting allogeneic pancreatic cancer cells alone or in sequence with cyclophosphamide in patients with advanced pancreatic cancer showed minimal treatment-related toxicity and induction of mesothelin-specific T-cell responses (Laheru, Lutz et al., 2008). In addition, cyclophosphamide-modulated immunotherapy resulted in prolonged overall survival in a gemcitabine-resistant population. Recently, a single institution phase II study of 60 patients with resected pancreatic adenocarcinoma was performed (Lutz, Yeo et al., 2011). This approach integrated with chemoradiation was safe and demonstrated prolonged overall survival in resected pancreas cancer. While this approach for pancreatic cancer is a safe and promising therapy, their clinical efficacy remains to be established. Further clinical evaluation of the approach in patients with pancreatic cancer is warranted.

8. Combined therapy of immunotherapy and chemotherapy

In established pancreatic cancer patients, the effect of immunotherapy alone is limited by the number of CTLs able to penetrate tumor and by the number of tumor cells expressing specific antigens. Even if large numbers of CTLs generated ex vivo were injected into the patients, CTLs cannot penetrate into tumor site because of tumor stroma. Moreover, in tumor site, Treg cells or MDSCs produce immunosuppressive cytokines such as IL-10 and TGF-β As a result, antitumor clinical responses may not induce in patients with advanced pancreatic cancer treated with immunotherapy alone.

Cytotoxic chemotherapy is well known to blunt immune responses, because of its toxicity for dividing cells in peripheral lymphoid tissue as well as the bone marrow. Indeed, several of the cancer chemotherapeutics agents such as cyclophosphamide (Weiner and Cohen 2002) and methotrexate (Weinblatt, Coblyn et al., 1985) are also used as immunosuppressants for the treatment of severe systemic autoimmune diseases. Therefore, the chemotherapeutic approach was considered to be inappropriate based on a widely held belief that the immunosuppressive effects of the chemotherapy would negate the efficacy of cancer vaccines (Zitvogel, Apetoh et al., 2008). However, increasing evidences have been mounting to suggest that immunotherapy has the possibility of achieving better success when used in combination with conventional chemotherapy (Gabrilovich 2007; Smith, Kasamon et al., 2010). Gemcitabine that is a standard cytotoxic agent for pancreatic cancer has been also generally considered immunosuppressive due to neutropenia and lymphopenia being common adverse side effects. There is increasing evidence, however,

that gemcitabine plays important roles in the induction of antitumor immune responses. Gemcitabine inhibited B cells (Nowak, Robinson et al., 2002) and CD11b+GR1+ MDSCs (Suzuki, Kapoor et al., 2005), the phenomenon that may skew antitumor immunity towards beneficial T‐cell responses (Qin, Richter et al., 1998). Moreover, gemcitabine treatment in patients with pancreatic cancer induced the proliferation of CD14+ monocytes and CD11c+ DCs (Soeda, Morita-Hoshi et al., 2009). To induce efficient therapeutic CTL responses, cross-presentation of TAAs by DCs is essential. Treatment of pancreatic cancer cells and DCs with gemcitabine results in enhanced cross-presentation of TAAs by DCs, CTL expansion, and infiltration of the tumor, all of which are associated with augmented CTL (Nowak, Lake et al., 2003a; Nowak, Robinson et al., 2003b; Dauer, Herten et al., 2005; Correale, Cusi et al., 2005). A recent report that chemotherapeutic agents caused up-regulation of cation-independent mannose 6-phosphate receptor (CI-MPR) expression on cancer cells and a concurrent increase in the uptake of granzyme B by activated CTLs also strongly suggests chemotherapy can function in synergy with induction of CTL responses to cure established pancreatic cancer (Ramakrishnan, Assudani et al., 2010).

These findings open a novel field of investigations for future clinical trial design, taking into account the immunostimulatory capacity of chemotherapeutic agents such as gemcitabine, and using them in combined chemoimmunotherapy strategies in patients with pancreatic cancer (Correale, Aquino et al., 2003; Nowak, Lake et al., 2003a; Correale, Cusi et al., 2005; Dauer, Herten et al., 2005; Correale, Del Vecchio et al., 2008). Now the immunostimulatory effects of gemcitabine have been confirmed in patients with cancer. In patients with pancreatic (Plate, Plate et al., 2005), nonsmall-cell lung (Levitt, Kassem et al., 2004) or colon (Galetto, Buttiglieri et al., 2003) cancer, standard cytotoxic agent, gemcitabine combined with recombinant cytokines or cancer vaccines could synergistically enhanced the frequency of tumor-specific CTL precursors. Therefore, patients with advanced pancreatic cancer have been treated by combination therapy of gemcitabine with peptide vaccine. For instance, both clinical and immune responses to personalized peptide vaccination combined with gemcitabine were evaluated in 21 patients with non-resectable pancreatic cancer (Yanagimoto, Mine et al., 2007; Yanagimoto, Shiomi et al., 2010). In this report, the reactive personalized peptides (maximum of 4 kinds of peptides) were administered with gemcitabine. Median overall survival time of all 21 patients was 9.0 months with a one-year survival rate of 38%. Immune boosting in both cellular and humoral responses was well correlated with overall survival. Combination therapy of a epitope peptide from vascular endothelial growth factor receptor 2 (VEGFR2) with gemcitabine was also conducted in 18 patients with metastatic and unresectable pancreatic cancer (Miyazawa, Ohsawa et al., 2010). The median overall survival time of all 18 patients who completed at least one course of the treatment was 8.7 months. Moreover, VEGFR2-specific CTL responses could be induced by the combination therapy. Similar findings were observed in 5 patients with inoperable locally advanced pancreatic cancer using gemcitabine, OK-432 stimulated DCs injected into the tumor sites, and intravenous infusion of lymphokine-activated killer (LAK) cells stimulated with anti-CD3 monoclonal antibody (Hirooka, Itoh et al., 2009). In this regimen, one patient had partial remission (PR) and 2 had long stable disease (SD) more than 6 months. Recently, we also reported that combination therapy of DC-based immunotherapies with gemcitabine/S-1 was effective in patients with advanced pancreatic cancer refractory to standard chemotherapy (Kimura, Imai et al., 2011). As WT1 is one of the excellent TAAs for the target of immunotherapy and is frequently expressed in pancreatic cancer cells (Oka, Tsuboi et al., 2004; Cheever, Allison et al., 2009), 38 out of 49 patients had

received vaccination with WT1 peptide pulsed DCs with or without combination of other peptides such as MUC1, CEA and CA125 in this report. Prior to this combination therapy, 46 out of 49 patients had been treated with chemotherapy, radiotherapy, heavy particle radiotherapy, or hyperthermia, but elicited no significant effects. In spite of these handicapped conditions, surprisingly, of 49 patients, 2 patients showed CR, 5 PR, and 10 SD, and median survival time was 360 days. We recently reported that gemcitabine sensitized the pancreatic cancer cells with WT1 specific T cell-mediated antitumor responses in vitro (Takahara, Koido et al., 2011), also supporting the significance of the combination therapy (Fig.5). In this study, gemcitabine treatment of human pancreatic cancer cells increased WT1 mRNA, and this increase was associated with nuclear factor kappa B (NF-κB) activation. Gemcitabine treatment also shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic WT1 peptide. Moreover, presentation of HLA-A*2402-restricted WT1 peptide increased in gemcitabine-treated pancreatic cancer cells. Indeed, we observed clinical response in a phase I clinical trial of combination therapy of WT1 peptide vaccine and gemcitabine (manuscript in preparation). Pancreatic cancer cells already, which have acquired gemcitabine resistance by the activation of NF-kB might be killed by WT1-specific CTLs. Assessment of the clinical response to the combined therapy of WT1 peptide vaccine and gemcitabine is presently underway.

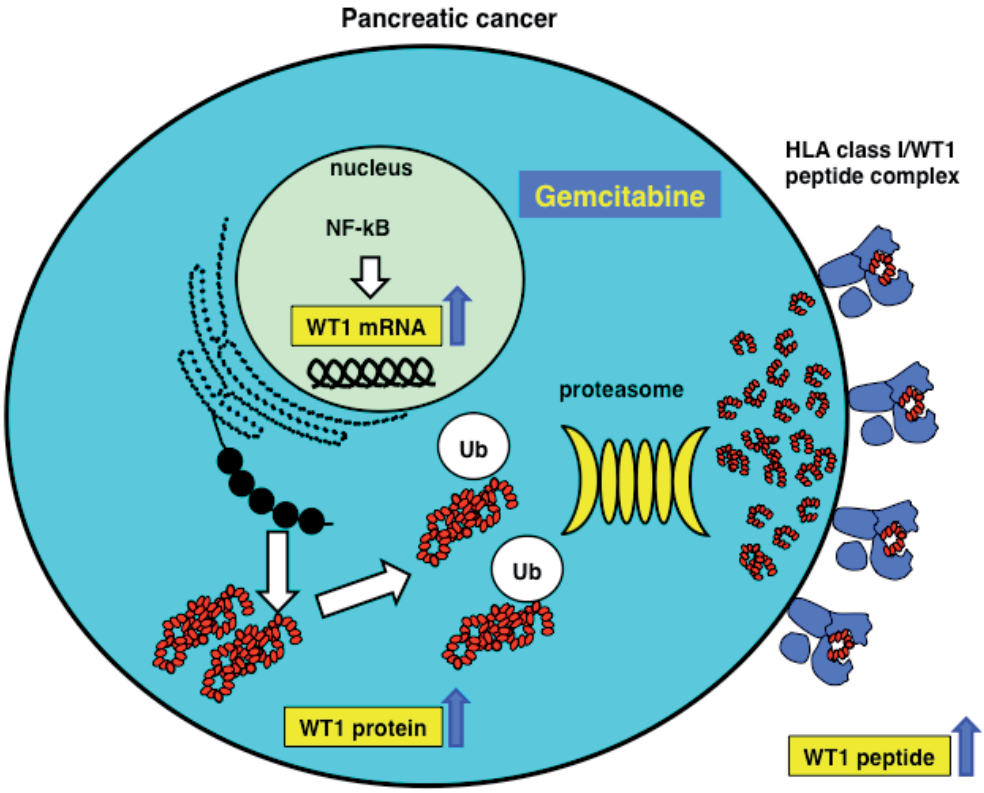

Fig. 5. Synergistic therapeutic antitumor effects of gemcitabine and WT1-specific CTLs. Gemcitabine enhanced WT1 expression in human pancreatic cancer cells and sensitized pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune responses.

Although the concept is still far from being firmly established, these reports may be sufficient to provide a platform for the combination of immunotherapy with chemotherapy. A combined approach of conventional therapies such as radiation or chemotherapy kill the bulk of tumor cells and CTLs that target TAAs may represent a promising approach for the treatment of patients with advanced pancreatic cancer. Evaluation is warranted to examine the effect of the combined approach on disease-free survival and overall survival.

9. Immunotherapy targeting cancer stem cells

It has been well known that the majority of patients with advanced pancreatic cancer that respond initially to standard chemotherapies ultimately undergo relapse due to the survival of small populations of cells with cancer-initiating/cancer stem cell (CSC) fraction (Wang, Li et al., 2011). These CSCs are a subpopulation of the tumor more capable than other cancer cells (CC) to self-propagate, initiate new tumors differentiate into bulk tumor, and therefore sustain tumor growth. It has been reported that pancreatic cancer cells resistant to chemoradiotherapy are rich in CSC fraction (Du, Qin et al., 2011). Moreover, CSCs could be

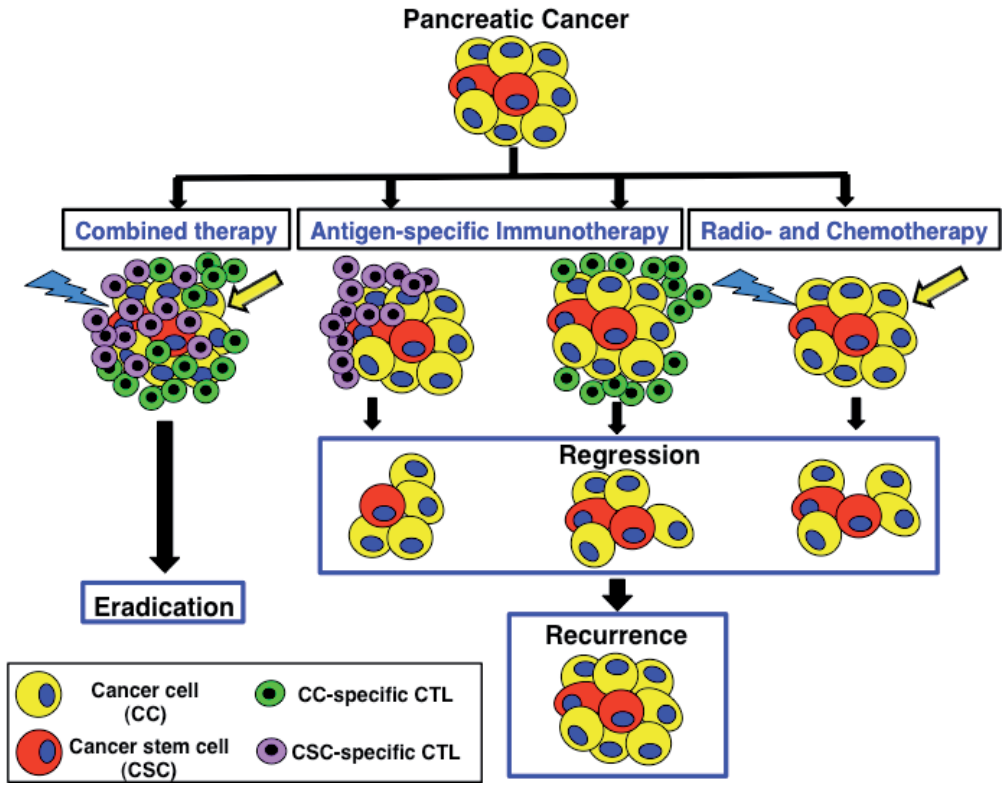

Fig. 6. Combination therapies of immunotherapy and standard radio- and chemotherapy. Currently applied standard therapies such as radio- and chemotherapy target bulk CCs that are less resistant than CSCs. This leads to initial regression of the tumor mass but eventually regrowth from residual CSCs. Combined therapies of standard therapies and immunotherapeutic approach targeting CSCs would cut off the rejuvenating supply of CSCs and resulted in tumor eradication.

expanded during the acquisition of gemcitabine resistance (Hong, Wen et al., 2009). Thus, targeted therapy against CSC fraction that is resistant to chemotherapy could be applied to overcome drug resistance in the treatment of pancreatic cancer (Fig. 6). Importantly, the tumors that develop chemotherapeutic drug resistance would still be a candidate target for immunotherapy. TAAs can be classified into two categories: i) CSC-specific antigens, such as SOX2 (Hong, Wen et al., 2009) or ALDH1A1 (Inoda, Hirohashi et al., 2011) and ii) shared antigens, such as CEP55 (Hirohashi, Torigoe et al., 2010), MUC1 (Engelmann, Shen et al., 2008; Weng, Song et al., 2010), or WT1 (Cheever, Allison et al., 2009; Sugiyama 2010) between CSCs and more differentiated subpopulations. Therefore, the development of strategies that target the CSC population by immunotherapy may be highly desirable. For example, DC-based cancer vaccine, γδ T cells, or natural killer (NK) cells killed human cancer stem cells (Pellegatta, Poliani et al., 2006; Todaro, D'Asaro et al., 2009; Pietra, Manzini et al., 2009; Weng, Song et al., 2010), in vitro. Success of these potential therapies will depend on how well immunological responses to CSCs can be modulated for example by vaccines upregulating antigen-processing and -presentation in DCs. Recently, we used fusions of DC and CSC to activate potent CSC-specific CTL responses and resulted in expression CTLs with elevated levels of IFN-γ and enhanced killing of CSCs in vitro (Weng, Song et al., 2010). Moreover, the classification of conclusive CSC markers followed by the identification of defined T cell-recognized CSC epitopes in the future may also lead to the clinical application of anti-CSC vaccination strategies.

10. Conclusion

The prognosis of patients with pancreatic cancer remains grim, and current thinking toward the development of curative therapy is likely to require eradication of the CSC population. A combined approach of conventional therapies such as radiation or chemotherapy kill the bulk of pancreatic cancer and CTLs that target CSC and CC fraction may represent a more promising approach for the treatment of patients with advanced pancreatic cancer. Clinical evaluation is warranted to examine the effect of the combined approach earlier in the disease course and in patients with less aggressive disease.

11. Acknowledgments

This work has been supported by Foundation for Promotion of Cancer Research, Mitsui Life Social Welfare Foundation, Grants-in-Aid for Scientific Research (B and C) from the Ministry of Education, Cultures, Sports, Science and Technology of Japan, Grant-in-Aid of the Japan Medical Association, Takeda Science Foundation, and Pancreas Research Foundation of Japan.

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14

The Role of Mesothelin in Pancreatic Cancer

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1. Introduction

Mesothelin (MSLN) is a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein and differentiation antigen. MSLN gets its name from typically limited expression in the mesothelial lining of the pleural cavity, where it was first identified in 1996 by Chang and Pastan[1]. The human MSLN gene (localized to 16p21)[2] encodes a 71-kDa precursor protein that is cleaved by furin-like proteinases to produce an amino-terminal 31 kDa soluble fragment, termed the megakaryocyte-potentiating factor (MPF), which is released to the extracellular fluid, and a carboxy-terminal 40 kDa membrane-bound fragment[1,3,4].

The normal function of MSLN has remained elusive. Following the initial report of megakaryocyte colony-forming activity[5], a mutant mouse model generated by homologous recombination showed that platelet numbers in both wild-type and MSLN null mice, were not changed, which suggested that MSLN is not required for megakaryocyte growth and differentiation *in vivo*[6]. These mice also showed no discernable phenotype, indicating that either MSLN does not play a significant role *in vivo* or that other molecules may be filling in for MSLN by playing a similar role in normal tissues. MSLN is predicted to have a superhelical structure made of ARM-type helical repeats, and is thereby predicted to function as superhelical lectins that bind the extracellular carbohydrate moiety of glycoproteins[7]. Supporting a potential role in cell-to-cell adhesion, it has been shown that MSLN interacts with mucin MUC16 to enhance cell-cell binding, which can play a role in leading to peritoneal metastatic dissemination of tumors[8].

Where the importance of MSLN is evident in the progression of cancers. MSLN is overexpressed in a variety of cancers, including mesotheliomas, stomach, and endometrial cancers, as well as in squamous cell carcinomas of the esophagus, lung, and cervix[9,10]. In addition, several studies have reported the overexpression of MSLN in virtually all human pancreatic ductal adenocarcinomas[11]. In tumors, MSLN may function as a mediator of cell survival under anchorage-independent conditions, where it facilitates anchorage-independent growth and confers resistance to anoikis[12].

2. Mesothelin in pancreatic cancers

Despite the fact that it is only the tenth most common type of cancer and accounts for only 6% of new cancer cases in North America, pancreatic cancer is the deadliest cancer, with the

worst prognosis of all solid tumors[13]. Due to its highly aggressive nature characterized by invasive growth and early metastasis, and compounded by late diagnosis and lack of effective therapies for treatment, pancreatic cancer remains the fourth leading cause of cancer-related deaths, 35,000 deaths from 42,000 new cases per year[3,14] and a 5 year survival rate of less than 5%[13,14].

In addition to its role in other cancers, MSLN is increasingly becoming established as a key factor in human pancreatic adenocarcinomas. The soluble fragment of MSLN, MPF, was first detected in the supernatant of the HPC-Y5 human pancreatic cell line in 1994 [5]. In fact, HPC-Y5 had the highest MPF activity of 64 cancer cell lines tested, implicating MPF as an important factor in pancreatic anomalies. The latest research confirms the MPF is in fact responsible for increased phosphorylation of ERK1/2, leading to a decreased rate of cell death and an increase in cell number[15].

MSLN expression has since been assessed in pancreatic cancers, where it is found to be overexpressed in a majority of pancreatic ductal adenocarcinomas[4,9,16], with little expression in normal pancreas and chronic pancreatitis[11]. In their studies, for instance, Argani *et al.* used serial analysis of gene expression (SAGE) as well as *in situ* hybridization, RT-PCR, and immunohistochemistry to show that MSLN is consistently overexpressed in 60 of 60 pancreatic tumors. Meanwhile, MSLN does not seem to be expressed in normal pancreatic tissues [4,17,18]. Recently, for instance, Glass *et al.* showed that MSLN 24 of 42 (57%) adenocarcinomas stained for MSLN, while only 0 of 16 non-carcinomas (0%) did so[19].

Exactly what the molecular mechanisms are which give rise to MSLN overexpression are not well documented. However, a recent study attributed MSLN overexpression in pancreatic cancers to an upstream enhancer element containing a transcription enhancer factor (TEF-1) dependent MCAT motif termed Canscript[2], although the presence of this factor was required but not sufficient for MSLN expression. The oncogenic transcription co-factor YAP1, normally phosphorylated and inactivated by the Hippo-YAP1 pathway, has been implicated in the activation of MSLN expression through the regulation of Canscript activity. Knocking down YAP1 expression in HeLa cells dramatically reduced endogenous MSLN expression and suppressed Canscript reporter activity; yet overexpression of YAP1 in HEK293 cells did not turn on MSLN expression, indicating that YAP1 may be necessary but not sufficient for MSLN overexpression[20]. Another study analyzed the methylation state of several pancreatic cancer-associated genes, and found that MSLN was hypomethylated in adenocarcinoma compared to its methylated state in normal pancreatic tissues, indicating an epigenetic event trigger is involved in MSLN overexpression[21].

Pancreatic cancer studies have revealed roles for MSLN in increasing aggressiveness of tumor cells, including enhancement of proliferation and migration. Li *et al.* found that overexpression of MSLN is also associated with an increased S-phase cell population in a cell cycle analysis. This resulted in a 90% increase in proliferation for MIA-PaCa2 cells overexpressing MSLN compared to vector controls[16]. Subsequent studies by Bharadwaj *et al.* elucidated a mechanism through which MSLN promotes proliferation of pancreatic cancer cells through alteration of Cyclin E as a result of constitutive activation of Signal Transducer and Activator of Transcription protein 3 (STAT3)[3]. Bharadwaj *et al*. have further shown that MSLN overexpression results in upregulation of growth/survival pathways through autocrine production of growth factors such as IL-6[22]. MSLN also induces in an increase in

NF-κB activation which leads to resistance to TNF-α-induced apoptosis[23], indicating a mechanism through which MSLN may help to increase survival of tumor cells in the highly inflammatory milieu evident in pancreatic cancer through Akt/PI3K/NF-κB Activation and IL-6 overexpression. MSLN overexpression results in secretion of high levels of IL-6, which could in turn be responsible for the cells' increased viability and proliferation under serum-reduced conditions through a IL-6/soluble IL-6R (sIL-6R) trans-signaling mechanism and the induction of the IL-6-STAT3 pathway[3,22].

MSLN overexpression has also been associated with increased metastatic potential in pancreatic cancers. *In vitro* experiments showed that MSLN increases pancreatic cancer cell migration by 300%, while *in vivo* results showed and increase in local and liver metastases following orthotopic injection, with control cells without MSLN expression showing no metastases[16]. Cancer antigen-125 (CA125), the circulating antigen encoded by the *MUC16* gene, has been identified as a marker for differential diagnosis of pancreatic mass lesions with an 88.2% positive predictive value for diagnosis of pancreatic tumors[24]. Taking into account that the high affinity of mesothelin-CA125 interaction might be the cause of intracavitary tumor metastasis[8,25], a recent study by Einama *et al.* determined that co-expression of these two factors plays a significant role in the acquisition of aggressive clinical behavior of pancreatic tumors, finding that co-expression of MSLN and CA125 correlated with unfavorable patient survival outcome [25].

3. Advances in diagnosis and treatment of mesothelin-overexpressing pancreatic cancers

MSLN is an attractive candidate for targeted therapy given its limited expression on normal tissues and high expression in tumors and the fact that it is expressed on the surface of cells. Immunostaining against MSLN has been demonstrated to be an effective adjunct to cytology for diagnosis of pancreatic adenocarcinoma[26], with a 90% accuracy rate in diagnosing pancreatic malignancies. In addition, the release of the MPF from the cell surface following furin cleavage makes it an attractive target for diagnostic detection.

Elevated circulating MSLN levels have been detected in patients with pancreatic disease[27]. Using ELISA, 73 of 74 (99%) patients with pancreatic adenocarcinoma were found to have elevated circulating levels of MSLN compared with none of 5 healthy controls[27]. Other approaches, including using multiplexed Proximity Ligation Assay (PLA) have been used to effectively detect levels of mesothelin-MUC16 complex in serum and plasma levels[28]. MSLN has also been used as a biomarker to test the efficacy of new technologies for early, minimally invasive diagnosis of pancreatic adenocarcinoma. In a study involving minimally invasive fine needle aspirations MSLN has helped to differentiate pancreatic adenocarcinomas from chronic pancreatitis with near 100% accuracy[29]. Another study utilized acoustic wave device-based immunosensors in molecular cancer biomarker detection in real-time, and effectively identified MSLN expression in three different pancreatic cancer cell supernatants, although further study is needed with this technology to determine its effectiveness in patient tissues[30].

Most recently, ELISA has been used not just to test for MSLN circulation in patients with advanced tumors, but for attempted early detection of pancreatic anomalies. A pancreatic ductal carcinoma transgenic animal model was established in rats using a Cre/loxP

controlled human K*ras* oncogene [13]. Using this system, Fukamachi *et al.* demonstrated that the rat homolog of human MSLN, *Erc* (expressed in renal carcinoma), could be detected in the serum of pre-symptomatic, pre-malignant pancreas lesions, opening the door to potential early diagnosis of mesothelin-induced pancreatic malignancies, as well as testing of early stage chemotherapeutic intervention to prevent progression of malignancies.

Potential treatments focusing on MSLN are already undergoing clinical trials. One example is use of a mouse-human chimeric antibody (MORAb-009), an IgG1kappa monoclonal antibody with an affinity of 1.5 nM for human MSLN containing the murine SS1 Fv for MSLN, which is currently being examined in a Phase II clinical trial[31,32]. This antibody prevents adhesion of mesothelin-bearing tumor cells to MUC16 positive cells and also elicits cell-mediated cytotoxicity on mesothelin-bearing tumor cells. A newer study using phage display has shown successful isolation of HN1, a human scFv, which recognizes a conformation-sensitive epitope of MSLN on cancer cells and promotes apoptosis by acting as an immunotoxin[31]. While this particular study focused on ovarian cancer treatment, it stands to reason that pancreatic cancer would make an effective target for future treatments with this antibody. MSLN has also been used as a targeting factor for pancreatic tumors in conjunction with quantum dot (QD) technology. QDs are semi-conductor nanocrystals which, when encapsulated in carboxyl-functionalized amphiphilic polymers form stable, micelle-like structures which form a potential platform for visualization and drug delivery to tumors[33]. Ding *et al.* conjugated MSLN-specific Ab to QD micelles and used them for effective targeted delivery to pancreatic cancer sites *in vitro* and *in vivo*[33]. The high level of overexpression of MSLN in pancreatic cancer cells and tumors compared to normal tissues allowed for selective targeting of QDs, indicating the potential of MSLN-targeted QDs or other MSLN-conjugated vehicles to serve as agents for tumor diagnosis, imaging, and treatment through drug delivery.

Another approach for MSLN focused therapy comes from studies involving SS1P (SS1(dsFv)PE38), a recombinant anti-mesothelin immunotoxin. SS1P was developed consisting of an anti-mesothelin Fv (SS1) fused to PE38, a 38-kDa portion of Pseudomonas exotoxin A[34,35], which kills cells upon internalization following binding to MSLN on the cell surface. A phase I study was conducted involving 34 patients, 2 of which had pancreatic adenocarcinoma[35]. The patients tolerated the doses given, indicating a potential for progressing to additional studies. Although results were encouraging in patients with ovarian cancer or mesotheliomas, no response was seen in the 2 pancreatic cancer patients. A second Phase I trial, this time involving continuous infusion of SS1P, was more recently conducted[36] to measure toxicity tolerance, also with promising results. Newer studies by the same group are currently examining methods for improving the efficacy of SS1P therapy by combining treatement with Taxol, which appears to limit the binding of SS1P with shed MSLN in the extracellular fluid rather than on the cell surface, thereby increasing the effectiveness of SS1P internalization and the killing of tumor cells[37]. Yet another toxin insertion approach used a a biodegradable nanoparticulate delivery system targeted specifically to mesothelin-overxpressing cell lines to deliver diphtheria toxin DNA, which effectively inhibited protein translation of targeted cells *in vitro*[38].

Finally, approaches using pancreatic cancer vaccines show that MSLN has great potential as an immunotherapeutic targets[10,39]. Johnston *et al.* showed that mesothelin-specific T cells can be induced in patients with pancreatic cancer. Their results indicated that mesothelin-

specific $CD4^+$ and $CD8^+$ T cells were generated from peripheral blood lymphocytes of 50% of patients with pancreatic cancer, up from only 20% of healthy individuals[27], and another study showed consistent induction of $CD8^+$ T cell responses to multiple MSLN epitopes in a small number of patients[40]. A study by Yokokawa *et al.* sought to define additional MSLN epitotpes capable of more efficiently activating T cells to lyse tumors[10]. Jaffee *et al.* carried out a phase I trial in patients with surgically resected adenocarcinoma of the pancreas was conducted using an allogeneic granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccines[39]. In a phase II trial, these results were confirmed through induction of mesothelin-specific $CD8^+$ T cell responses following exposure to different MSLN epitopes, leading to a correlation of the posttreatment induction of mesothelin-specific T cell responses with improved overall response [41].

The latest attempts at immunotherapy with MSLN involved vaccination with virus-like particles (VLPs) to induce protective antiviral immune responses against MSLN, yielding promising results. Li *et al.* investigated the effect and mechanism of chimeric VLPs that contain human MSLN (VLP-hMSLN) as a candidate vaccine for controlling pancreatic cancer progression in an orthotopic pancreatic cancer mouse model[16]. In the study, VLP-hMSLN vaccination inhibited tumor progression in C57BL/6J mice, and increased mesothelin-specific antibodies and CTL activity and decreased regulatory T cells, resulting in reduced tumor progression and prolonged survival. Most recently, dendritic cells transduced with full-length MSLN cDNA-encoding adenoviral vectors have been shown to elicit mesothelin-specific cytotoxicity against pancreatic cancer cells *in vitro*, through activation of both $CD8^+$ T cells and $CD4^+$ helper T cells[42], suggesting the therapeutic potential of using MSLN-targeted DC vaccines in future clinical applications.

4. Conclusion

MSLN is an important molecule overexpressed in a variety of cancerous human malignancies, and in particular has been identified as a biomarker of pancreatic cancers. The high expression of MSLN in pancreatic tumors compared with its limited expression in normal tissues makes it an interesting candidate for targeted therapies and diagnostic screening. In addition, MSLN has been shown to play important roles in proliferation, survival, and metastatic potential of pancreatic tumors where it is overexpressed. While much progress has been made in understanding the molecular mechanisms that give rise to MSLN-associated pancreatic cancer pathogenesis, further studies are needed to truly elucidate the functions and effects of this molecule with regards to what still remains the deadliest of human cancers.

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15

Establishment of Primary Cell Lines in Pancreatic Cancer

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is by far the most common type of tumor in the exocrine pancreas, accounting for 85% to 100% of all malignant pancreatic tumors (Kloppel et al., 2004). During the last 30 years, little improvement in the prognosis of patients with PDAC has been achieved (Jemal et al., 2009). A better understanding of the biological nature of this neoplasm might improve the prognosis of patients. For this purpose, permanent cultured cell lines are helpful, since their convenience of use facilitates a variety of experiments (Ku et al., 2002). However, the amount of viable tumor-derived material is limited. The majority of research in PDAC has been done in a few cell lines; only 19 pancreatic carcinoma cell lines are broadly available for research. In addition to their small number, permanent cell lines have another disadvantage: the long culture times leave the cells prone to genetic drift (Kato et al., 1999; McQueen et al., 1991).

To obtain a greater phenotypic heterogeneity of the disposable cell lines, and to circumvent the use of "old" cell lines, it is advantageous for research laboratories that focus on pancreatic cancer to establish their own primary carcinoma cell lines (Ruckert et al., 2011). Before using these cell lines in basic research, the origin of the cell line should be confirmed to exclude cross-contamination by existing cell lines. This is important, because some studies suggest that 15–20% of the cell lines used in experiments have been misidentified or contaminated with another cell line, which is also true for primary cell lines (Cabrera et al., 2006; Drexler et al., 1999). A characterization of the cell lines is further necessary to analyze and document the typical biochemical and pathophysiological features of the tumor cell lines (Henderson et al., 1996). In the present article we give information on how to isolate and characterize primary pancreatic cancer cell lines.

2. Techniques for the isolation of primary pancreatic cancer cell lines

The principle of tissue culture was established by Wilhelm Roux in the 19th century. However, it took nearly 60 years before the establishment of the first human cancer cell line (Gey et al., 1952). The first report on the culture of pancreatic cancer cell lines was in l963 (Dobrynin, 1963). Cells that are cultured directly from a subject are known as *primary cell lines* and different techniques have been used to establish such pancreatic cancer cell lines.

Some of these primary cells can be cultured successfully over a long time period and are then named *permanent* or *immortal cell lines*.

The most frequent used techniques are explant culture (where the culture grows from a solid tumor sample), xenografting, enzymatic digestion and culture of malignant bodily fluids. In the following, we describe these techniques in detail.

2.1 Explant cultures

The two most frequently used explant culture techniques are the "mechanical dissociation" and the "outgrowth method". To obtain sterile conditions, the tumor specimen should be rinsed three times in wash medium.

Most scientist use the mechanical dissociation to establish pancreatic cancer cell lines (**Table 1**). In this technique after washing, the specimen is transferred to a sterile petridish and cut up into smaller pieces about 1-2 mm^3 using sterile scalpel and forceps. The fragments and the spilled cells are then placed in a tissue culture flask and medium is added. The production of proteolytic factors (Diamantidis et al., 2008; Mahadevan et al., 2007) and augmented cell motility (Hotz et al., 2007; Rückert et al., 2010) enables pancreatic cancer cells to actively leave the tissue fragment. Complexes of tumor cells can be seen within the medium after 24 h (**Figure 1, C and D**). The presence of these "STC´s" (small trabecular complex) is generally a good predictive parameter for the successful isolation of primary tumor cell lines and under standard cell culture conditions cancer cell colonies will grow, as well as other contaminating cell types such as fibroblasts or lymphocytes (Rückert et al., 2011).

The other explant culture technique is the outgrowth method. As in the mechanical dissociation- technique, little pieces of the solid tumor are produced by dissection of the tumor sample with an sterile scalpel. Those little fragments are placed within a 6-well plate and left to dry until the pieces stick on to the surface. Medium is then added cautiously. Tumor cells as well as fibroblast will grow out of the tissue fragment (**Figure 1, A and B**; for a pictured tutorial see Rückert et al., 2011).

2.2 Xenografts

Xenografting of tumor samples into athymic mice is a way to establish pancreatic tumor lines with a good success rate and is frequently used by scientist for this purpose (Hotz et al., 2000). By repeated passages of the tumor lines in nude mice the cells are said to become more aggressive. Some of these tumor lines are used as source for the establishment of cell lines by either explant cultures or enzymatic digestion (Dexter et al., 1982; Yachida et al., 2011) (**Table 1**).

2.3 Enzymatic digestion

Enzymatic digestion can be used to free cancer cells from adherent connective tissue and produce a suspension of cells. For this purpose different enzymes are used, such as trypsin (Kaku et al., 1980) or collagenase type IV (Chifenti et al., 2009; Kalinina et al., 2010). However, this method is laborious and rarely used to establish pancreatic cancer cell lines (**Table 1**).

Author and year	Name	Method used	Medium	Characterisation
(Dobrynin et al., 1963)	CaPa	Explant culture	Medium 199, 5% human serum, 5% FCS	M, DD, chr
(Lieber et al., 1975)	Panc-1	Explant culture	DMEM, 10% FCS	M, DD, chr, xeno, FA
(Owens et al., 1976)	Hs 700T HS 766T	Enzymatic digestion	DMEM, 10% FCS	M, DD, chr, plating efficiency
(Yunis et al., 1977)	MIA PaCa-2	Explant culture	DMEM, 10% FCS, 2.5% horse serum	M, DD, chr, HC, FA, EM
(Akagi & Kimoto, 1977)	HCG-25	Ascites w/ medium	RPMI 1640, 10% FCS	M, DD, chr, xeno, HC, FA, EM, plating efficiency
(Fogh et al., 1977)	Capan-1	n.n.	RPMI 1640, 15% FCS	M, DD, HC, xeno, FA
(Grant et al., 1979)	Ger	Explant culture	(Ham's F12 + Eagle's) or DMEM, 10% FCS, amino acids, 1 mM glutamine, AA, 50 tzg/ml soya-bean trypsin inhibitor	M, DD, chr, xeno, FA,TM
(Kaku et al., 1980)	QCP-1	Enzymatic digestion	Medium 199, 10% FCS	M, DD, chr, HC, FA
(Morgan et al., 1980)	COLO 357	Explant culture	RPMI 1640, 20% FCS	M, DD, chr, HC, FA, plating efficiency
(Chen et al., 1982)	AsPC-1	Ascites w/ medium	RPMI 1640, 10% FCS	M, HC, chr, xeno, FA
(Metzgar et al., 1982)	HPAF	Ascites w/ medium	EMEM, 10% FCS	xeno
(Dexter, 1982)	RWP1 RWP2	Xenograft	RPMI 1640, 20% FCS, buffered	M, DD, chr, HC, xeno, FA, plating efficiency
(von Bulow et al., 1982)	PancTuI-I	n.n.	n.n.	
(Kyriazis et al., 1983)	SW-1990	Explant culture	L-15-CI	M, DD, chr, HC, xeno, FA, plating efficiency
(Yamaguchi et al., 1983)	HPC-Y1	n.n.	RPMI 1640, 10% FCS	M, xeno, TM
(Okabe et al., 1983)	T3M-4	Xenograft	F10, 15% FCS, AA	M, DD, chr, HC
(Kyriazis et al., 1986)	Capan-2	n.n.	RPMI 1640, 15% FCS, AA	M, DD, chr, HC, xeno, FA, plating efficiency

Author and year	Name	Method used	Medium	Characterisation
(Yamada et al., 1986)	PSN-1	Xenograft	RPMI 1640, 10% FCS	M
(Yamaguchi et al., 1986)	HPC-YT	n.n.	RPMI 1640, 10% FCS	M, DD, chr, xeno, FA
(Tan et al., 1986)	BxPC-3	Explant culture	RPMI 1640, 20% FCS, buffered	M, DD, chr, HC, xeno
(Kobari et al., 1986)	PK-1 PK-8 PK-9 PK-12 PK-14 PK-16	Explant culture	MEM, 20% FCS	M, DD, chr, HC, xeno, TM, doubling time
(Iwamura et al., 1987)	SUIT-2	Explant culture	DMEM, 5% FCS	M, DD, xeno, TM, EM
(Sujino et al., 1988)	JHP-1	Ascites w/ medium	n.n.	M, DD, chr, xeno, TM, plating efficiency
(Drucker et al., 1988)	SU.86	Explant culture	RPMI 1640, 20% FCS	M, DD, HC, chr, xeno, TM
(Nagata et al., 1989)	FA6	Xenograft	RPMI 1640, 10% FCS	FA
(Frazier et al., 1990)	MDA Panc-3	Explant culture	(L-15 + DMEM), 16 kg/ml glutathione, 5 kg/rnl insulin, 5 pg/ml transferring, 5 ng/ml selenium, 5 ng/ml EGF, 10% FCS, AA	M, DD, chr, HC, EM
(Fujii et al., 1990)	SOJ	Explant culture	RPMI 1640, 10% FCS	M, DD, chr, xeno, TM
(Schoumacher et al., 1990)	CFPAC-1	Explant culture	RPMI 1640, 10% FCS	IHC, xeno, FA
(Ikeda et al., 1990)	KP-1N KP-2 KP-3	Xenograft	Daigos T, 10%FCS	IHC, xeno, TM
(Yamaguchi et al., 1990)	HPC-Y0 HPC-Y1 HPC-Y5 HPC-Y9 HPC-Y11 HPC-Y15 HPC-YP HPY-YS HPC-Y25	Explant culture	RPMI 1640, 10% FCS	M, DD, IHC,chr, xeno, FA

Author and year	Name	Method used	Medium	Characterisation
(Chen et al., 1990)	PC-1 PC-2	Xenograft	Ham´s F12, 15% FCS	M, DD, chr, IHC, xeno, TM
(Elsasser et al., 1992)	PA-TU-8988S PA-TU-8988T	Explant culture	DMEM, 10% FBS, 10% Horse serum, 2 mM glutamine, AA	M, DD, chr, HC, xeno, EM
(Nishimura et al., 1993)	HuP-T3 HuP-T4	Ascites w/ medium	MEM, 10% FBS, 1% amino acids, 1% sodium pyruvate	M, DD, xeno, TM
(Elsasser et al., 1993)	PA-TU-8902	Explant culture	DMEM, 10% FCS, 2 mM glutamine	M, DD, chr, IHC, xeno, TM, EM
(Kalthoff et al., 1993)	PT45	n.n.	n.n.	n.n.
(Vila et al., 1995)	IMIM-PC-1 IMIM-PC-2 SK-PC-1 SK-PC-3	Explant culture	E4, 10% FCS	M, IHC, TM, FA
(Heike et al., 1995)	MZ-PC-1	Pleural effusion w/ medium	CMRL, 15% FCS, 2 mM glutamine, 1% amino acids	M, IHC, xeno, EM
(Lehnert et al., 1999)	A818	Explant culture	RPMI 1640, 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, AA	TM, FA
(Kato et al., 1999)	KMP-1 KMP-2 KMP-3 KMP-4 KMP-5 KMP-6	Explant culture	RPMI 1640 + Ham's F12, 20% FCS, AA	M, DD, chr, IHC, xeno, TM, seeding efficiency
(Hou et al., 1999)	PC-EN	Xenograft	n.n.	M, DD, chr, mut, IHC, xeno, TM
(Fralix et al., 2000)	UK Pan-1	Explant culture	DMEM, 10% FCS	M, DD, chr, mut, xeno, FISH
(Ku et al., 2002)	SNU-213 SNU-324 SNU-410 SNU-494	Explant culture	ACL-4, 5% FCS	M, DD, chr, mut, IHC
(Eisold et al., 2004)	FAMPAC	Explant culture	RPMI 1640, 10% FCS, AA	M, DD, chr, mut, xeno, TM
(Kawano et al., 2004)	SUIT-4	Ascites w/ medium	DMEM, Ham´s F12, AA	M, DD, chr, mut, IHC, xeno, FA

Author and year	Name	Method used	Medium	Characterisation
(Starr et al., 2005)	p34	Pleural effusion w/ medium	DMEM, 10% FCS, 2 mM glutamine, AA	M, DD, chr, IHC, xeno, FA
(Kong et al., 2007)	SPH	Ascites w/ medium	n.n.	M, DD, chr, xeno, TM
(Chifenti et al., 2009)	PP78 PP109 PP117 PP161	Enzymatic digestion	RPMI 1640, 1% glutamine, 10% FCS, AA	M, DD, STR, chr, mut, IHC, FA
(Kalinina et al., 2010)	PaCa 5061	Enzymatic digestion	RPMI 1640, 10% FCS, AA, 50 nmol/ml transferrin, 0.01 µg/ml insulin, 0.01 µg/ml EGF, 0.01 µg/ml basic FGF	M, DD, IHC, xeno, FA, TM, FISH
(Rückert et al., 2011)	PaCaDD-43 PaCaDD-60 PaCaDD-119 PaCaDD-135 PaCaDD-137	Explant culture	Dresden-Medium	M, DD, STR, mut, FA
(Yachida et al., 2011)	a99	Xenograft	DMEM, 20% FCS	M, DD, chr, mut, xeno, colony formation

(DD= doubling rate/ growth curve; M= morphology; STR= standard tandem repeat/DNA-fingerprint; chr= chromosomal analysis; mut= mutational analysis; xeno= xenograft; FA= functional analysis; HC= histochemistry; IHC= immunohistochemistry; EM= electron microscopy, TM= tumor marker; AA= antibiotics).

Table 1. Human pancreatic tumor cell lines (adapted from Iwamura & Hollingsworth, 1998). This list is not exhaustive. Some of the cell lines are available at ATCC (www.lgcstandards-atcc.org) or the German Collection of Microorganisms and Cell Cultures (www.dsmz.de)

2.4 Processing of malignant pleural effusion or ascites

This method is frequently used to obtain primary cancer cell lines. Although the success rate is not higher than in other methods many scientist favour this technique as material is easily obtainable. For establishing cancer cell lines with this method, bodily fluids are added to variable amounts of cell culture medium (Akagi et al., 1977; Chen et al., 1990; Nishimura et al., 1993) (**Table 1**).

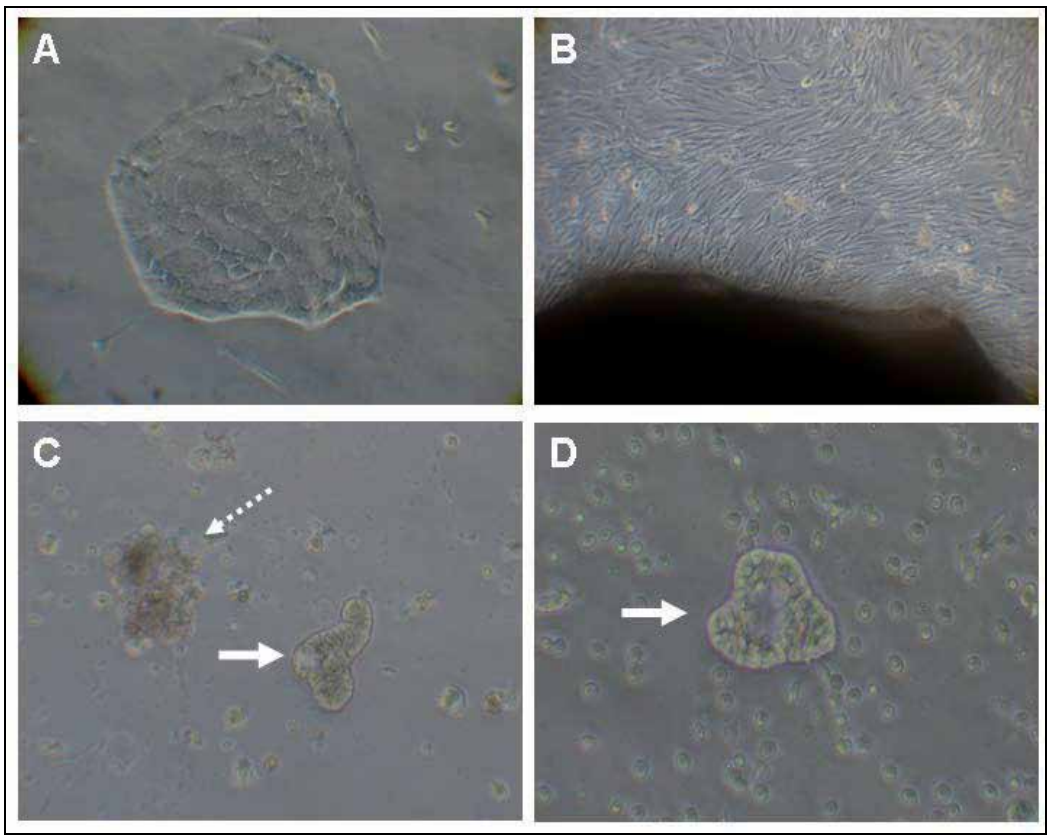

Fig. 1. Typical morphology of a primary pancreatic cancer colony (**A**). Fibroblasts growing out of a tissue fragment (**B**). Figures **C** and **D** depict complexes of cancer cells (full arrow) which the author calles "STC´s" (small trabecular complex). In (**C**) there is also a cluster of mesenchymal cells (interrupted arrow).

2.5 General remarks on the establishment of primary cell lines

The processing of samples should be as fast as possible. The success rate for the establishment of cell lines is considerably shortened if processing starts more than 2 hours after harvesting of the sample.

Parenchymal cells in culture are generally obvious due to their close cell-cell contacts and their tendency to grow in colonies (**Figure 1, A**). However, parenchymal cells are often overgrown by cells from the stromal compartment (**Figure 1, B**). Those fibroblasts can be removed by different techniques. One possibility is to mechanically remove fibroblasts by a sterile cannula under phase contrast microscope. Another method uses a solution of trypsin to enzymatic remove fibroblasts. This enzymatic removement is possible because tumor cells adhere much more firm to the surface than fibroblasts do.

The successful establishment of primary cell lines is often impeded by contamination by fungi and bacteria. For this purpose we advise to always use antibiotics within the cell culture media. A way to protect cell lines from fungal infection is reported in Rückert et al,

2011. Cell lines should be cryoconserved early and often during the early passages. As a general rule, cultures of pancreatic tumor cell lines should be split at high density (l : 2) (Iwamura et al., 1998; Rückert et al., 2011).

3. Cell culture conditions and medias

The majority of pancreatic cancer cell lines are grown *in vitro* in a basic culture medium such as DMEM or RPMI 1640 substituted with foetal calf serum (FCS) in varying percentages (**Table 1**). However, there are also successful isolations using other media. Generally, after a primary cell culture is established it should be assessed if growth might be optimized by a different media and/or a different ratio of FCS. Some pancreatic tumor cells can be adapted to growth in reduced serum (0.5% or less) or serum free conditions**.**

Cell cultures are generally maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium should be replaced every 3 d in established cultures.

To guarantee the availability of primary cell lines for the further experiments, all cell lines should be cryoconserved. Storage of cryotubes should always be performed in liquid nitrogen.

4. Characterisation of primary tumor cell lines

Characterization of primary cell lines should be conducted to *proof the origin* from the parent tumor and to *proof the absence of cross-contamination*. Only by this the researcher can be sure that *in vitro* studies with a certain cell line can be related to a distinct tumor and its clinicophysiological properties. Most malignant tumors are heterogeneous in morphology and biological properties, e.g. degree of differentiation, malignant transformation, and metastatic properties. This fundamental facet of all solid tumors is called tumor heterogeneity (Heppner, 1984). Therefore it is essential to further *define the cytostructural and pathophysiological characteristics* of the cell line.

For these purposes different standards have been defined, which will be further explained below (Henderson & Kirkland, 1996; Iwamura & Hollingsworth, 1998).

4.1 Morphology and cytostructural characteristics

4.1.1 Growth pattern

Pancreatic tumors are generally categorized according to morphological criteria as grades of differentiation and invasiveness. Many cell lines exhibit morphological characteristics that are consistent with the grades of differentiation reported for the tumor from which the cell line was derived. In cell lines, morphology is normally assessed by phase contrast microscopy, but some authors also use electron microscopy or histochemical staining.

Features that should be described are:

- the growth pattern (e.g. epithelial, disorganized, mesenchymal)
- size of the cells (e.g. homogenous, inhomogenous, small, big)
- form of the cell (e.g. round, polygonal, elongated, swirly)
- size and form of the nucleus (e.g. small, big, ovoid, round)

Some examples of growth patterns of primary cells are given in **Figure 2**.

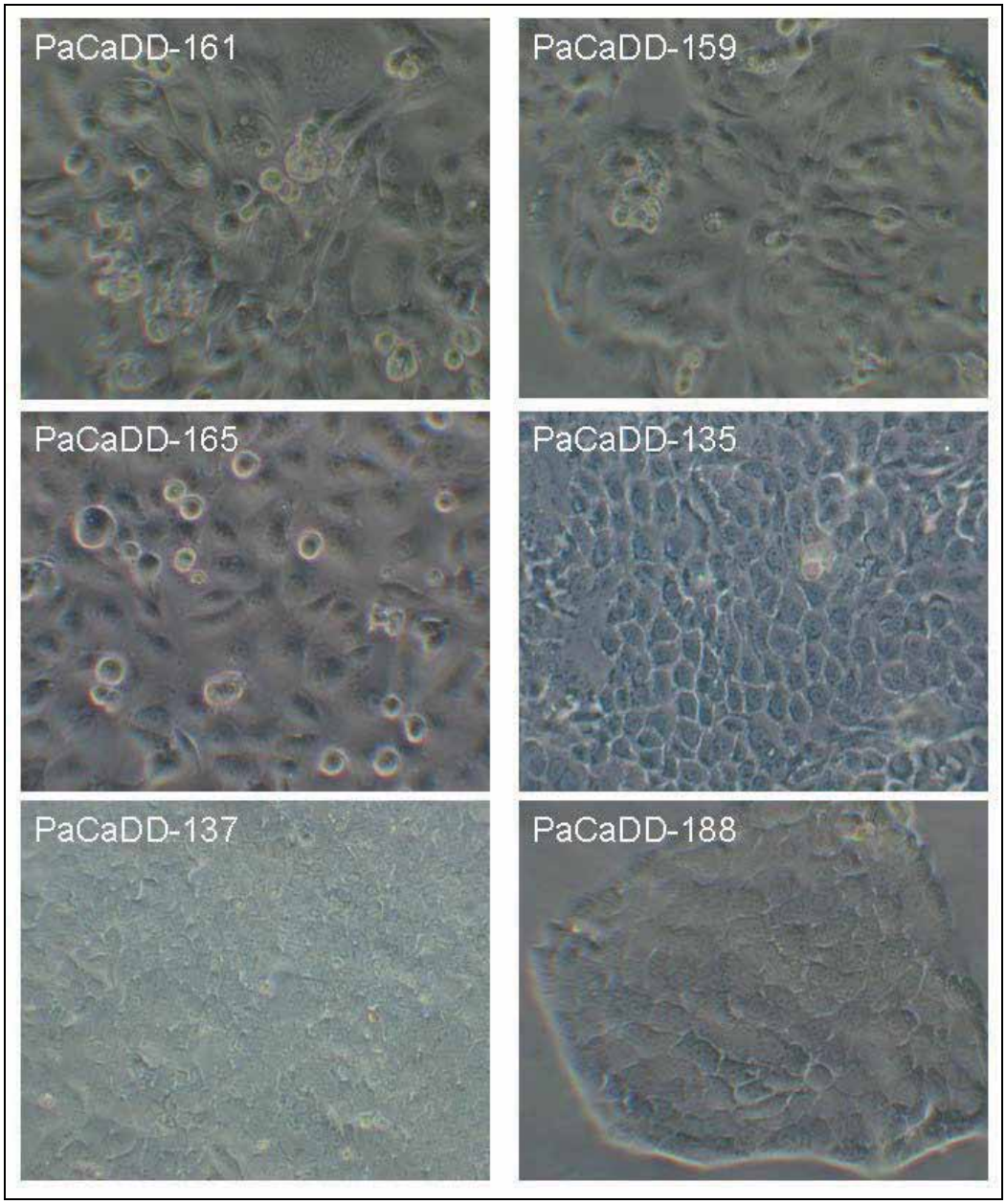

Fig. 2. Examples of the morphology and growth pattern of the primary carcinoma cell lines from our research lab as seen with phase contrast microscopy (Details from 40x, pictures by Felix Rückert).

4.1.2 Immunohistochemistry

Cell surface antigen characteristics can be used to discriminate pancreatic cancer cells from contaminating cell types like stromal and immune cells. The immunohistochemical phenotype can be compared to the native tumor tissue and thereby further proof its origin. Typical markers for pancreatic cancer are CK 8/18, E-cadherin, ezrin, p53, SMAD4, and Vimentin (Rückert et al., 2011).

4.2 Pathophysiological characteristics

4.2.1 Doubling time

The doubling time is an important feature of each cell line, because it can be correlated with an important pathophysiological parameter: aggressive growth. Furthermore, conclusion can be drawn on how often medium should be changed and in which ratio cell lines should be split.

The cell doubling time can be determined by plating a certain number of viable cells in to a well. Cells should then be counted at 24 h intervals for 7 d in triplicate. The doubling time of the cell population can then be calculated from the logarithmic growth curve by the following formula:

$$\vartheta = \lg N - \lg N0/\lg 2\ (t-t0),$$

with doubling time = $1/\vartheta$

Of course there are other possibilites to define the growth rate e.g. BrdU-staining or ki-67 staining.

4.2.2 Tumorigenicity

One notable feature of pancreatic cancer, which is amenable to study through the use of cell lines, is invasiveness and metastasis. Tumorigenicity can be measured by assessing the tumor formation of cell lines in athymic mice (Henderson & Kirkland, 1996). This can be done by orthotope (pancreas) or ectope (subcutaneous) injection of tumor cells. The number of cells required ranges from 100,000 to 2,000,000 cells. Pancreatic cancer cell lines should form a tumor within 3-4 weeks. If the cell line has tumorigenic potential, the progression of tumor volume/time as well as the ability to form metastasis should be documented.

4.3 Chromosomal and mutational analysis

Chromosomal and mutational analyses are used to proof the origin and the uniqueness of each cell line. They give further evidence on the role of mutations for the pathophysiology of the tumor. The origin of the cell lines, however, can also be proofed by other techniques as microsatellite analyses (Rückert et al., 2011).

4.3.1 Chromosomal analysis

Chromosomal analysis has long been the standard method to proof the uniqueness of an certain cell line. Classically, karyotype analysis was undertaken by chromosome banding.

By this method numerical and structural chromosomal aberrations could be identified. However, the chromosomal origins of markers, subtle translocations, or complex chromosomal rearrangements were often difficult to identify with certainty (Schrock et al., 1997). Because of this, new methods are recommended like the spectral karyotyping (SKY) or the comparative genomic hybridisation (array CGH).

During SKY, chromosomes are hybridized simultaneously with 24 chromosome-specific painting probes. The measurement of defined emission spectra allows for the definitive discernment of all human chromosomes in different colors (Schrock, 1997). The array CGH is a method to analyse copy number changes (gains/losses) in the DNA content of a given cell. By this, gains or losses of genes in tumor chromosomes compared to normal DNA can be detected (Wessendorf et al., 2002).

4.3.2 Mutational analysis

Pre-malignant and malignant tumors evolve by clonal expansion of mutant cells that have either a reproductive or survival advantage over other cells in the tissue. This competitive advantage is acquired by the overexpression of oncogenes and inactivation of tumor suppressor genes. Numerous studies have found a relatively unique molecular fingerprint in PDAC that is comprised of frequent alterations of four genes: kRAS, $p16^{INK4A}$, DPC4 and p53 (Bardeesy et al., 2002; Krautz et al., 2011). Because of the importance of these genes for the pathophysiology of PDAC the mutational analysis of these genes is recommended.

5. Conclusion

Basic research in cancer is absolutely dependent on cancer cell lines. The establishment of primary pancreatic cancer cell lines in laboratories with focus on pancreatic cancer has considerable advantages and can be done with reasonable expense. We hope that the present chapter encourages more scientists to start establishing own cell lines.

6. Acknowledgment

The authors welcome requests for their cell lines (Felix.Rueckert@uniklinikum-dresden.de). We like to thank Silke Zeugner, Heike Berthold, Doro Sturm and Trixi Jahnke for technical support. We want to thank the Wilhelm Sander Foundation (2009.039.1) and the MedDrive38 program of the Medical Faculty of the Technische Universität Dresden, Germany, for the support of our work.

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16

Disruption of Cell Cycle Machinery in Pancreatic Cancer

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1. Introduction

This chapter discusses the role of cell cycle machinery in initiation and progression of pancreatic cancer. Normal pancreatic cells—their types, organization, and functions—are first described to characterize the environment in which cellular transformation and tumor expansion occurs. The epidemiology and histology of pancreatic cancer is then briefly presented to emphasize the urgent need for earlier diagnosis and more effective treatments. Current efforts towards this goal are focused on understanding the disease at the molecular level, so the hallmarks of cancerous cells are discussed with respect to the progression model of pancreatic cancer development. Because the pancreas is composed of various cell types with different genetic backgrounds and regulatory systems, identifying the cell in which cancer originates is of utmost importance. Molecular mechanisms of normal proliferative control are then presented so that mechanisms by which they are disrupted can be appreciated. Particular attention is paid to how signaling transduction pathways and the cell cycle machinery cooperate to make cell fate decisions at the Restriction point. This analysis sets the stage for evaluating the role of cell cycle control mechanisms in transformation of the initiating cell in pancreatic cancer. The chapter concludes by arguing that genetic alterations associated with pancreatic cancer indicate disrupted cell cycle control mechanisms play a central role in disease development and progression.

2. Pancreatic tissue organization and cellular function

Evaluating the role of cell cycle machinery in pancreatic cancer requires understanding the architecture and cellular organization of this dual-function gland. The pancreas is an approximately six inch long cylindrical organ in the abdomen, located between the stomach and the spine (Romer & Parsons, 1977).

The endocrine component is composed of clusters of alpha, beta, and PP (pancreatic peptide) cell types that form structures called the islets of Langerhans (Jain & Lammert, 2009). These cells produce metabolic hormones involved in energy metabolism. Major cell types and their organization are summarized in Figure 1.

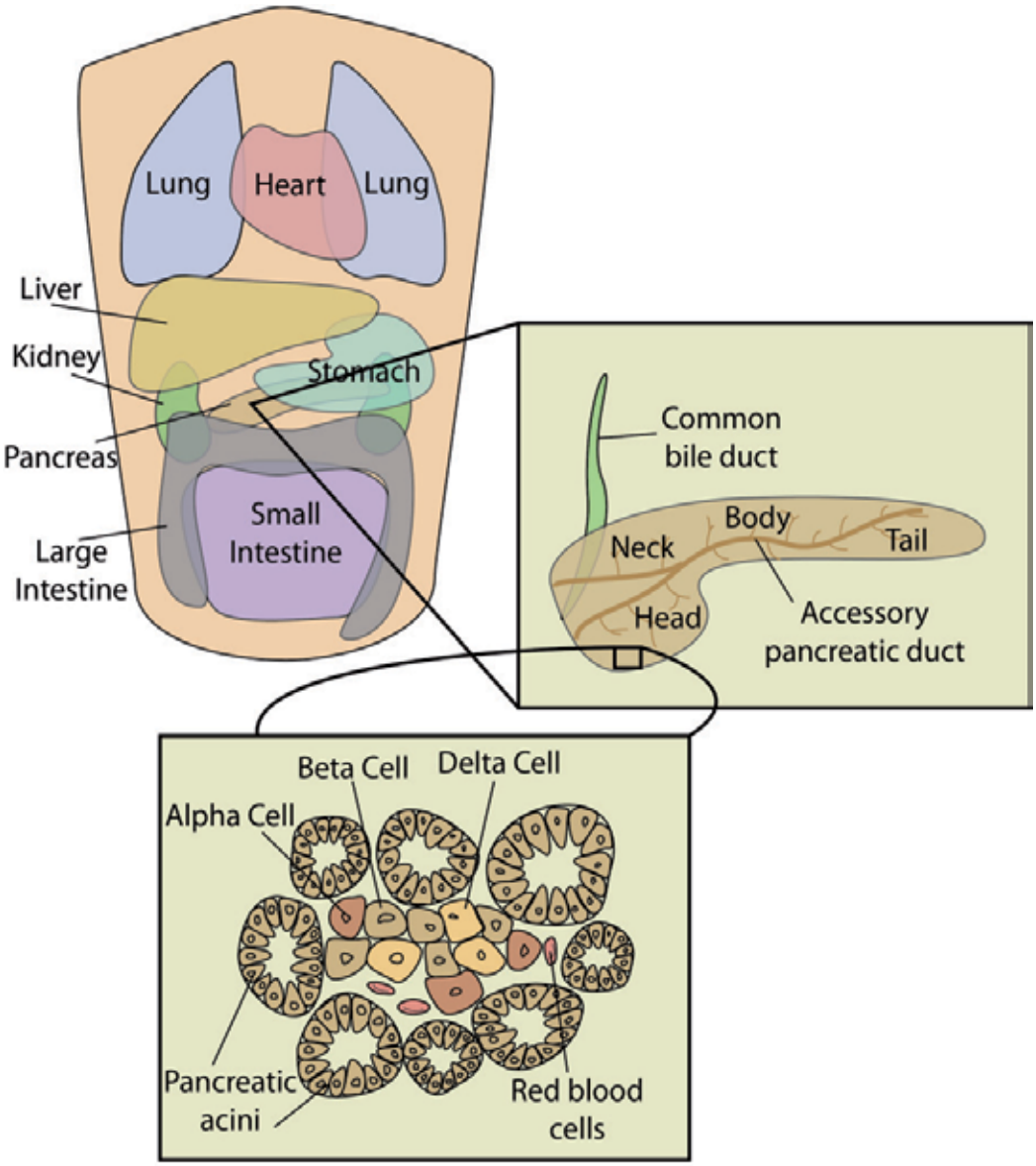

Fig. 1. **Global View of Pancreas.** The pancreas is located in the abdomen behind the stomach. It is composed of four areas: The head, neck, body, and tail. It is comprised of two types of parenchymal tissue: The islets of Langerhans, composed of alpha, beta, and gamma cells are in charge of endocrine signal and hormone detection, while the pancreatic ancine are in charge of exocrine signaling and production of digestive enzymes.

It plays major roles in the vertebrate hormonal (endocrine) and digestive (exocrine) systems (Jain & Lammert, 2009, Means & Leach, 2001). The pancreas contains two different types of parenchymal (i.e. functional) tissue that is of endodermal origin (Gittes, 2009). Most of its mass is clustered acinar cells that synthesize digestive pro-enzymes (Means & Leach, 2001).

The most prevalent type of pancreatic cancer is infiltrating ductal adenocarcinoma, which appears to initiate in distinct subsets of cells within the exocrine tissue (Maitra, A & Hruban, 2008). However, other cell types can participate and/or be affected by the disease. This includes "enabling cells", which are local, untransformed populations that can contribute to disease development. Pancreatic stellate cells, for instance, are stromal cells recruited by the tumor to help create an environment promoting disease progression (Vonlaufen, *et al.*, 2008).

2.1 Endocrine function

Endocrine function is mediated by groups of cells called the islets of Langerhans, which secrete essential peptide hormones regulating energy metabolism into the bloodstream (Jain & Lammert, 2009). The pancreas contains approximately 1 million of these cell clusters, each composed of four different cell types distinguished by their secretatory role. The alpha and beta cells work together to maintain blood sugar levels. Alpha cells produce glucagon to promote release of stored glucose in response to an unfed state (Gromada, *et al.*, 2007). In contrast, β cells generate insulin in response to eating so that incoming glucose can be utilized by body tissues (Collombat, *et al.*, 2010). These functionalities are fine-tuned by somatostatin secreted from δ cells (Brink, 2003). PP cells are so called because they produce pancreatic polypeptide that helps regulate endocrine and exocrine secretions, control hepatic glycogen levels, and participate in regulation of gastrointestinal secretions (Lonovics, *et al.*, 1981). Insulin and glucagon are rapidly disseminated by a capillary network that is connected to blood vessels via layers of endocrine cells (Jain & Lammert, 2009, Means & Leach, 2001). Less than 10% of pancreatic cancers originate in endocrine cells. Nevertheless, these cells could play an important secondary role in more common ductal adenocarcinomas via their ability to produce hormones affecting cell fate decisions. In addition, their extensive capillary network could be exploited by metastasizing tumor cells.

2.2 Exocrine function

The majority of pancreatic cancers (>90%) are infiltrating ductal adenocarcinomas of the exocrine system (Maitra, A & Hruban, 2008). Thus, identifying the potential cell types involved and their normal function is essential for evaluating how cell cycle machinery contributes to cancer development. Exocrine function is mediated by clusters of acinar cells (called acinus) that secrete bicarbonate ions and digestive pro-enzymes (Means & Leach, 2001). These products are transported in the pancreatic juice to the duodemun by a ductal system lined with a layer of mucinous columnar epithelial cells. Exocrine function is under control of the hormones gastrin, cholecystokinin and secretin, which are secreted by gastrointestinal cells in response to physical distension and food intake (Jean, 2008).

The alkaline bicarbonate secreted by centroacinar cells regulates pH in the small intestine by neutralizing the acidic chyme arriving from the stomach (Freedman & Scheele, 1994). Centroacinar cells also secrete mucins, a family of high-molecular-weight, heavily glycosylated proteins known primarily for forming biological gels (Nagata, *et al.*, 2007). They are involved in signaling, barrier formation, lubrication, and the immune response via binding and/or blocking pathogens (Hollingsworth & Swanson, 2004). Overexpression of mucin proteins (e.g. MUC1) occurs in many different types of cancers, including pancreatic (Moniaux, *et al.*, 2004). Based on their unique genetic background, centroacinar cells have

been implicated as the potential cell of origin giving rise to ductal adenocarcinomas (Singer & Niebergall-Roth, 2009, Stanger & Dor, 2006).

Digestive pro-enzymes for breaking down fats and protein are secreted into the lumen of the acinus by basophilic cells, so-called because they tend to stain intensely with basic dyes (Singer & Niebergall-Roth, 2009). These cells contain large cytoplasmic secretory granules in which digestive pro-enzymes are maintained in an inactive state, a method of regulatory control that is essential to prevent auto-degradation of the cell and subsequent development of a condition called acute pancreatitis (Waldthaler, *et al.*, 2010). This type of cellular damage increases the risk of pancreatic cancer, an observation that provides insight into identity of the originating cell and how it is transformed (Suda, *et al.*, 2007).

3. Pancreatic cancer

Epidemiology studies have revealed pancreatic cancer runs in some families and is associated with specific genetic mutations (Chakraborty, *et al.*, 2011, Greer, *et al.*, 2009, Lowenfels & Maisonneuve, 2006, Shi, *et al.*, 2009). As is the case for all cancers, pancreatic cancer arises when a normal cell is transformed by accumulated genetic alterations into a cancer cell that seeks to duplicate itself at the expense of the organism. If this transformed cell can escape internal and external fail-safe mechanisms, obtain nutrients, and successfully activate its proliferative program, it can eventually form a more complex mass of cancerous cells. Continued growth of the tumor depends on space availability and promoting growth of blood vessels (angiogenesis) to supply nutrients and remove waste. Further tumor growth can result in loss of pancreatic function, impairment of local blood vessels and organs, and finally metastasis and secondary tumor formation at distal parts of the body. An unfortunate characteristic of pancreatic cancer is its propensity for metastasis early in tumor growth, which is likely responsible in part for its aggressive nature (Bardeesy & DePinho, 2002).

Pancreatic cancer is not exceptionally rare, with approximately 43,000 cases in 2010, making it the tenth-most-common form of the disease (ACS, 2007, ACS, 2010, NCI, 2010). However, 37,000 of those died, illustrating its high mortality rate and ranking pancreatic cancer as the fourth leading cause of cancer fatalities worldwide (ACS, 2007, ACS, 2010, NCI, 2010). The lack of early symptoms results in delayed diagnosis and a higher likelihood of metastasis, thus dramatically limiting treatment options and outcome (Hansel, *et al.*, 2003). The percent of patients alive one year after diagnosis is about 25%, while the five year rate is a depressing 6% (Hariharan, *et al.*, 2008). The five year survival increases substantially with early detection and surgical removal of the tumor (approximately 22%), emphasizing the importance of developing better diagnostic tools and markers (Benson, *et al.*, 2010). Even with early detection and conventional treatment, however, pancreatic cancer is still quite deadly. The ineffectiveness of standard therapy suggests its development and aggressiveness might involve unique molecular features.

The pancreas is composed of head, body, and tail sections, with cancer most commonly occurring in the head region (see Figure 1) (Romer & Parsons, 1977). The majority of cases are malignant adenocarcinomas arising in the exocrine component (Maitra, A & Hruban, 2008). Typically, the cancer initiates in the pancreatic ducts (infiltrating ductal adenocarcinoma), or less frequently in the acini (acinar adenocarcinoma). It presents as a

dense mass with crennolated extensions into surrounding tissue. Histological analysis reveals a neoplastic epithelium composed of poorly differentiated, gland-forming cells that illicit a very strong growth of fibrous and/or connective tissue around the neoplaisa (i.e. a desmoplastic reaction) (Hartel, *et al.*, 2004, Maitra, Anirban, *et al.*, 2006). A much smaller subset of exocrine pancreatic tumors present as adenosquamous carcinomas, squamous cell carcinomas, and giant cell carcinomas (Hruban & Zamboni, 2009). Metastatic endocrine cancers (also called islet cell tumors) are much less common and only account for approximately 1% of pancreatic cancers (Spiegel & Libutti, 2010).

3.1 Molecular events underlying cellular transformation

Cancers are recognized as such despite diverse physiological presentation because they all share a limited set of underlying causative characteristics. These so-called "hallmarks of cancer" are the distillation of extensive efforts to understand how a normal cell is transformed into a cancerous version (Hanahan & Weinberg, 2000).

Fig. 2. **Hallmarks of Cancer.** Genetic insults to the pancreatic cells can result in the accumulation of the six hallmarks associated with cancer. The relative percentage of transformed cells types in the pancreas is also shown.

For this reason they are the predominant paradigm for understanding its molecular basis and developing better diagnostics and treatments. In order to evaluate how disrupted cell cycle control specifically contributes to pancreatic cancer, it is first necessary to appreciate why it has been designated as a fundamental hallmark of the disease.

3.2 Hallmarks of cancer

Normal cells perform specific tasks and exist for the greater good of the organism. For this reason, their proliferative capability is a double-edged sword that must be highly constrained by internal checkpoints and external signals from other cells or the microenvironment (Bogenrieder & Herlyn, 2003). Cancer cells evade these constraints via accumulated genetic alterations, resulting in a selfish cell whose allegiance is now to its own survival and expansion. A multi-cellular organism protects itself by making cell duplication dependent on externally generated signals. Positive growth-promoting factors are required for proliferation while negative growth-inhibitory factors must be withdrawn. Two hallmarks of cancer are therefore self-sufficiency in growth signals (i.e. cell division in the absence of mitogenic factors) and insensitivity to growth-inhibitory factors (i.e. cell division

despite the presence of anti-mitogenic signals) (Hanahan & Weinberg, 2000). Extensive analysis of cell signaling pathways has identified myriad ways in which genetic alterations can satisfy these two criteria (Brognard & Hunter, 2011). Typically, multiple biological targets must be compromised due to redundancies and control mechanisms that evolved to prevent deregulated signaling.

Self-sufficiency in growth signals and insensitivity to growth-inhibitory factors is not sufficient to generate a transformed cell capable of tumor formation. Most cells have an internal clock that limits their replicative potential so that they can only duplicate a fixed number of times before entering a senescent or non proliferative state (Hornsby, 2005). As will be discussed in more detail below, stem cells (and cell populations with stem cell-like characteristics) are an important exception to this rule and likely play a central role in the initiation of pancreatic cancer. The mechanism of this clock centers on telomere maintenance, the process whereby chromosome ends are protected from degradation (Yang, Q., 2008). Loss of telomere protection occurs after a fixed number of duplications, sending a signal that causes cells to exit the proliferative cycle and enter a senescent state (Yibin, *et al*., 2008). Cancer cells typically overcome this internal checkpoint—for instance by hyperactivation of the telomere synthesizing enzyme telomerase—to acquire the proliferative capacity needed for tumor formation (Artandi & DePinho, 2010). Telomere maintenance is also disrupted in pancreatic cancer, but in a complex manner that varies with disease progression (van Heek, *et al*., 2002). This pattern provides insight into its origins and development.

Cells have additional mechanisms to prevent inappropriate proliferation and mount a protective response should it occur. In the event of irreparable damage or grow-promoting mutations, for example, the cell can initiate an apoptotic pathway resulting in its death and deconstruction (Wyllie, 2010). A major hallmark of cancer is, thus, evasion of apoptosis (Hanahan & Weinberg, 2000).

Once immortalized, the transformed cell can proliferate and begin to generate cancer cells, comprising the bulk of the tumor. However, cellular expansion can only occur up to a certain point as determined by available space and nutrients. A hallmark of cancer is, therefore, formation of new blood vessels (angiogenesis) so nutrients can be obtained and delivered throughout the tumor and so waste products can be removed (Hanahan & Weinberg, 2000). As a result, the tumor can further increase in size and complexity, compromising not only the affected organ itself but also nearby blood vessels and tissues.

The final hallmark of cancer is perhaps most responsible for threatening organism survival. Even with angiogenesis, tumor size will eventually be constrained by physical barriers. As a consequence, the tumor is subjected to selective pressure, driving invasion of surrounding tissue and metastasis to distal locations (Hanahan & Weinberg, 2000). In this process, cancer cells detach from the primary tumor and secrete enzymes (e.g. MMPs) that allow passage through the extracellular matrix (Singh, *et al*., 2002). They can then migrate and start secondary tumors in surrounding tissue or in distal areas by commuting through the body's highways (lymph and hematopoietic systems). Once a tumor has metastasized, the potential for successful therapeutic intervention is severely reduced, as is the case with pancreatic cancer (Bardeesy & DePinho, 2002).

3.3 Progression model of pancreatic cancer development

The current view of pancreatic cancer development is summarized in a progression model wherein the temporally ordered accumulation of genetic mutations drive transitions through a series of pre-cancerous legions culminating with infiltrating ductal adenocarcinoma (in the majority of cases) (Koorstra, *et al.*, 2008a). These steps were defined by histology of precancerous lesions at various stages that are collectively described as pancreatic intraepithelial neoplasias (PanINs) (Koorstra, *et al.*, 2008b). They are further subdivided into PanIN-1A, PanIN-1B, and PanIN-2/3 based on distinct histology and genetic background (Koorstra, *et al.*, 2008b, Koorstra, *et al.*, 2008a). PanINs present as microscopic lesions situated in the smaller pancreatic ducts (Maitra, Anirban, *et al.*, 2005). Genetic analysis of these distinct cell populations revealed ordered accumulation of alterations associated with the more advanced adenocarcinoma (Koorstra, *et al.*, 2008a). The types of genetic insults present in pancreatic cancer are quite diverse and include large chromosomal alterations such as breaks, duplications, deletions, fusions, and translocations (Campbell, *et al.*, 2010). There is also CpG island methylation of promoters, telomere disruptions, and microsatellite instability, along with specific mutations in important oncogenes and tumor suppressors (Lin, *et al.*, 2011, López-Casas & López-Fernández, 2010, Welsch, *et al.*, 2007). Precursor lesions display an increasing proliferation rate as they progress towards an adenocarcinoma (Koorstra, *et al.*, 2008b).

From a molecular biology perspective, three pertinent questions are: 1) What type of cell undergoes transformation? 2) What genetic elements are disrupted? 3) What biological activities are compromised and how do they contribute to transformation? Each of these questions will be addressed, with particular emphasis on the role of cell cycle machinery. The first task is to consider the cell type in which pancreatic cancer originates, since its genetic makeup and regulatory systems will dictate how to interpret genetic alterations associated with the disease.

3.4 Identity of the cell initiating tumor formation

Identifying the cell in which cancer originates is essential for understanding the consequences of genetic alterations and their effect on cell cycle control. This initiating cell has not yet been definitively described, but we can speculate it accumulates genetic mutations that, if not resolved (via repair or cell death), culminate in the hallmarks of cancer. The average age of onset for infiltrating ductal adenocarcinoma is approximately 73 years, indicating it develops in the mature organ (Greer, *et al.*, 2009, Lowenfels & Maisonneuve, 2006). One obvious candidate for transformation is the differentiated acinar ductal cell itself, which could be induced to re-enter the cell cycle and proliferate in opposition to internal and environmental cues. Such a transition seems quite daunting, because the mature cell must first de-differentiate to a more primitive state with proliferative capacity via genomic reorganization and altered gene expression. Secondly, this reverted cell must be induced to proliferate inappropriately, which likely requires disruption of different biological pathways. Thirdly, it is likely that fail-safe systems unique to each transition would have to be inactivated. Despite these hurdles, centroacinar cells are a possible candidate because they reside at the junction between acini and ducts and are the only differentiated cells in the developed pancreas with activated signaling pathways associated with proliferation (Miyamoto, *et al.*, 2003). Thus, fewer genetic changes might be required in this background to generate the initiating transformed cell.

An alternative possibility is that cancer arises from transformation of a pancreatic stem cell. This model posits that the originating cell in cancer development is either a stem cell or has stem cell-like characteristics (Stanger & Dor, 2006). Their defining feature is unlimited self-renewal capabilities. Normal stem cells play key roles in development and tissue maintenance by dividing asymmetrically to give one progeny with self-renewing capacity and another that commits to a differentiation pathway (Leeb, *et al.*, 2011). Cancer stem cells are thought to follow the same process, except that their asymmetrical division generates cancer cells comprising the bulk of the tumor (Clevers, 2011, Stanger & Dor, 2006). Transformation of a cell with proliferative capacity seems more likely because it already exhibits one or more of the hallmarks of cancer. Evidence suggests that stem cells only make up 0.1-1% of the tumor cell population and show greater resistance to chemotherapy and radiation compared to their progeny (Bomken, *et al.*, 2010). Consistent with this idea, subpopulations of pancreatic cancer cells have been isolated that can initiate a new tumor when implanted in mice (Reya, *et al.*, 2001). Given that disease arises in the mature organ, a likely candidate for initial transformation would be a pancreatic adult stem cell. Such a cell type has not yet been identified, but their presence in other organs makes a pancreatic version a distinct possibility (Li, *et al.*, 2007). These observations could help explain the well-known unresponsiveness of pancreatic cancer to traditional chemotherapies (Wang, Zhiwei, *et al.*, 2011c).

A third possibility is involvement of what are called facultative progenitor cells located in the acinar environment (Leach, 2005). In the case of pancreatic injury (e.g. acute pancreatitis), differentiated cells can be recruited back into the proliferative cycle to replace old or damaged cells (Raimondi, *et al.*, 2010). Such cells exhibit a more "stem cell-like" phenotype and hence are more susceptible to transformation. There is experimental evidence supporting this idea. Acute pancreatitis is a risk factor for pancreatic cancer, and, in a chemically induced version of this disease, acinar cells de-differentiate to replenish the cell supply (Guerra, *et al.*, 2007, Jensen, *et al.*, 2005). In addition, growth-factor stimulation can cause acinar cells to undergo an abnormal transdifferentiation event to generate what is called an acinar-to-ductal metaplasia (Husain & Thrower, 2009). Taken together, these observations strongly implicate facultative progenitors as the initiating cell in pancreatic cancer. While further efforts to definitively characterize the relevant cell type are required, armed with the above possibilities we can now discuss the basics of proliferative control mechanisms and the important role of the cell cycle machinery.

4. Molecular mechanisms of proliferative control

The cell cycle is an experimentally based, theoretical construct describing the stages through which a cell proceeds to generate a faithful copy of itself (Vermeulen, *et al.*, 2003). These discrete steps must be transversed in sequential fashion with DNA replication in S phase followed by chromosome segregation and division in M phase. Gap 1 and Gap 2 phases separate DNA replication from chromosome segregation. They are also important for increasing cell mass (e.g. protein synthesis) and monitoring S and M phases to ensure accurate transmission of the genetic material. Progression through the cell cycle is controlled by a diverse group of molecular components collectively called the cell cycle machinery (Suryadinata, *et al.*, 2010). Disruption of this regulatory network is a fundamental event required for the expansion of cancer cells (Hanahan & Weinberg, 2000). Understanding how

this machinery functions at the molecular level and is linked to cell fate decisions is therefore crucial to evaluating its role in development and progression of pancreatic cancer.

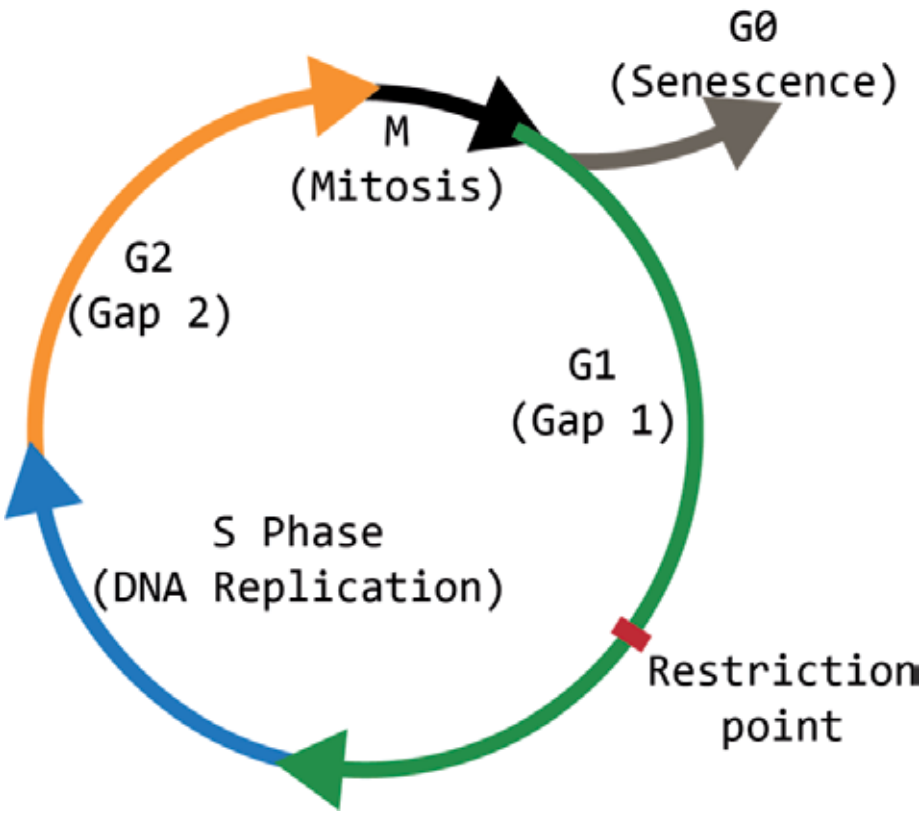

Fig. 3. **The Cell Cycle.** The stages of the cell cycle (G1, S, G2, M) that take place during the duplication of the cell. The Restriction point, the regulatory checkpoint of the cell cycle after which the cell is committed to replication is shown, along with the option of senescence.

4.1 Cell fate decisions

It is essential to evaluate proliferative capacity within the context of cell function and contribution to the organism as a whole. During development, proliferation is transcendent, since exponential cell division is required to generate large number of cells. Once maturity is reached, proliferative capacity is utilized less frequently (e.g. tissue maintenance and repair). A classic example is the hematopoietic system, where immortal stem cells generate progeny destined to become distinct functional cells in the blood (Heike & Nakahata, 2004). As these cells proceed along differentiation pathways and initiate specific genetic programs, their capacity for division diminishes (Congdon & Reya, 2008). External information combined with internal preparedness are key components determining cell fate decisions. Cell cycle machinery is intimately involved in this process because it coordinates and interprets incoming signals to decide whether to proliferate or adopt an alternative fate. This decision is called the Restriction point, and its disruption is an essential event in cellular transformation (Blagosklonny & Pardee, 2002).

A major challenge for the cell is properly interpreting external signals, establishing and maintaining connections between signal and cell cycle systems, and maintaining control of proliferative potential. For example, withdrawal of growth factors or anti-mitogenic signals will cause a tissue culture cell to exit the proliferative cycle and enter a quiescent state (Zetterberg, *et al.*, 1995). Such cells can be induced to re-enter the cell cycle by growth factor addition, which rapidly activates signal transduction cascades that communicate this information to the nucleus (Pomerening, 2009). A key pathway in this regard is Ras/Map kinase, which transmits a proliferative signal to the nucleus that jump-starts the cell cycle machinery and initiates the gene expression program required for cell duplication (Chang, *et al.*, 2003, Coleman, *et al.*, 2004, Takuwa & Takuwa, 2001). In addition, it is imperative that the nucleus alert signaling systems that their information has been received and properly acted upon. An example of such feedback will be discussed in more detail below. The Ras/Map kinase pathway and its regulation of cell cycle machinery play a key role in initiation and development of pancreatic cancer (Caldas & Kern, 1995, Moskaluk, *et al.*, 1997). Understanding normal functioning of the major cell cycle components and their connection to signal transduction pathways is therefore essential to elucidating how and why they are disrupted in the disease state.

4.2 Cell cycle machinery

Regulation of cell cycle progression is designed to ensure DNA replication and chromosome segregation occur in response to the proper signals, proceed in the required temporal order, and are carried out accurately (Suryadinata, *et al.*, 2010). The cell cycle machinery that control events can be promoters, inhibitors, or evaluators of cell cycle progression but in all cases are responsive to internal and extracellular signaling pathways (Novák, *et al.*, 2010). The six major types of activity regulated by this machinery include: 1) Establishment of ordered biochemical pathways responsible for sequential progression through the cycle; 2) Assembly/disassembly of required structures (e.g. formation of DNA origins of replication, transcription start sites, chromosome segregation sites, telomeres, etc); 3) Regulation of nanomachines controlling production (DNA/RNA polymerases, ribosomes, lipid production etc); 4) Communication of outcomes (e.g. informing signaling components that transmitted information has been received); 5) Monitoring fidelity of ongoing or completed tasks (e.g. mechanisms ensuring cell cycle events are carried out in an accurate and timely fashion); 6) Self-regulation of activities (e.g. cell cycle components often regulate themselves or each other to drive cell cycle transitions and maintain ordered progression).

Cyclin-Dependent Kinases (CDKs) phosphorylate specific protein substrates at serine/threonine residues to initiate specific events (e.g. DNA replication) and drive cell cycle transitions (Malumbres & Barbacid, 2005). Regulating CDK activity is therefore crucial, as indicated by the multiple distinct and redundant pathways controlling its function. The CDK subunit alone lacks kinase activity, so it must bind a cell cycle-specific cyclin subunit and undergo both phosphorylation and dephosphorylation at unique sites to be activated (Harper & Adams, 2001). Functional cyclin-CDK complexes can be inhibited by phosphorylation/dephosphorylation, cyclin degradation, and by tight binding of small inhibitory proteins such as members of the CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and the INK family ($p15^{INK4b}$; $p16^{INK4a}$; $p18^{INK4c}$ and $p19^{INK4d}$) (Ekholm & Reed, 2000, Morgan, 1997, Pavletich, 1999, Sheaff, 1997, Wang, Q., *et al.*, 2011a). The CDK inhibitors are typically

thought to function as tumor suppressor proteins (Sherr, C. J. & Roberts, 1995). Genetic analysis of pancreatic cancer reveals both cyclin and CDK inhibitors are commonly disrupted in the disease, either directly or by alteration of upstream signaling pathways (Chen, Jinyun, *et al.*, 2009b, Gansauge, *et al.*, 1997, Kornmann, Marko, *et al.*, 1998a, Lee, *et al.*, 2009, Schutte, *et al.*, 1997).

4.3 The restriction point

The Restriction point represents an operationally defined transition in G1 when the cell decides whether to proceed with the proliferative cycle or withdraw and adopt an alternative fate (Blagosklonny & Pardee, 2002). The cell cycle machinery makes the decision based on evaluation of external signals and internal preparedness. Before the Restriction point, cell cycle progression is dependent on mitogen stimulation and thus represents a period in which the cell is still receiving information and evaluating its ability to successfully divide (Blagosklonny & Pardee, 2002, Sheaff & Roberts, 1998). After the Restriction point, cell cycle progression no longer requires growth factor stimulation and the cell is committed to completing the proliferative cycle (Blagosklonny & Pardee, 2002, Sheaff & Roberts, 1998). Mitogen signaling performs three main functions: 1) It establishes and maintains extracellular contact with the cell, transmitting the need for duplication; 2) It activates and communicates with cell cycle machinery to drive progression through the cycle; and 3) It initiates the gene expression programs required for cell duplication.

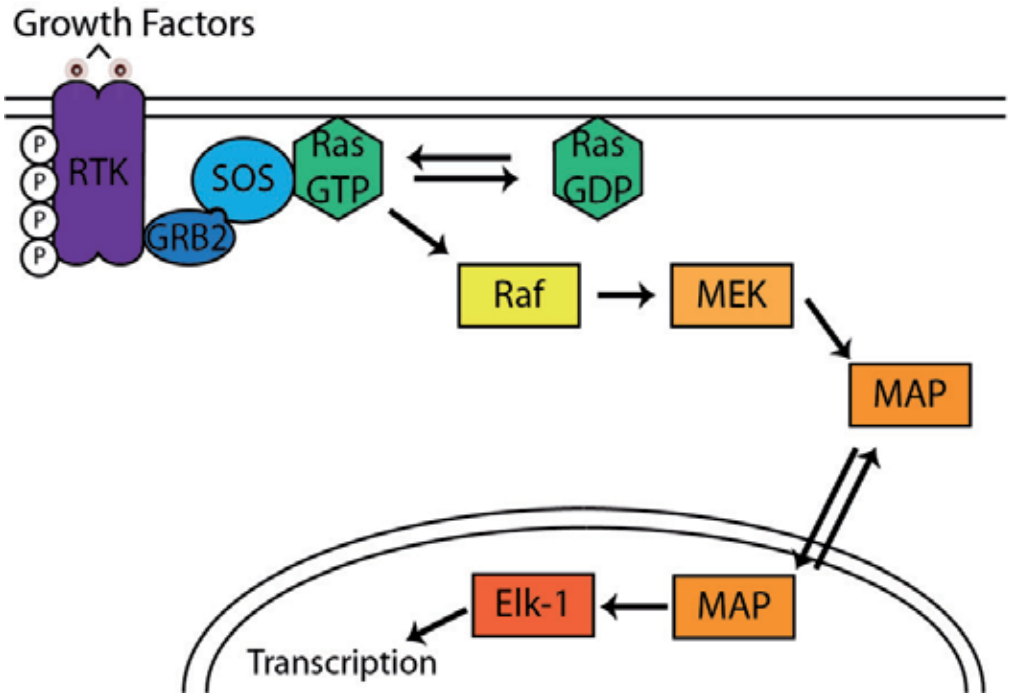

Fig. 4. **The Ras/Map Pathway.** External growth factors bind to the receptor tyrosine kinase (RTK) and initiate the transduction of the signal down the protein chain, the end result of which is the transcription of factors required for initiation of the cell cycle and cellular division.

To elucidate the molecular events describing the Restriction point, recall how tissue culture cells exit the cell cycle in response to mitogen withdrawal. Re-stimulating these quiescent cells with growth factors causes a rapid activation of various signal transduction cascades, including the Ras/Map kinase pathway (see Figure 4). In normal cells, this process typically involves transient accumulation of active Ras-GTP, which presumably reflects successful communication with the nucleus. While growth factor binding to receptor tyrosine kinases is responsible for the rapid accumulation of Ras-GTP, it does not explain the transient nature of Ras activation. Work from our lab suggests activation of the Map kinase cascade initiates a negative feedback loop that indicates successful transmission and so prevents further Ras activation (Moeller, *et al.*, 2003).

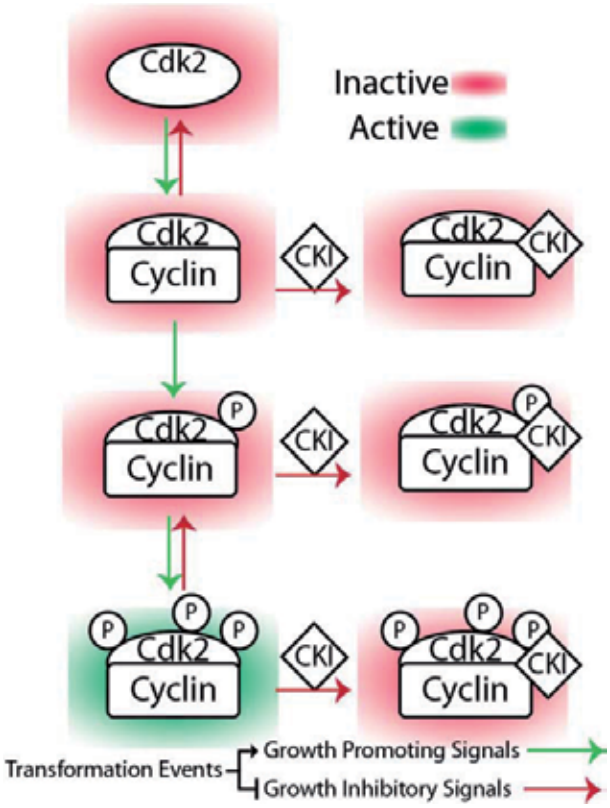

Fig. 5. **CDK2 Regulation.** Mitogen stimulation eventually leads to the activation of Cdk2, a important late G1 and S phase regulator through the production and subsequent binding of cyclins follow by phosphorylation of the complex. Inhibitory signals can lead to dephosphorylation of the complex, the degredation of the complex, or it be bound by cyclin kinase ihhibitors (CKI).

Map kinase is translocated into the nucleus, where it initiates transcriptional programs required for cell cycling and duplication. It also phosphorylates the CDK2 inhibitor p27kip1, resulting in its export from the nucleus. Cytoplasmic p27 targets GRB2 and prevents its interaction with the guanine nucleotide exchange factor SOS, thereby preventing formation of the GRB2-SOS complex that recruits Ras to the membrane. These observations led us to

hypothesize that p27 plays an important role in a negative feedback loop ensuring that: 1) The successful transmission of external information to the nucleus is communicated to the signaling system; 2) The magnitude and duration of the signal is properly maintained and regulated to prevent activation of fail-safe checkpoints and/or hyperproliferation. As will be discussed below, our more recent evidence suggests disruption of this pathway could be relevant to the initiation and progression of pancreatic tumorigenesis.

In addition to opening lines of communication, an early response to growth factor stimulation is synthesis of cyclin D and activation of CDK4/CDK6 (Ladha, *et al.*, 1998, Ohtsubo & Chibazakura, 1996). Cyclin D-CDK4/6 phosphorylates the retinoblastoma protein (pRb), a well known tumor suppressor that binds and inhibits the transcription factor E2F (Knudsen & Knudsen, 2008). As a result, E2F is released and mediates transcription of genes whose products are required for cell cycle progression, mass increase, and DNA replication (Chen, Hui-Zi, et al., 2009a, Nevins, 2001). Activity of cyclin D-CDK4/6 complexes is negatively regulated by the tight binding inhibitor p16 (Sherr, C. J. & Roberts, 1999, Wang, Q., et al., 2011a). In pancreatic cancer, cyclin D1 overexpression and p16 inactivation are very common events, emphasizing the importance of disrupting G1 progression to disease development (Chen, Jinyun, et al., 2009b, Fry, et al., 2008, Gansauge, et al., 1997, Kornmann, Marko, et al., 1998a, Schutte, et al., 1997). A major goal of E2F liberated by cyclin D-CDK4/6 is synthesizing cyclin E, which binds and activates CDK2 to continue progression through G1 and prepare for the S phase transition (Roberts & Sherr, 2003, Sheaff & Roberts, 1998). Cyclin E-CDK2 further phosphorylates pRb and releases E2F to make more cyclin E, thus establishing a positive feedback loop. The result is a burst of cyclin E-CDK2 activity that is thought to drive the transition from mitogen-dependent to mitogen-independent cell cycle progression (Sheaff & Roberts, 1998).

It will be argued below that compromised Restriction point control is the major focus of genetic alterations in pancreatic cancer development.

Another positive feedback loop is established as cells approach the G1/S phase transition. Cyclin E-CDK2 phosphorylates and inactivates its own inhibitor, p27kip1, resulting in a burst of cyclin E-CDK2 activity (Sheaff, et al., 1997). As a consequence cyclin A-CDK2 is generated to propel cells into S phase and monitor its progression (Woo & Poon, 2003). After accomplishing its goals, cyclin E-CDK2 initiates its own destruction by phosphorylating cyclin E and targeting it for proteasomal degradation (Clurman, et al., 1996). Once DNA is replicated, the cell transitions into G2 where the accuracy of DNA synthesis is evaluated and the cell prepares for chromosome segregation (Clarke & Gimenez-Abian, 2000). Cyclin B-CDK1 is in control during these processes (Kishimoto & Okumura, 1997). After division the system resets and cells must again evaluate internal and external signals to decide whether to continue the proliferative cycle or withdraw and adopt an alternative fate (Sheaff & Roberts, 1998). In the case of transformed cells, the default decision is generally proliferation.

5. Cell cycle machinery in development and progression of pancreatic cancer

The previously discussed hallmarks of cancer—self-sufficiency in growth signals, insensitivity to growth-inhibitory factors, unlimited replicative potential, evasion of apoptosis, angiogenesis, and metastasis—identify the biological processes which must be genetically altered to generate a transformed pancreatic cell (Hanahan & Weinberg, 2000).

As the discussion of cancer cell origins hopefully made clear, however, differentiated quiescent cells can sometimes acquire some of these characteristics during normal organism functioning. If such a facultative progenitor cell initiates pancreatic cancer, then genetic alterations likely affect the remaining uncompromised biological systems. This section will argue that a major consequence of genetic mutations in pancreatic cancer is accelerating G1 progression and disrupting Restriction point control via deregulation of the cell cycle machinery. Proliferative control mechanisms can be disrupted in one of two ways: 1) Directly, by mutation of the machinery itself; 2) Indirectly, by disruption of upstream signaling pathways. As is always the case with cancer, enhanced genetic instability underlies accumulation of transforming mutations (Negrini, *et al.*, 2010). One such genetic alteration occurring very early in pancreatic cancer is telomere abnormalities (Gisselsson, *et al.*, 2001, Hong, *et al.*, 2011, Kobitsu, *et al.*, 1997, van Heek, *et al.*, 2002).

5.1 Telomere abnormalities

Most cells have limited replicative potential determined by the rate of telomere loss (Hornsby, 2005). Chromosome ends present a special challenge to DNA replication, since the directionality of DNA polymerase and the subsequent removal of RNA primers means that genetic material is lost each round unless special precautions are taken (Gilson & Géli, 2007). The resulting ends become "sticky" and potentially interact, resulting in aberrant recombination events and chromosome breakage during anaphase. This repeated cycle of chromosome fusion and breakage has been implicated in genetic abnormalities such as amplifications and deletions that contribute to transformation (Murnane, 2010). Chromosome ends are therefore protected by TTAGGG repeats which are enzymatically added to the ends by an enzyme called telomerase (Osterhage & Friedman, 2009).

In many types of cancer telomerase is inappropriately activated in order to maintain the ends and prevent cell cycle exit (Artandi & DePinho, 2010). In the case of pancreatic cancer, however, telomere shortening appears to be a very early event in the formation of PanIN precursor lesions (Bogenrieder & Herlyn, 2003, Hong, *et al.*, 2011). It results in greatly enhanced genomic instability that causes global genome rearrangements and facilitates accumulation of subsequent point mutations. Telomerase appears to be re-activated if these lesions progress and become ductal adenocarcinomas, perhaps to reduce genome rearrangements that threaten cancer cell viability (Hong, *et al.*, 2011). The early onset of telomere shortening in pancreatic cancer suggests that the affected cell may not be subject to limited replicative potential, further evidence supporting a stem cell origin for the disease.

5.2 Mutational activation of ras signaling

K-ras appears to be the major proto-oncogene mutated in pancreatic cancer (~90% of cases), acting as an initiating event occurring very early in pre-cancerous lesions (Caldas & Kern, 1995, Moskaluk, *et al.*, 1997). The high likelihood of K-ras disruption strongly suggests that it is also disrupted in the remaining 10% of cases, albeit by different mechanisms. As will be discussed below, work from our laboratory on the CDK inhibitor p27kip1 has identified a novel pathway by which this could occur. Ras is a member of the GTPase family that plays a key role in receiving and transmitting extracellular signals to the nucleus, where they modulate gene expression and make cell fate decisions (Takuwa & Takuwa, 2001). It is

recruited to the membrane upon activation of receptor tyrosine kinases by association with GRB2-SOS (Chang, *et al.*, 2003). This complex activates Ras by converting it to the GTP-bound form, which initiates signal transmission via activation of the Map kinase cascade (see Figure 4) (Coleman, *et al.*, 2004). The ability to turn off signaling is crucial, so Ras is a GTPase that can hydrolyze GTP to GDP (Bernards, 2003). Ras regulation therefore centers in large part on controlling GTP hydrolysis and GDP dissociation to achieve the proper degree and duration of downstream signaling.

Sequencing of primary pancreatic cancer samples revealed that K-ras mutations tend to target codon 12 and inactivate GTPase activity (Caldas & Kern, 1995, Moskaluk, *et al.*, 1997) . The resulting K-ras-GTP continues downstream signaling in the absence of upstream effectors and in spite of inhibitory signals. Although mutated K-ras is in its active GTP bound form, it may still need to be localized to the membrane in order to initiate downstream signaling (Weise, *et al.*, 2011). Thus, other regulatory events help determine the level and duration of downstream signaling from mutated K-ras. For this reason, additional mutations affecting Ras regulation are also observed. These results illustrate how disrupting key signaling pathways requires the synergistic effects of multiple genetic disruptions due to fail-safe mechanisms and checkpoints that have evolved to prevent cell transformation (Hanahan & Weinberg, 2000).

Signaling pathways affected to be activated by mutant K-ras are the Raf-Map kinase cascade, PI3K-AKT, and RalGDS, with each making a distinct contribution to development of the transformed cell (Calvo, *et al.*, 2010). As discussed above, Ras/Map kinase plays a key role in promoting cell cycle re-entry and progression. PI3K-AKT is involved in cell survival, while RalGDS is one of several Ras-regulated guanine-nucleotide exchange factors that activates Ral A and B GTPases (Carnero, *et al.*, 2008, Ferro & Trabalzini, 2010). Ral proteins regulate key cellular processes such as endocytosis, exocytosis, and actin organization, as well as contributing to regulation of gene expression (Carnero, *et al.*, 2008, Ferro & Trabalzini, 2010). A number of additional genetic disruptions appear to be required for mutant K-ras induced transformation. Of particular interest is excess cyclin D1, itself a downstream target of Ras that is commonly mutated in the disease state (Fry, *et al.*, 2008, Gansauge, *et al.*, 1997, Kornmann, Marko, *et al.*, 1998a). Given the pleotropic effects of Ras signaling, further work is required to completely describe its contribution to transformation.

5.3 Mutational activation of other proto-oncogenic signaling pathways

The Ras/Map kinase pathway is only one of twelve core signaling pathway disrupted in the disease (Jones, *et al.*, 2008). It is beyond the scope of this chapter to discuss each pathway in detail, other than to note that the consequences of their disruption likely mimic, enhance, or synergize with K-ras mutations to drive transformation and cancer progression. Involvement of Notch and hedgehog signaling deserves special mention, as these critical pathways are best known for maintaining cells in an undifferentiated state during development (Kelleher, 2011, Ristorcelli & Lombardo, 2010). In the adult organism, these pathways are involved in tissue homeostasis via maintenance of tissue stem cell populations. During injury, there is a transient induction and expansion of Hedgehog or Notch dependent stem cell populations to replace damaged or lost cells (Siveke, *et al.*, 2008). In the mature pancreas, Notch signaling is restricted to centroacinar cells, suggesting they

might be the originating cancer cell (Miyamoto, *et al.*, 2003). Support for this idea comes from mice with conditional knockout of the PTEN tumor suppressor in the pancreas, which develop ductal metaplasias resulting from expansion of CACs (Hill, *et al.*, 2010). Similarly, expression of Notch components is elevated in PanIN lesions and invasive cancer (Wang, Zhiwei, *et al.*, 2011b). Mutational activation of these pathways cooperates with K-ras mutations throughout development of pancreatic cancer, from generating precancerous PanIN lesions to involvement in tumor maintenance and metastases. Both the Hedgehog and Notch pathways appear to be disrupted by ligand overexpression rather than direct mutational targeting of pathway constituents.

5.4 Mutational inactivation of tumor suppressors

Temporal disruption of specific tumor suppressors is also observed in the PanIN progression model and again seem to converge on enhancing genomic instability and/or disrupting Restriction point control. For most cancers, mutations in the tumor suppressor p53 or one of its regulatory components (e.g. ARF) are present in the majority of cases (Sherr, C. J., 1998). p53 is the central transcriptional regulator responding to all types of cell stress. It induces expression of proteins to stop the cell cycle and determine if damage can be repaired; if not, it helps activate the apoptotic pathway (Muller, *et al.*, 2011). Inactivation of this pathway is so common because it contributes to cell immortalization and allows accumulation of further genetic mutations (Hanahan & Weinberg, 2000). It is somewhat curious that p53 mutations are observed in only 50% of pancreatic cancers, and they tend to occur later in the progression model (Morton, *et al.*, 2011). This observation (as was the case with telomere shortening), suggests the initiating cell might already be immortal or at least more resistant to apoptosis. Mutation of p53 could therefore contribute something else to development of pancreatic cancer. A central target of p53 is p21cip1, a CDK2 inhibitor that blocks cell cycle progression in G1 phase (Doucas, *et al.*, 2006, el-Deiry, 1998). Thus, its mutation could enhance the rate of G1 progression and passage through the Restriction point.

The p16INK4A CDK inhibitor specifically targets cyclin D and is inactivated in >80% of pancreatic cancers (Gansauge, *et al.*, 1997, Kornmann, Marko, *et al.*, 1998a). This suggests enhanced activity of cyclin D-CDK4/6 complexes makes an important contribution to development of pancreatic cancer, consistent with the common overexpression of cyclin D1 (Fry, *et al.*, 2008). The mechanism of p16 loss is diverse, involving homozygous deletion (40%), intragenic mutation followed by inactivation of the second allele (40%), and methylation inactivation of the promoter (10-15%) (Gansauge, *et al.*, 1997, Kornmann, Marko, *et al.*, 1998a). The locus encoding for this gene is unique in that it also encodes for p14ARF, an alternative reading frame gene product that plays a key role in activating p53 by preventing its degradation (Sherr, C. J., 2001). Specific inactivation of p16 plays a greater role in pancreatic cancer than loss of ARF function, as mutations have been characterized that compromise p16 while leaving ARF intact (Jeong, *et al.*, 2005, Maitra, A & Hruban, 2008). As discussed above, this observation is intriguing since loss of ARF is the major alternative pathway for inactivating p53 (Sherr, C. J., 2001). Preferential targeting of p16 further illustrates the importance of compromising Restriction point control via disruption of the cyclins and CDKs controlling G1 progression.

Other identified mutations support this idea. SMAD4 is a cytoplasmic transcription factor involved in G1 cell cycle arrest mediated by the TGFB pathway (Yang, Guan & Yang, 2010). It is mutated ~50% of pancreatic cancers (Blackford, *et al.*, 2009). Its normal function involves association with SMAD2/3 and translocation into the nucleus where it mediates transcriptional activation of growth inhibitory genes like p27kip1 (Yang, Guan & Yang, 2010). Again, these results can be interpreted as modulating the cell cycle machinery controlling G1 progression and the Restriction point.

5.5 Animal models of pancreatic cancer

Genetically engineered mice have been developed in which genes of interest can be expressed in the pancreas using organ-specific promoter constructs. Mutant K-ras expression in the mature ductal epithelium gave little phenotype and no precursor lesions, suggesting these are not the cells of origin (Brembeck, *et al.*, 2003). However, the K-ras mutation was able to induce carcinogenesis when combined with chemically induced pancreatic injury (Guerra, *et al.*, 2007). Under these conditions mature differentiated cells are directed to re-enter the cell cycle to replace lost cells. This result strongly supports the idea of a stem cell or facultative progenitor cell as the originating cancer cell.

In contrast, mice developed a spectrum of precursor lesions when oncogenic K-ras was expressed from its endogenous promoter in the developing pancreas (Guerra, *et al.*, 2007). Only 10% developed into metastatic adenocarcinoma, however, and it required a long latency period. Similar results were observed when mutant K-ras was specifically expressed during development in acinar cells under control of the elastase promoter (Hruban, *et al.*, 2006). Ras-induced senescence may be responsible for the lack of tumor formation, underscoring the importance of cooperating mutations (Dimauro & David, 2010). This hypothesis was confirmed by the much greater penetrance in mice with combinations of mutations (K-ras plus p53 or p16) (Wang, Zhiwei, *et al.*, 2011b). Under these conditions, the putative cell of origin responds to mutated K-ras by differentiating along a ductal lineage (hence the ductal adenocarcinomas). Inactivating the tumor suppressors p16 or p53 alone did not give an obvious phenotype or precursor lesions, suggesting K-ras acts as an initiator mutation (Wang, Zhiwei, *et al.*, 2011b). Similarly, conditional Smad4 or TGFB receptor deletions were not sufficient to induce mPanIN lesions or cancer, although they clearly cooperated with K-ras to induce pancreatic neoplasia (Bardeesy, *et al.*, 2006).

5.6 Centrality of disrupted restriction point control

Altered G1 progression and Restriction point control appear to be key events in development of pancreatic cancer based on mutations involved and the putative cell of origin. The central role of cell cycle disruptions can be modeled by considering mutations in the context of the progression model. Greater than 90% of low-grade PanIN lesions show shortened telomeres, providing a mechanism for rapidly generating genetic alterations required for cell transformation (Hong, *et al.*, 2011, van Heek, *et al.*, 2002). K-ras mutation is one of the earliest abnormalities and is likely an initiating event, being present in 36% of PanIN-1A, 44% of PanIN-1B, and 87% of PanIN-2/3 precursor lesions (Caldas & Kern, 1995, Moskaluk, *et al.*, 1997). That such a dramatic alteration occurs so early is interesting, since expressing mutated K-ras in animal models typically results in senescence or apoptosis as a

protective response to loss of proliferative control (Dimauro & David, 2010, Overmeyer & Maltese, 2011). Taken together, telomere shortening and K-ras activation point to the initial transformation involving a stem cell or facultative progenitor that re-enters the cell cycle in response to injury/tissue maintenance.

Such cells likely already possess characteristics of cancer cells such as immortality and replicative potential. Thus, mutations are not required to drive cells back into the proliferative cycle from a quiescent state. Their predisposition to immortality means tumor suppressors like p53 or ARF need not be immediately inactivated, while telomere shortening is tolerated and beneficial since it induces genomic instability. What then is the limiting factor in transformation and expansion of the cancer cell? It is becoming increasingly apparent that the kinetics of stem cell cycling are quite different that those of somatic cells in culture (Nacusi & Sheaff, 2007, Neganova & Lako, 2008). Although they have limitless replicative capacity, stem cells generally take much longer to duplicate (i.e. doubling time of days). If the initiating cell in pancreatic cancer replicates so slowly, a major consequence of K-ras mutation might be to speed up the duplication rate. The overall time required for cell cycling is determined in large part by the rate of G1 progression (the longest phase of the cell cycle), so mutations would be expected to speed up this process and ensure passage through the Restriction point (Salomoni & Calegari, 2010).

One way mutant K-ras might increase the proliferative rate is by increasing activity of cell cycle components involved in the rate limiting step(s) for G1 progression (see Figure 7). Experiments in tissue culture cells suggest that overexpressing G1 cyclins or otherwise increasing CDK activity (e.g. blocking its inhibition) can accelerate G1 phase (Roberts & Sherr, 2003, Sherr, C. J. & Roberts, 1999). Similarly, cyclin D1 is commonly overexpressed and its inhibitor p16 inactivated in pancreatic cancers (Chen, Jinyun, *et al.*, 2009b, Fry, *et al.*, 2008, Gansauge, *et al.*, 1997, Kornmann, Marko, *et al.*, 1998a, Lee, *et al.*, 2009, Schutte, *et al.*, 1997). Experiments using pancreatic cancer cell lines show blocking activity of excess cyclin D retarded cell growth and reduce their ability to generate tumors in nude mice (Kornmann, M., *et al.*, 1998b). Various drugs (e.g. celecoxib, green tea constituents) displaying efficacy against pancreatic cancer cell lines often target excess cyclin D (Tseng, *et al.*, 2002). Disruptions during the early stage of mitogen dependent cell cycle progression leading up to the Restriction point therefore appear critical to development of pancreatic adenocarcinoma. One obvious consequence would be to enhance cyclin D/CDK4/6 activity, suggesting Rb phosphorylation and inactivation is crucial to transformation of the initiating cell. While these early events could certainly accelerate G1 progression, the transition to mitogen independence reflected in the Restriction point is also likely to be rate limiting. Thus, cyclin E-CDK2 activity is likely to be enhanced given its role in this process (see Figure 7) (Sheaff & Roberts, 1998). Evidence in support of this hypothesis comes from analyzing the contribution of K-ras mutation on proliferation of pancreatic cancer cell lines.

Using small molecule inhibitors of MEK, Gysin et al. found that cells arrested in G1 due to increased expression of the tumor suppressor p27kip1 (Gysin, *et al.*, 2005). As a consequence, CDK2 was inhibited, Rb was not phosphorylated, and the E2F activity required for G1 progression was absent. These results indicate that inhibiting p27 expression is a major role of K-ras mutation, thereby enhancing CDK2 activity and accelerating G1 progression. As described below, we propose that an additional rationale for decreasing p27 is to ensure continued activation of the mutated K-ras pathway driving transformation. In

contrast, MEK inhibition had little effect on CDK4 activity, suggesting K-ras mutation is not responsible for effects on early G1 progression (Gysin, *et al.*, 2005). The obvious candidates for affecting this period are the aforementioned cyclin D1 overexpression and inactivation of p16. It should be noted that there are also mechanisms through which increasing cyclin D1 levels could contribute to enhanced CDK2 activity. Additional support for the importance of CDK2 comes from analysis of OGF signaling, which negatively regulates progression of pancreatic cancer (Fan, *et al.*, 2008). It does so by inducing expression of the CDK2 inhibitor p21cip1, further evidence that accelerating G1 progression is a key step in disease development (Fan, *et al.*, 2008).

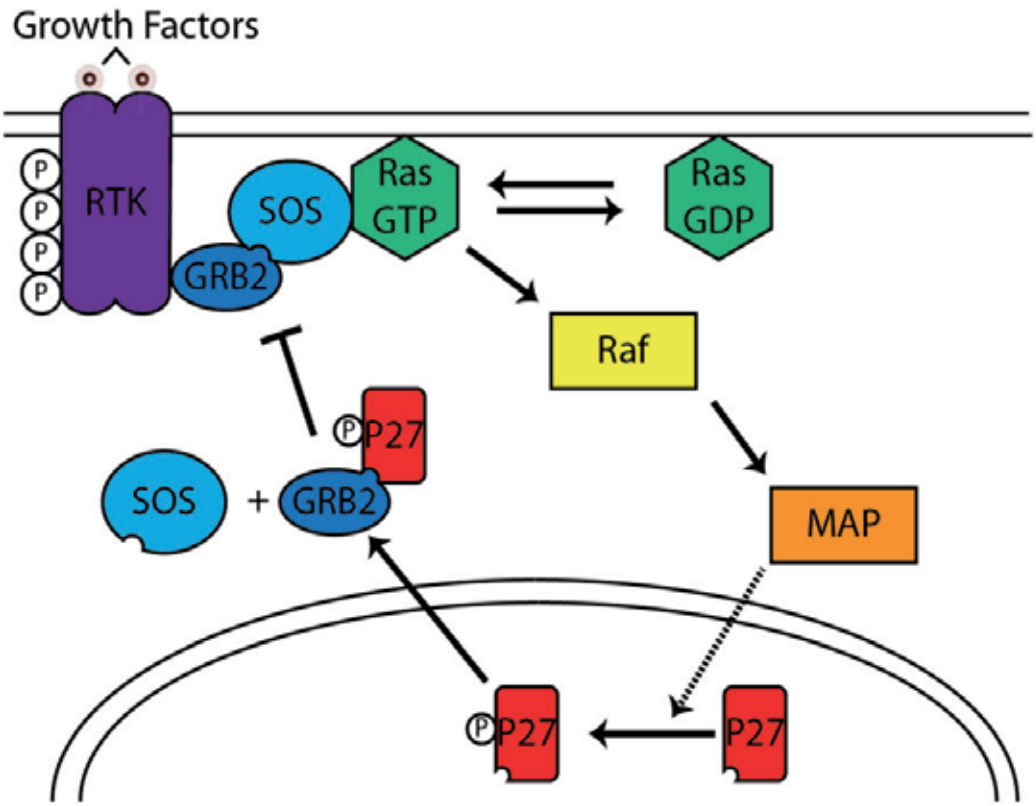

Fig. 6. **Ras/Map Regulation by p27.** Mitogen stimulation of the Ras/Map pathway leads to the phosphorylation and export of p27 from the nucleus. This exported P-p27 binds competitively for Grb2 against SOS. The dissociation of the Grb2/SOS complex leads to the down regulation of the Ras/Map pathway signal.

In addition to its effects on early G1 phase, overexpressed cyclin D1 is known to bind and sequester p27 (Sherr, C. J. & Roberts, 1999). This would indirectly enhance CDK2 activity and hence progression through the Restriction point. Work in our lab has shown that p27 also functions in a negative feedback loop regulating Ras activation by GRB2 (see Figure 6) (Moeller, *et al.*, 2003). If cyclin D1 were to sequester p27, this negative feedback would be disrupted, thereby contributing to sustained K-ras signaling and accelerated cell cycle

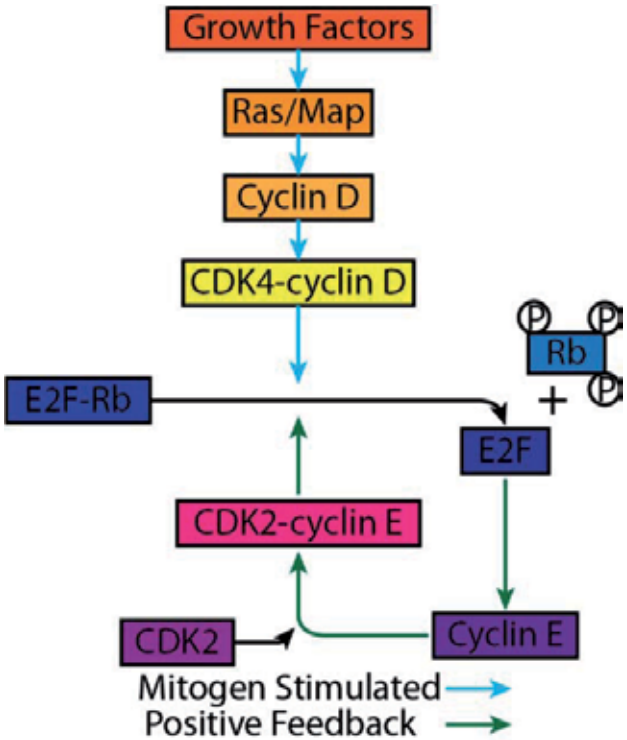

Fig. 7. **E2F Activation and Positive Feedback**. Mitogen stimulation leads to the phosphorylation and removal of the cell cycle regulating protein Rb from the E2F-Rb complex. E2F goes on to activate cyclin E, whose complex with CDK2 further activates more E2F by Rb phosphorylation. This self-sustained postive feedback marks the transition from mitogen stimulated to self-sustained cell cycle progression.

progression. We have recently investigated this possibility in breast cancer cells (which often overexpress cyclin D1) and found that it preferentially sequestered the phosphorylated p27 that is exported to inhibit GRB2 (submitted). We are now investigating whether the excess cyclin D1 in pancreatic cancer cell lines causes a similar disruption in p27 regulation of the Ras pathway. These observations could help explain why cyclin D1 overexpression is necessary for the oncogenic effects of K-ras in pancreatic cancer (Fry, *et al.*, 2008). Given that the activation of facultative progenitor cells in response to injuries is normally transient, inappropriate K-ras signaling could also play an important role in preventing their withdrawal from the cell cycle. This hypothesis is consistent with results showing that constitutive K-ras activation is required for maintenance of pancreatic cancer (Caldas & Kern, 1995).

6. Conclusions

Given the aggressiveness and poor prognosis of pancreatic cancer compared to many other forms of the disease, it is worthwhile to consider what makes it unique. This chapter provides an evaluation of mutational changes and disrupted biological function within the context of cancer hallmarks and identity of the originating cell. We argue that mutations

directly and indirectly affecting G1 progression and the Restriction point are crucial to development of the pancreatic cancer cell. This analysis will hopefully stimulate further research into methodologies for treating the disease by targeting disruptions in or activity of the relevant cell cycle machinery. This approach may offer greater specificity and fewer side effects than previously expected. Increasing evidence provides tantalizing clues that proliferative control mechanisms in normal and transformed cells might be different (Moeller & Sheaff, 2006). Mice lacking cyclins and CDKs, for example, can still develop normally (Sherr, Charles I. & Roberts, 2004). The dispensability of some cell cycle machinery in normal development and untransformed cells is in stark contrast to its apparent necessity in pancreatic cancer. Thus, the cell cycle machinery and its activities may represent viable therapeutic targets with unanticipated specificity for preferentially inhibiting proliferation of the pancreatic cancer cell.

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17

Glycans and Galectins: Sweet New Approaches in Pancreatic Cancer Diagnosis and Treatment

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1. Introduction

Pancreatic cancer is one of the tumors with worst prognosis. Its low survival rate is due to late diagnosis because of the lack of symptoms when the tumor initiates, being frequently diagnosed when it metastasizes to other organs. Thus, new early diagnostic biomarkers are an urgent need to improve pancreatic cancer survival rates. Aberrant protein glycosylation is common in tumoral cells, involving changes in glycosyltransferases and glycosidases that could be mediated by inflammatory cytokines and growth factors. These alterations are functionally important in cancer progression influencing cell migration and adhesion, metastatic capability and immune escape. These changes in protein glycosylation during tumor progression can lead to alterations in membrane proteins clustering and lectin binding, conferring functional advantages to tumoral cells. In this regard, differential reactivity towards endogenous lectins, especially galectins, has been reported in several cancers. Galectins are involved in a variety of biological processes including tumor growth and malignant transformation. This chapter focuses on the specific alterations in protein glycosylation and galectin expression and binding during pancreatic cancer progression, as well as their potential use as prognostic biomarkers and therapeutic targets. Interestingly, we have characterized the importance of the interaction between a glycoprotein (tissue plasminogen activator, tPA) and Galectin-1 (Gal-1) in pancreatic cancer, suggesting that strategies targeting this interplay might result in successful treatments.

2. Glycosylation in cancer

2.1 Glycans: General features

Glycosylation is one of the most common post-translational modifications and nearly half of all proteins in eukaryotes are glycosylated (Spiro, 2002). Glycans (oligosaccharides from glycoproteins) are classified considering their linkage to the protein backbone in N-Glycans (bound to the amide side chain of Asn) and O-Glycans (bound to the hydroxyl of Thr or Ser).

Studies focused on the carbohydrate moiety of proteins are methodologically complicated due to the extremely high diversity and flexibility of these structures. N-glycan content at

one particular site is frequently miscellaneous. Their structural diversity embraces the number and nature of monomeric units, their position, anomeric configuration and branching. Glycoproteins display site-occupancy heterogeneity (macroheterogeneity), which refers to the diversity on the presence or absence of glycan chains in specific aminoacids. Moreover, not all N-linked glycan sites are occupied. Apart from this source of variation, glycoproteins also present site-specific heterogeneity (microheterogeneity), which describes differences found regarding the carbohydrate content and structure present in a single glycosylation site.

Glycosylation of proteins can affect their folding, enhance solubility, intracellular trafficking, localization, secretion and rate of degradation (Hakomori, 2002). Apart from conferring specific properties to proteins themselves, glycans significantly affect protein/protein interactions, preventing the non-specific ones. In this direction, they mediate accurate cell/cell communication and signal transduction as well as the interaction between a cell and the extracellular milieu and soluble signaling molecules. Carbohydrate structures are key in many cell biological functions and indeed eighteen different types of congenital disorders of glycosylation (CDG) have been genetically defined (Freeze & Aebi, 2005).

2.2 Altered glycosylation in cancer

Typically, cancer has been associated with gain-of-functions in oncogenes or loss-of-function in tumor suppressor genes. However, there are many other mechanisms responsible for orchestrating all the events triggering cancer stepwise progression. In spite of the marked physiological glycan heterogeneity, cancer progression and metastasis have been characterized by significant alterations of the carbohydrate signature. Indeed, aberrant glycosylation is one of the cancer cell hallmarks (Varki et al., 2009), and certain structures are well-known markers of tumor development (Hakomori, 2002; Lau & Dennis, 2008). Besides, changes in glycosylation are presented not only by cancerous cells but also by cells surrounding the tumor (Rabinovich & Toscano, 2009). This specific pattern of glycosylation linked to neoplasia might affect protein functionality significantly, altering cell behavior in many different ways. Distinctive glycosylation profiles favor or impede interactions with different proteins.

Some of the best characterized glycan specific alterations in cancer are a general increase in sialic acid content, an increase in glycan branching and overexpression of specific carbohydrate antigens like sialyl Lewis antigens (SLe^a and SLe^x) (Fig.1). The tight regulation of enzymes during protein glycosylation is crucial and indeed, the population of sugars attached to each glycosylated site depends on the cell type in which the glycoprotein is expressed and in the physiological status of the cell. Inflammatory cytokines and growth factors such as IL-1β, TNF-α, IL-6 and EGF, mediate changes in concentration of glycosyltransferases and glycosidases, altering the proportion of the glycoforms present in a particular glycoprotein.

Glycan alterations are functionally important in cancer progression by affecting cell proliferation and survival, adhesion and migration, angiogenesis and metastatic capability, as well as the immune escape. For instance, a very common feature in cancer is the increased activity of β1-6-N-acetylglucosaminyltransferase V (GlcNAcT-V or MGAT5), which is in charge of β1-6 branching of both O and N-glycans. As a functional example of this fact,

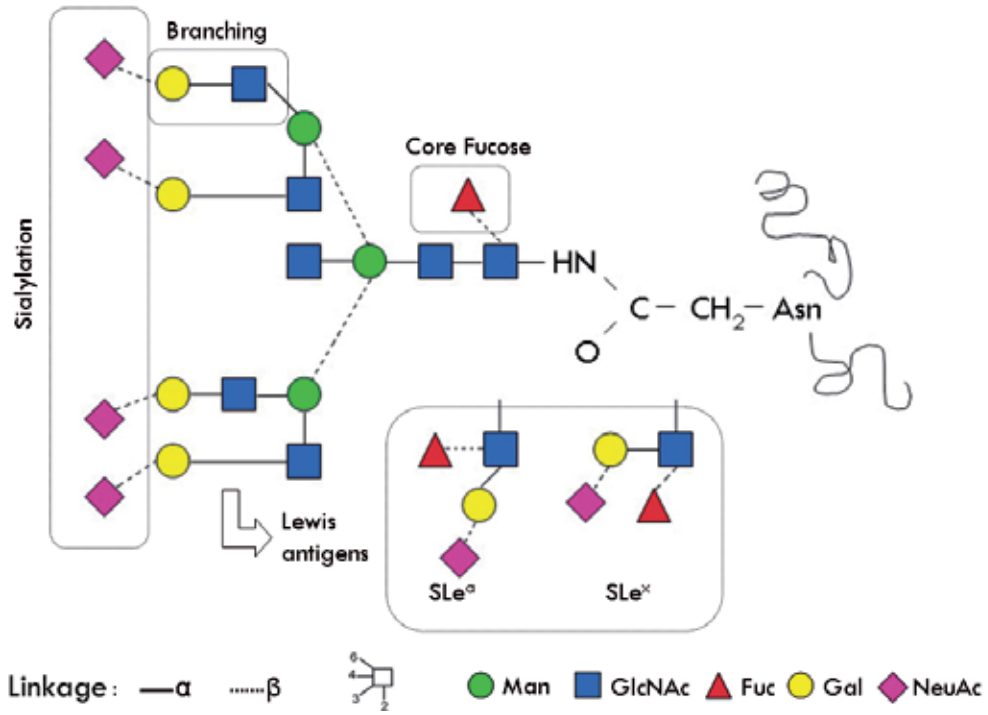

Fig. 1. Most frequent N-glycan altered patterns observed in tumorigenesis. Common features of cancer glycosylation include sialylation, increased β-1,6-branching, core fucosylation and sialyl-Lewis antigens.

increased branching in the β_1 subunit of $\alpha_5\beta_1$ integrin due to enhanced MGAT5 expression, inhibits integrin clustering, reducing the attachment of cancer cells to fibronectin and thus inducing migration (Guo et al., 2002). This enzyme is also involved in enrichment of the SLe^x group, which confers cells the ability to extravasate and metastasize. *In vivo*, progression of mammary tumors in MGAT5 knockout mice is significantly impaired (Granovsky et al., 2000). Various factors including oncogenes as Src, Her-2/neu, H-Ras, and V-sis and known cancer altered signaling pathways as Ras-Raf-Ets regulate MGAT5 transcription. What still remains to be determined is whether changes in glycosylation are a cause or a consequence of transformation. Cytokine regulation of glycosyltransferase activity suggests that signaling from the tumor microenvironment can be the responsible for cancer-associated glycosylation.

2.3 Glycosylation in pancreatic cancer

Specific alterations in pancreatic cancer glycoproteins have been described, such as increased N-glycan branching and increased fucosylation and sialylation (Zhao et al., 2007). Importantly, some of the aberrantly glycosylated proteins have been suggested as biomarkers (Lacunza et al., 2007; Okuyama et al., 2006; Peracaula et al., 2008). Lectin antibody microarrays have been used to detect unique glycosylation patterns in pancreatic cancer serum in high throughput strategies (Li et al., 2009; Wu et al., 2009). These assays proved efficient specificity and sensitivity and shed some light in distinguishing between

pancreatic cancer and chronic pancreatitis, a matter that has been for long unresolved. Major alterations in glycan-linked gene expression associated to pancreatic cancer epithelial to mesenchymal transition *in vitro* have been also reported (Maupin et al., 2010).

Data proposing some of the causes of altered glycosylation have emerged. Proinflammatory stimuli such as IFNγ, TNFα and IL-1α, in pancreatic cancer cells are responsible for altering Muc1, Muc5AC and Muc16 glycosylation in a cell type specific manner (Wu et al., 2009), and indeed, cytokine secretion has also been considered in pancreatic cancer diagnosis (Fearon et al., 1999; Wigmore et al., 2002).

One of the current pancreatic tumor markers is the monoclonal antibody CA19-9 (Ferrone et al., 2006), whose epitope is the SLe^a antigen in gangliosides and mucins. SLe^a physiologically functions in the extravasation of lymphocytes from the bloodstream by interacting with selectins on endothelial cells. In accordance with these data, its expression on the surface of pancreatic cancer cells has been linked to metastasis spread to other tissue sites (Aubert et al., 2000). Nevertheless, CA19-9 generally does not have the specificity and sensitivity required for general screening, being frequently restricted to monitor patient's progress after surgery. RNase-1 was long ago proposed as a tumor marker in pancreatic cancer but both its levels and its activity in serum failed in diagnosis. However, differences in glycosylation in this protein exist, finding neutral structures in healthy pancreas whereas charged structures (such as SLe^x and SLe^a antigens) and a significant increase in core fucosylation and sialylation are observed in pancreatic cancer (Peracaula et al., 2003). Increased core fucosylation is a general cancer feature and it is also common in pancreatic cancer. Serum haptoglobin and other acute phase proteins are also found to be more core fucosylated specifically in pancreatic cancer (Okuyama et al., 2006; Sarrats et al., 2010).

3. Galectins in cancer

3.1 The galectin family: Main features

Galectins belong to the lectin family of proteins, which are highly evolutionary conserved finding their members in all animal kingdoms and even in plants, fungi and viruses. All the proteins of the family share two main features: high affinity for β-galactosides and a well conserved carbohydrate recognition domain (CRD) of 130 aminoacids (Barondes et al., 1994). However, each galectin has a specific carbohydrate binding preference, as a result of their ability to accommodate different saccharides attached to galactose.

15 galectins have been described in mammals (11 of which are expressed in humans) and they can be structurally clustered in three groups (Fig.2): 1) Prototype galectins (1, 2, 5, 7, 10, 11) consist of a single CRD with a short N-terminal sequence; 2) Tandem-repeat galectins (4, 6, 8, 9) are composed of two differents CRDs joined by a short linker peptide sequence; and 3) Chimaeric galectins (Gal-3) have an extended N-terminal tail containing a consensus nine aminoacid residue-repeat rich in Pro, Tyr and Gly.

Galectins are differently distributed in animal tissue and its expression is modulated during differentiation and tissue development, changing in some physiological and pathological conditions (Yang et al., 2008), such as in cancer (Danguy et al., 2002). Galectins are secreted by a non-canonical pathway and display a wide variety of intra and extracellular functions.

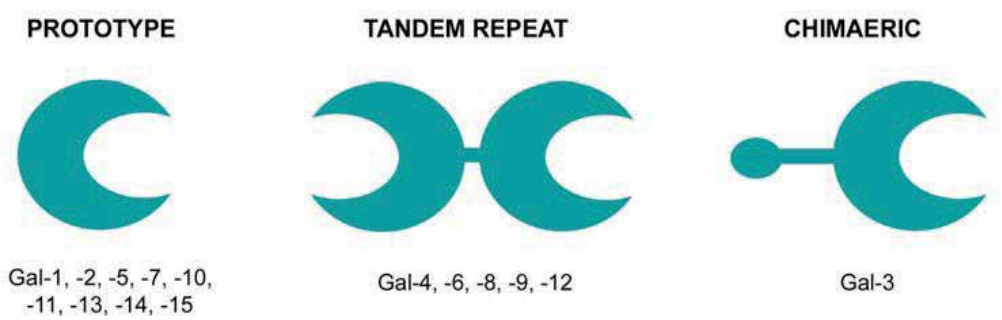

Fig. 2. Galectin structural classification. Prototype galectins (Gal-1,2,5,7,10,11,13,14,15) have one CRD domain. Tandem repeat galectins (Gal-4,6,8,9,12) are composed of two different CRD. The only chimaeric galectin (Gal-3) has an extended N-terminal domain.

3.2 Gal-1: Structure and functions

The first protein discovered in the human galectin family was Gal-1 (Couraud et al., 1989; Gitt & Barondes, 1986), which is encoded by *LGALS1* gene located in chromosome 22q12-13.1. Splicing of its four exons results in a 0.6 Kb transcript that is translated into a protein of 135 aminoacids, without suffering any post-translational modification. Gal-1 expression might be modulated by histone acetylation and promoter methylation.

Gal-1 is a symmetrical dimer of 14.5 KDa subunits and it has a β-sandwich "jelly-roll" conformation involving two parallel β-sheets, which form a central hydrophobic core holding both amino and carboxy-terminus of each monomer. Gal-1 CRD has a binding grove that allows the presence of a tetrasaccharide (A, B, C and D). C site includes the eight

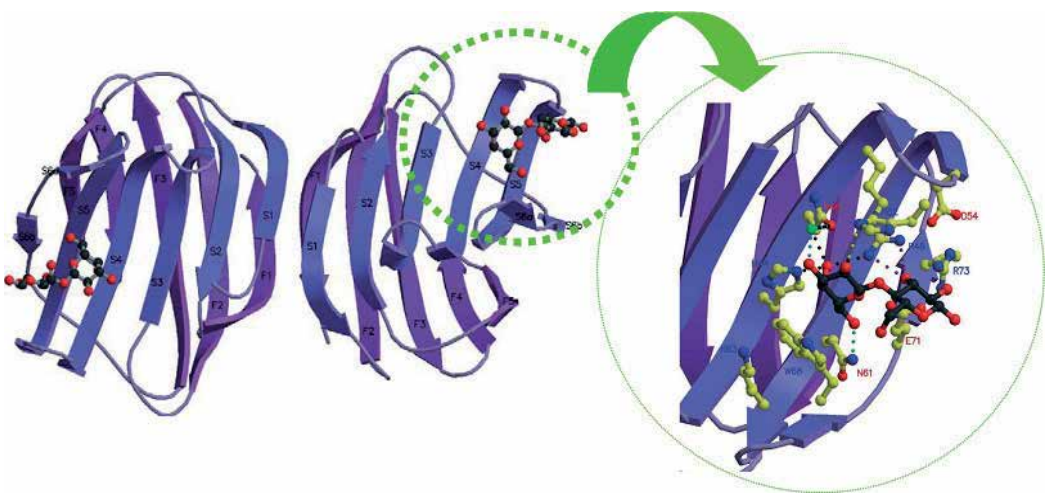

Fig. 3. Human dimeric Gal-1 jelly-roll structure complexed with lactose. Ribbon diagram prepared with MOLSCRIPT. Five-stranded (F) and six-stranded (S) sheets of each monomer are labelled in the image and the aminoacids involved in lactose binding are highlighted in the enlargement (His44, Asn46, Arg48, His52, Asn61, Trp68, Glu71 and Arg73). Adapted from (Lopez-Lucendo et al., 2004).

so well conserved aminoacids responsible for galactose binding (Fig.3), and this is common among all galectins. The rest of the sites are involved in galectin recognition specificity. Both Gal-1 and Gal-3 typically lodge a terminal LacNAc in site C-D but binding is inhibited by the presence of NeuAcα2-6 in the galactose located in B. Functional differences and binding avidities between Gal-1 and Gal-3 suggest the existence of additional determinants of binding specificity.

Gal-1 is found in the cytoplasm, membrane, extracellular matrix (ECM) and nucleus, being involved in a wide variety of cellular functions through its ability to recognize many different proteins (Elola et al., 2005). Extracellular functions depend on Gal-1 lectin activity whereas intracellular functions are usually independent and involve protein/protein interactions.

3.3 Role of galectins in cancer

Galectins have been reported to be clear modulators of tumor progression (Liu & Rabinovich, 2005) and their heightened expression usually correlates with tumor clinical aggressiveness and metastasis. Several members of the family have been involved in tumor progression, being Gal-1 and Gal-3 the best characterized ones (Danguy et al., 2002; Yang et al., 2008). These proteins display important functions in several aspects of cancer biology including cell adhesion, migration, tumor transformation, apoptosis, cell cycle progression, angiogenesis and immune response regulation. Indeed, galectin inhibitors have been well considered for cancer therapy (John et al., 2003; Sorme et al., 2003; Zou et al., 2005).

Gal-1 expression has been identified as a prognostic factor for tumor progression in many different neoplasms (Demydenko & Berest, 2009). Gal-1 involvement in tumor progression is focused on different aspects: neoplastic transformation, tumor cell proliferation and survival, angiogenesis, metastasis and evasion from the immune response (Fig.4).

Inhibition of Gal-1 expression impairs transformation in glioma cells (Yamaoka et al., 2000). Among all Gal-1 partners, H-Ras could be the one closer linked to tumor transformation (Paz et al., 2001) although this interaction is lectin independent. Gal-1 is also very important in fibroblast activation in different tumor settings (Fitzner et al., 2005; Masamune et al., 2006), and indeed, Gal-1 knockdown in cancer associated fibroblasts inhibits *in vivo* tumor progression (Wu et al., 2011).

Gal-1 effects in cell proliferation are controversial. It is mitogenic in several cell types, such as in mammalian vascular cells and hepatic stellate cells, but it is also able to hamper cell growth in other cell types, such as in stromal bone marrow cells. Intracellular Gal-1 can induce not only cell cycle arrest but also apoptosis in cancer cells. Gal-1 concentration seems to be key when deciding the final outcome: high doses (μM) of Gal-1 inhibit cell proliferation independently of its lectin activity whereas low doses (nM) are mitogenic through its ability to recognize carbohydrates (Adams et al., 1996). Apart from this dose response effect, the cell type and cell activation status, the distribution of monomeric versus dimeric forms and Gal-1 compartimentalization, might be also affecting the overall result on cell cycle progression.

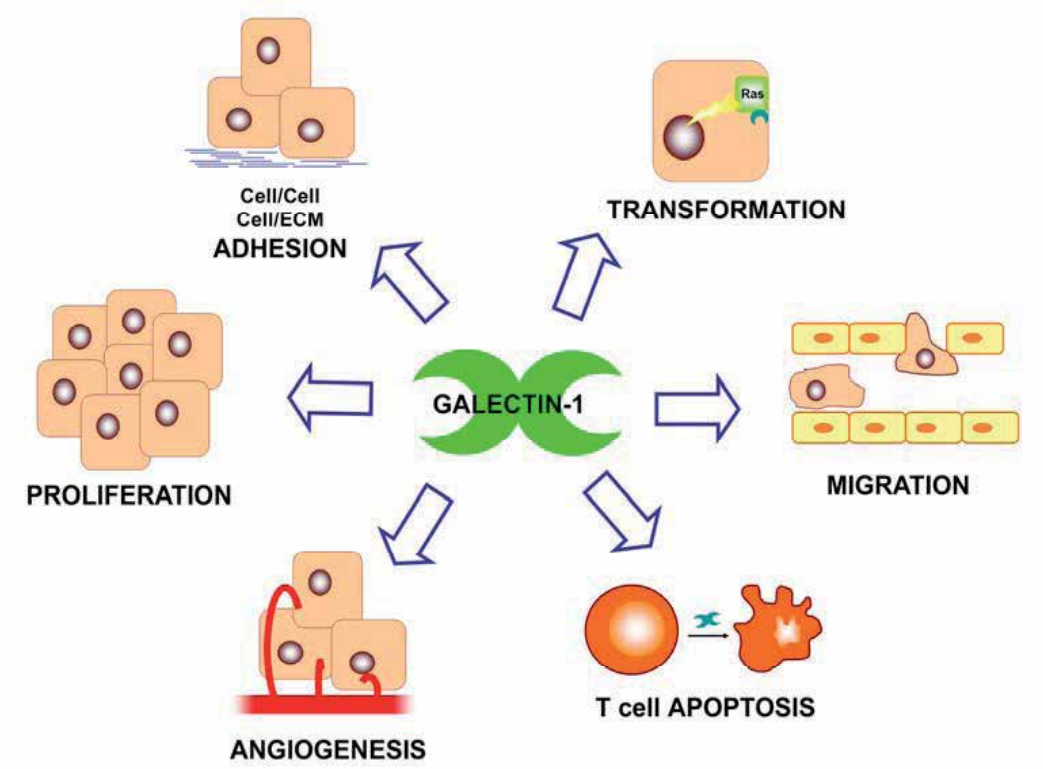

Fig. 4. Gal-1 is involved in many different tumor progression events. Gal-1 participates in cell transformation, proliferation, adhesion, migration, angiogenesis and T cell apoptosis.

Gal-1 has been actively involved in the long range dissemination of tumoral cells or metastasis (Camby et al., 2006), as it participates in adhesion, migration, motility and invasion. Gal-1 can decrease tumor cell adhesion to the ECM, resulting in cell detachment from primary sites and invasion. Alternatively, the dimeric nature of Gal-1 allows crosslinking integrins on the cell surface of tumoral cells to proteins on the ECM, mediates tumoral cell/cell interactions favoring aggregation and their interaction with endothelial cells, facilitating tumor cell dispersion on the blood stream and establishment at distal sites during metastasis. In addition, Gal-1 has been also involved in invasion through adhesion independent mechanisms by upregulating well known ECM degradators like MMP-2, MMP-9, or by reorganizing the actin cytoskeleton through Cdc42 or RhoA upregulation.

Gal-1 also plays a key role in angiogenesis as it is able to stimulate the growth of vascular endothelial cells. The lectin is overexpressed in activated tumor endothelium and it is involved in endothelial cell function (by NRP-1 interaction and VEGFR-2 activation). Gal-1 deficiency impairs tumor growth and angiogenesis *in vivo* (Le Mercier et al., 2009; Thijssen et al., 2006). Moreover, Gal-1 modulates the expression of BEX2 and several hypoxia related genes involved in angiogenesis. Paracrine mechanisms involving the uptake by endothelial cells of Gal-1 secreted from tumoral cells have been linked to endothelial cell activation and tumor angiogenesis stimulation, through Ras and Erk1/2 activation (Thijssen et al., 2010).

Finally, Gal-1 is involved in the tumor immune response promoting an immunosuppressive environment at tumor sites by inhibiting full T cell activation, triggering T cell growth arrest and apoptosis and protecting the tumor by negatively regulating Th1 and proinflammatory cytokines. These effects are mediated by Gal-1 recognition of cell surface glycoproteins present on T cell membranes such as CD2, CD3, CD7, CD43 and CD45 (Galvan et al., 2000; Pace et al., 1999).

3.4 Galectins in pancreatic cancer

In pancreatic cancer, Gal-1 and Gal-3 are found to be overexpressed (Berberat et al., 2001; Chung et al., 2008; Grutzmann et al., 2004; Schaffert et al., 1998).

Gal-3 expression is faint in ductal cells of normal pancreas but it is high in intrapapillary mucin neoplasms, chronic pancretatitis, cancerous pancreatic tissue and metastatic cells, suggesting its role in cancer cell proliferation and metastasis formation. However, decreased Gal-3 expression has been linked to advanced stage, tumor de-differentiation and metastasis in ductal adenocarcinomas, implying a fine tuned regulation of its levels in different steps of tumor progression. Gal-3 secreted by pancreatic cells plays a role in pancreatic stellate cell proliferation and in pancreatic cancer cell proliferation and invasion *in vitro*. A negative correlation between anoikis and Gal-3 presence has been established, too. Besides, the interaction between Gal-3 and Muc4 has been proven to be functional to dock tumor cells to the endothelial surface, what might present a possible mechanism to explain Gal-3 involvement in metastasis.

Gal-1 has found to be overexpressed in pancreatic tumors compared to normal tissue (Berberat et al., 2001; Grutzmann et al., 2004; Iacobuzio-Donahue et al., 2003; Shen et al., 2004) (Fig.5). Interestingly, its expression levels correlate not only with histology but also with T stage, N stage and global AJCC stage of pancreatic cancer disease (Chung et al., 2008), suggesting that Gal-1 might also participate in tumor progression and that its presence does not seem to be a random event. Gal-1 expression by immunohistochemical analysis has been reported to be mainly restricted to the ECM and fibroblasts in and around the cancer mass, but not to pancreatic cancer cells, suggesting its importance in the so characteristic desmoplastic reaction. Gal-1 is also found in the stroma of PanIN-2 and PanIN-3 (Pan et al., 2009) and in chronic pancreatitis (Wang et al., 2000).

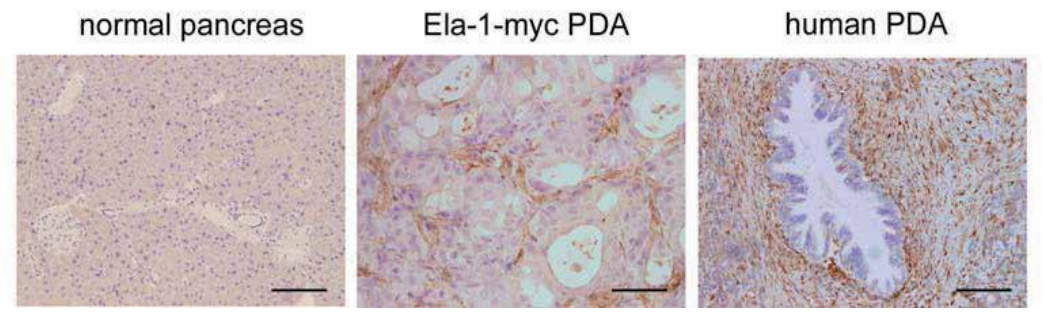

Fig. 5. Gal-1 is overexpressed in precursor lesions and pancreatic cancer. Gal-1 immunohistochemistry in mouse pancreatic normal tissue, in Ela-1-myc pancreatic ductal adenocarcinoma (PDA) lesions and in human pancreatic cancer tissue. Scale bars correspond to 200 µm.

Interestingly, although Gal-1 did not appear in the list of genes consistently misregulated in pancreatic cancer that were gathered in 12 core signaling pathways (Jones et al., 2008), 54 of the genes found overexpressed encoded secreted or cell surface proteins, putative and already known Gal-1 binding targets, like laminin. Thus, Gal-1 overexpression might be involved in the functional outcome of these overrepresented molecules, playing a role in some of the key identified signaling pathways such as homophilic cell adhesion, integrin signaling and regulation of invasion. Gal-1 could have been excluded from the reported list because this important global genomic analysis was based on tumoral epithelial cells, leaving out the stroma, whose population seems to be the one predominantly affected by Gal-1 increased levels.

Gal-1 could be involved in tumor progression in pancreatic cancer by remodeling the ECM in the formation of the desmoplastic reaction. Indeed, Gal-1 is able to induce activation (increased collagen synthesis), proliferation and chemokine production (MCP-1 and CINC-1) of pancreatic stellate cells, through Erk1/2, Jnk, NF-κB and AP-1 activation. At the same time, activated pancreatic stellate cells secrete Gal-1, which can be acting autocrinely and might be also regulating the tumor immune response (Fitzner et al., 2005; Masamune et al., 2006).

As it has been described above, Gal-1 displays a wide variety of biological functions which bring up a high degree of complexity when trying to understand its involvement in cancer. Thus, Gal-1 might not always tilt the balance in the same direction. In pancreatic cancer cells, for example, stable transfection of the tumor suppressor p16/Ink4a can induce Gal-1 expression and its affinity for the fibronectin receptor, resulting in increased susceptibility towards anoikis (Andre et al., 2007). Another Gal-1 antitumoral role is presented by the fact that it is downregulated in gemcitabine resistant pancreatic cancer cells (Kuramitsu et al., 2010). The ability of Gal-1 to induce opposite effects regarding proliferation and adhesion, as well as its reduced expression found in some tumors (Choufani et al., 1999), hint at Gal-1 as a double side coin and question its nature as a protumoral molecule. Many variables might be influencing the final outcome, such as cell type and activation status, Gal-1 levels and localization, as well as its quaternary structure.

3.5 Gal-1 establishing protein/glycan interactions

Gal-1 interactions involving its CRD domain and lectin activity are involved in many of Gal-1 important functions (Table 1). N-glycans from cell surface glycoproteins are the major ligands for Gal-1 and Gal-3, although they also bind to mucins, proteoglycans and the ECM. Although both proteins have high affinity for β-galactosides and indeed they share many interacting partners such as CD45, laminin, fibronectin and integrins, a fine specificity level results in binding differences. The general rule is that Gal-3 prefers repeating lactosamine units whereas Gal-1 recognizes independent lactosamine disaccharides with low affinity (Kd=50 μM) but deeply increases avidity when presented in multiantennary repeating units (Kd=5 μM) and when the lectin is surface bound to cell membranes or to the ECM. Indeed, Gal-1 is involved in microdomain (lattice) formation within membranes by crosslinking ligands in a glycoside cluster effect that greatly increases its affinity. However, as a matter of fact, Gal-1 is able to recognize only about 1/40 of the total N-glycans present in human serum glycoproteins (Kita et al., 2007), and around 1/8 of the sites supposed to be galectin specific. It is believed that part of Gal-1 specificity is mediated by additional binding sites

Gal-1 partners	Biological context	Functional Outcome
CA-125	cervical cancer cells	Gal-1 export to cell surface
CD2/CD3	T cells	T cell activation and apoptosis
CD4	T cells	Unclear
CD43, CD45	T cells	Gal-1 induced T cell death (depending on specific receptor glycosylation). Redistribution of the receptors in the cell surface.
CD7	T cells	Induction of apoptosis
CEA	colon carcinoma cells	Unclear
Chondroitin sulphate	SMC	Incorporation of ECM components important for SMC
Fibronectin	placenta ovary carcinoma cells	Control of cell adhesion
1B2 glycolipid	olfactory neurons	Adhesion between adjacent axons and with the ECM resulting in olfactory axon fasciculation
Glycoprotein 90K	melanoma cells	Formation of multicell aggregates
GM1 ganglioside	neuroblastoma cells	Sialidase dependent cell growth inhibition
HBGp82	brain	Unknown
INTEGRINS		
$\alpha_1\beta_1$, $\alpha_7\beta_1$	SMC skeletal myocytes	Intracellular signaling leading to adhesion, FAK activation, migration
$\alpha_5\beta_1$	colon, breast, ovarian, hepatocellular carcinoma cells,	Antiproliferative effects, induction of anoikis
$\alpha_M\beta_2$	macrophages	Possibly crosslinking receptors or affecting receptor-ligand binding affinity
Laminin	placenta, smooth muscle cells, leydig cells	Assembly of ECM, adhesion, migration, apoptosis
LAMP-1, LAMP-2	ovarian, colon carcinoma cells	Tumor cell adhesion and metastasis
Mucin	gastrointestinal tract	Protection from the epithelial surface
NRP-1	endothelial cells	Signaling pathway activation, migration and adhesion
Pre-B cell receptor	B cells	Cell differentiation, adhesion
Osteopontin, vitronectin	SMC	Adhesion, ECM assembly
Thrombospondin	SMC	Adhesion

Table 1. Proteins that are known to interact with Gal-1 through their CRD. Description of the best characterized Gal-1 interactors, specifying the biological context in which the interaction has been identified, and the consequent functional outcome. SMC: smooth muscle cells. Detailed references can be found at (Camby et al., 2006).

recognizing more than the canonical galactose. Thus, the particular structural context of galectin binding sites depicts a complex scenario and impairs stating generalizations. For instance, Gal-1 is able to induce T cell death by binding a glycan ligand without lactosamine units, that is very abundant but less preferred (Hernandez et al., 2006). Normally though, Gal-1 recognition capacity is deeply influenced by specific conditions regarding carbohydrate content and linkage. Minor alterations in N-glycan chains have been reported to influence Gal-1 binding in such a way that changes the overall biological outcome (Andre et al., 2007). Cell type specific expression patterns of several proteins and their glycans can modulate different Gal-1 mediated effects (Gu et al., 1994; Moiseeva et al., 1999). Particular glycosylation structures are known to mask glycans to Gal-1, which impede Gal-1 induced T-lymphocyte (Liu & Rabinovich, 2010) and cancer cell (Valenzuela et al., 2007) death. For instance, in contrast to Th1 and Th17 cells, Th2 cells are protected from Gal-1 induced apoptosis by presenting α2-6 sialylation of cell surface glycoproteins (Toscano et al., 2007).

In the ECM, Gal-1 displays high affinity for laminin, fibronectin, thrombospondin, vitronectin, osteopontin and glycosamine glycans such as chondroitin sulfate (Table 1). Depending on the cell type and cell activation status, these interactions finally lead to a pro-adhesive or an anti-adhesive effect.

In the cell membrane, Gal-1 has many interactors resulting in very different effects (Table 1). Glycosylated cell surface receptors are closely linked to the adhesive properties mediated by Gal-1. For instance, Gal-1 interaction with $\alpha_7\beta_1$ integrin interferes with integrin/laminin binding and controls cell adhesion. Gal-1 interaction with NRP-1 has been involved in migration and adhesion of endothelial cells (Hsieh et al., 2008). Gal-1 can also function as a regulator of the immune response through its interaction with CD7, CD45 and CD43. Moreover, Gal-1 has also been involved in cell growth inhibition through its interaction with $\alpha_5\beta_1$ integrin, GM1 ganglioside or the glycoprotein 90K/MAC-2BP. Gal-1 can also recognize HBGp82 in the brain, CA125 in ovarian cancer cells, LAMP-1, LAMP-2 and CEA in colon carcinoma cells and 1B2 glycolipid in olfactory axons.

4. tPA: Connecting galectins and cancer protein glycosylation?

4.1 tPA: General features

Our group has recently characterized how an interaction between Gal-1 and a glycosylated protein –tPA- is involved in pancreatic cancer progression (Roda et al., 2009). tPA is mainly synthesized by endothelial cells, but it has also been detected in the central nervous system, being secreted by neurons and glial cells and it can also be produced by keratinocytes, melanocytes and various tumor cells. tPA best documented role is the conversion of plasminogen into plasmin, which degrades fibrin clots in blood vessels after thrombosis through a well-orchestrated process involving several regulators. Besides, tPA is also involved –by its catalytic activity- in the activation of growth factors and matrix metalloproteinases in the ECM (Fig.6). In addition to these proteolytic activities, we and others have demonstrated that tPA can exert catalytic-independent functions in different cell types, including neurons (Medina M.G. et al., 2005), kidney fibroblasts (Hu et al., 2006) and tumors (Ortiz-Zapater et al., 2007).

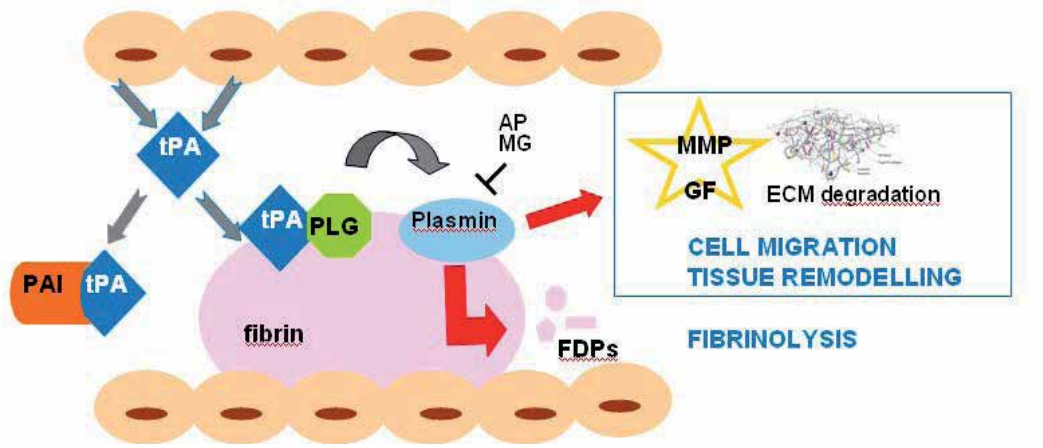

Fig. 6. tPA physiological functions. The best documented role for tPA is the activation of the zymogen plasminogen into plasmin, which degrades fibrin clots in blood vessels after thrombosis. Moreover, tPA is also involved in activation of matrix metalloproteinases and growth factors and in the ECM degradation, participating in cell migration and tissue remodeling events.

tPA is a glycoprotein of 527 aminoacids and around 70 KDa, depending on specific glycosylation. tPA is synthesized as a single-chain protein but it is quickly hydrolyzed by plasmin, forming a two-chain structure maintained together by a disulfide bond. Structurally, apart from a typical signal peptide and a prosequence, tPA is formed by 5 different autonomous domains, which are encoded by separate exons or sets of exons: 1) The fibronectin type I domain in the amino terminus, which mediates fibrin affinity; 2) An EGF-like domain which is probably involved in cell surface receptor binding; 3) Two kringle regions with a triple looped structure, with a high degree of homology with plasminogen kringle domains and 4) A serine protease domain with the active site residues His322, Asp371 and Ser478.

Due to the size of tPA and the presence of glycosylated chains in the molecule, the complete structure of the protease remains still undetermined. However, the detailed structure has been revealed for some of the domains by NMR or X-Ray diffraction as for the fibronectin type I domain (Downing et al., 1992), EGF domain (Smith et al., 1995), kringle 2 (Byeon et al., 1991; Byeon & Llinas, 1991; de Vos et al., 1992) and the catalytic domain (Lamba et al., 1996; Renatus et al., 1997a; Renatus et al., 1997b).

Glycosylation differences describe two different tPA isoforms (type I and type II), displaying species, cell and site-specific patterns of these post-translational modifications. In type I tPA, 4 glycosylation sites are occupied in separate domains, which play a role in different biological and pathological tPA functions: an O-linked fucose in Thr61 (EGF domain) and three N-linked carbohydrate chains; an oligomannosidic structure highly conserved between species at Asn117 (in kringle 1), and two complex and hybrid type structures at Asn184 (in kringle 2) and Asn448 (in the catalytic domain). Type II tPA lacks the glycosylation at Asn184 and this absence allows the conversion of single-chain to two-chain tPA, through plasmin mediated cleavage of the polypeptide backbone between Arg275 and Ile276. The presence of glycan chains at site Asn184 affects the structure of the

glycan population at Asn448, being two-chain tPA a more active tPA regarding clot lytic activity and fibrin-binding capacity.

4.2 Role of tPA and tPA receptors in pancreatic cancer

tPA overexpression correlates with poor prognosis in several cancers. In pancreatic cancer studies, tPA is found to be highly expressed in well differentiated human pancreatic cancer cultures and overexpressed in 95% of pancreatic ductal adenocarcinomas (PDAs), being absent in normal pancreas (Paciucci et al., 1996, 1998; Ryu et al., 2002) (Fig.7).

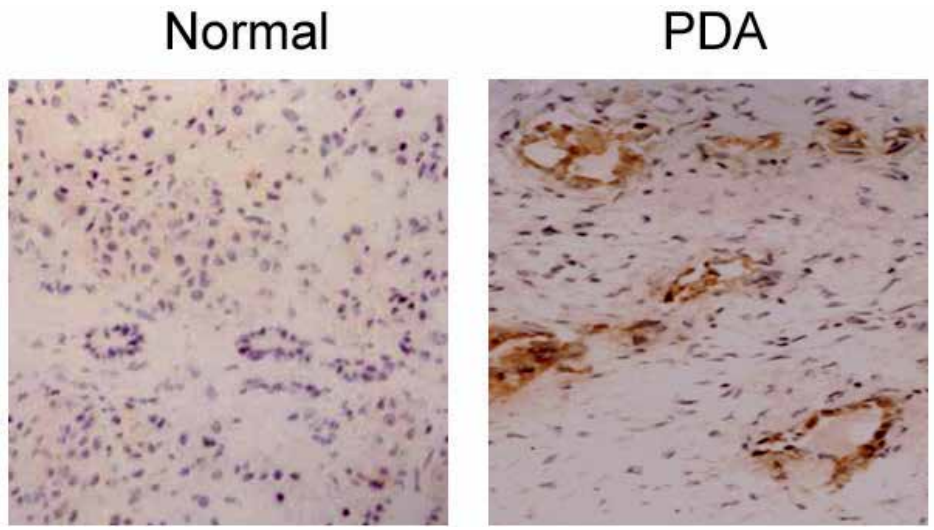

Fig. 7. tPA is overexpressed in human pancreatic cancer. tPA expression assessed by immunohistochemistry in normal pancreas (normal), showing no tPA expression, whereas, in human pancreatic ductal adenocarcinoma (PDA), high expression levels of tPA are detected in ducts.

In vitro and *in vivo* studies have determined that tPA contributes to pancreatic cancer progression by increasing cell invasion, Erk1/2 phosphorylation, cell proliferation and angiogenesis (Aguilar S et al., 2004; Diaz et al., 2004; Paciucci et al., 1998). These effects are mediated through tPA interaction with different cell membrane receptors. In this regard, EGFR is overexpressed in pancreatic cancer and it has been demonstrated to participate in tPA effects in cell proliferation (Hurtado et al., 2007; Ortiz-Zapater et al., 2007). AnxA2 -the best characterized tPA receptor and its major receptor in endothelial cells- has also been clearly involved in tPA-mediated pancreatic cancer cell invasion, proliferation and angiogenesis (Diaz et al., 2004; Ortiz-Zapater et al., 2007). Nevertheless, AnxA2 does not seem to be the only functional tPA pancreatic cancer receptor as its interaction with the protease only explains part of the tPA found in the cell membrane (Diaz et al., 2004; Ortiz-Zapater et al., 2007). These data and the fact that AnxA2 seems to be inappropriate as a target for pancreatic therapy due to its important physiological functions in blood coagulation homeostasis moved us to find new tPA receptors that could be involved in tPA protumoral functions in pancreatic cancer. As described in the next section, we have recently demonstrated that Gal-1 is a new functional tPA receptor (Roda et al., 2006, 2009).

4.3 tPA/Gal-1 interaction: Glycosylation involvement and role in pancreatic cancer

Interaction between tPA and Gal-1 was first identified in total tumoral pancreatic cell lysates by affinity capture with tPA-sepharose followed by 2D- electrophoresis (Roda et al., 2006). However these data did not prove whether tPA/Gal-1 interaction was direct or mediated through other proteins. In a more recent work, using recombinant proteins and surface plasmon resonance, we proved that tPA/Gal-1 interaction was direct and specific (Roda et al., 2009). Furthermore, Gal-1 was able to increase tPA mediated plasmin generation, suggesting interesting functional outcomes from their interaction.

Taken into account that 1) galectins are lectins with high affinity for β-galactosides, 2) Gal-1 binds galactose, and lactose with even higher affinity, through its CRD, and 3) tPA is a glycoprotein, we hypothesized that tPA and Gal-1 interaction was N-glycan mediated. In order to know whether that was the case, surface plasmon resonance was used to determine if carbohydrates were able to interfere with this interaction. Galactose (in a dose dependent manner) and lactose (with even higher effectiveness), inhibited tPA/Gal-1 interaction (Roda et al., 2009). Proving galactose specificity, neither glucose nor cellobiose was able to do so. These data demonstrated that the Gal-1 CRD was involved in tPA interaction and as expected, pointed at galactose in a β-anomeric position as its high affinity epitope.

Importantly, our results showed that this Gal-1/tPA interaction was not only relevant *in vitro,* but also *in vivo* where the lectin was actively involved in tPA induced Erk1/2 activation, proliferation, migration and invasion. tPA/Gal-1 effects were not restricted to pancreatic cells but were also found in tPA-mediated protumoral effects in fibroblasts from the tumor stroma, demonstrating the important role for tPA/Gal-1 interaction in the epithelial/fibroblast crosstalk and in pancreatic cancer tumor progression (Roda et al., 2009).

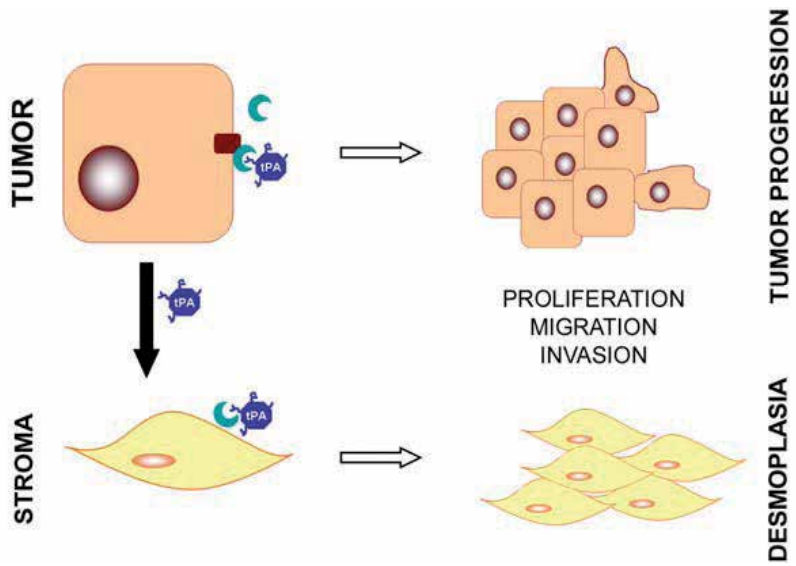

Fig. 8. Gal-1 is acting as a functional tPA receptor in pancreatic cell lines and fibroblasts. Gal-1 in pancreatic cancer cells can activate Erk1/2, induce proliferation, migration and invasion by binding to tPA in an autocrine fashion. Gal-1 can also act in a paracrine fashion over fibroblasts, triggering the same pathological effects that could be involved in the desmoplastic reaction.

Thus, we propose a model in which tPA secreted from pancreatic epithelial cells could act both in a paracrine and in an autocrine manner. In the latter, it would bind to Gal-1 in the cell surface of pancreatic tumoral cells triggering Erk1/2 activation and subsequent proliferation, as well as invasion. These events would be favoring tumor progression. On the other hand, tPA could bind in a paracrine fashion to Gal-1 from fibroblasts, what would induce the same events but in this mesenchymal cell line, leading to the desmoplastic reaction (Fig.8).

5. Glycans and Gal-1 in pancreatic cancer diagnosis and therapy

5.1 Glycans in diagnosis and therapy

Serum glycoproteins constitute the most frequent family of current tumor markers (Ludwig & Weinstein, 2005). Among others, two main characteristics highlight glycans in diagnosis: their altered structure upon tumorigenesis and the fact that they are frequently found in secreted proteins, what might facilitate their accessibility in the clinics. The most frequently used glycoproteins in this context are highly glycosylated mucins like CA19-9, CA125, CA27-29, CA15-3 but other proteins like PSA, AFP, CEA, RNase1 and hCG-β have also been considered (Peracaula et al., 2008).

Glycans have been closely involved in several events driving tumor progression, so therapeutic strategies targeting them have been studied with special attention (Dube & Bertozzi, 2005; Fuster & Esko, 2005). For example, the carbohydrate moiety of growth factor receptors is key in the regulation of cell signaling towards proliferation. Besides, several molecules like mucins, proteoglycans and gangliosides, modulate growth factor receptor activity through their glycan structures. Thus, different approaches directed to these glycans have already been designed and are being tested in the clinics such as peptide-based vaccines and monoclonal antibodies against mucins or gangliosides. The possibility of altering glycan synthesis and their maduration has also been proposed. One of the most studied effects of glycans over tumor development is their role in invasion due to their structurally altered presence in proteins well known for their effects upon adhesion and migration like E-cadherin, integrins, syndecans, proteoglycans and hyaluronan. Therefore, several strategies with the aim to block tumor specific patterns of glycosylation have been planned such as the inhibition of GnTV (responsible of increased β1,6-branched N-glycans) or polysialyltransferases. The reduction of tumor angiogenesis has also been addressed through glycan-based therapy by the use of modified heparin fragments or compounds inhibiting heparanase. Anti-selectin antibodies or mimetics of selectin ligands have been proposed to be useful against metastasis.

5.2 Gal-1 in diagnosis and therapy

Galectins are overexpressed in many different tumors and their expresssion has been related to poor prognosis suggesting their possible use as markers for diagnosis (Lahm et al., 2001; Rabinovich, 2005; Salatino et al., 2008). Indeed, Gal-1 detection in serum has been proven to be useful to monitor tumor progression and clinical severity in patients with head and neck squamous cell carcinoma (Saussez et al., 2008) and ovarian carcinoma (Allen et al., 1993).

In pancreatic cancer therapy, Gal-1 fulfills interesting requirements to be considered for targeting such as not being expressed in normal pancreas, increasing drug selectivity. Moreover, the use of Gal-1 inhibitors is particularly appealing because Gal-1 knockout mice are viable and fertile and do not show overt abnormalities (Poirier & Robertson, 1993), probably due to redundant functions from other members of the galectin family. Nevertheless, the dichotomous effects of Gal-1 must be well considered for efficient targeting, as depending on many intrinsic and extrinsic factors, the lectin can exert contrary effects (mitogenic or antiproliferative and pro or anti-adhesive). That is so the case that even Gal-1 and Gal-1 mimetic compounds have been also proposed for anticancer therapy (Fischer et al., 2005). Thus, special attention must be paid concerning Gal-1 conformation, quaternary structure, oxidation state, concentration, subcellular localization, ability to establish protein/protein or protein/glycan interactions, target cell type and presence of specific glycan receptors with certain glycosylation signatures, among others. Another interesting aspect to take into account for the use of Gal-1 in cancer therapy is its role as a master regulator of the immune response. Indeed, downregulating Gal-1 expression inhibits migration and restores susceptibility to apoptosis and so to cytotoxic drugs, making its inhibition a promising target in cancer therapy (Salatino et al., 2008; Rabinovich, 2005).

Finally, it has been reported that the huge stromal reaction accompanied with an important lack of angiogenesis impairs drug delivery and cause pancreatic cancer resistance. The stroma has been shown to be decisive in tumor progression, which can be inhibited maintaining a normal context. Different stromal cells have been under the scope for therapy as they are more accessible to pharmacological agents and genetically stable, which makes them less prone to acquire resistance. Indeed, therapies targeting other molecules involved in the desmoplastic reaction and vasculature have proven to improve efficiency delivery of gemcitabine in a pancreatic cancer mice model (Olive et al., 2009). Interestingly, silencing Gal-1 results in increased chemotherapy toxicity in glioblastoma cell lines (Le Mercier et al., 2008; Puchades et al., 2007). Gal-1 importance in tumor microenvironment immunosuppression is also considered in treatment. As a matter of fact, Gal-1 inhibition as adjuvant with vaccine immunotherapy significantly reduces breast tumor progression in mice (Stannard et al., 2010).

6. Conclusion

Overall, this context provides us with a whole universe of possibilities that might help in the design of new diagnosis markers and therapies directed to hamper tumor development. Still, the huge versatility of most of the molecules containing a glycan fraction forces research to deeply evaluate the molecular mechanisms affected upon targeting in order to avoid undesirable secondary effects that might prevent their use in treatment. Regarding Gal-1, the same precautions must be taken, considering the vast amount of partners and biological outcomes to which it is link. This complexity impairs analyzing the role of molecules independently and requires that each and every interaction is studied in detail. In this sense, a much finer approach in cancer therapy would result from targeting specific protein/protein interactions instead of individual proteins.

Our work has made an important contribution by specifically deciphering the relevance of Gal-1 interaction with a glycosylated protein – tPA- in the context of pancreatic tumor progression. Our data add valuable knowledge to enable a better understanding of

pancreatic cancer molecular biology. The relevant functional outcomes from Gal-1/tPA interplay open the door to new therapeutic strategies targeting the complex without interfering with tPA and Gal-1 independent physiological functions. Therefore, we stand for tPA/Gal-1 interaction as a promising target for pancreatic cancer, which could delay or even revert tumoral progression in this devastating disease.

7. Acknowledgement

This work was supported by grants from Plan Nacional de I+D, Ministerio de Educación y Ciencia (SAF2005-00704 to P.N) and from Instituto de Salud Carlos III-FEDER, Ministerio de Ciencia e Innovación (PI080421 to PN). NMB was supported by a predoctoral fellowship from the Ramón Areces Foundation. The authors would like to acknowledge Ricardo Gutiérrez-Gallego for critical reading of the manuscript and helpful suggestions.

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The Adhesion Molecule L1CAM as a Novel Therapeutic Target for Treatment of Pancreatic Cancer Patients?

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor disease with a still dismal prognosis for the patients. Since the tumor is mostly detected only in an advanced stage when the tumor has already metastasized, therapeutic options are quite limited. Moreover, this tumor is characterized by a profound resistance towards cytostatic drugs essentially hampering chemotherapy and reducing survival times of PDAC patients. The expression of the adhesion molecule L1CAM (CD171) has been recently reported to be associated with a chemoresistant and migratory phenotype of PDAC cells. L1CAM is a member of the immunoglobulin superfamily and has been initially found to play a role during the development of the nervous system. Meanwhile, L1CAM has been detected in numerous cancer tissues including PDAC and its elevated expression correlates with poor prognosis for the patients. Moreover, L1CAM has been shown to play an important role in different cellular processes involved in tumorigenesis such as cell migration and invasion, proliferation, survival and chemoresistance. Accordingly, inhibition of L1CAM by means of RNA interference or antibody-mediated blockade markedly reduced migration and proliferation of tumor cells and increased their chemosensitivity. Several preclinical studies e.g. in mouse model sytems for PDAC already demonstrated considerable anti-tumor effects with significantly reduced tumor outgrowth and tumor cell dissemination along with prolonged survival. Altogether, these data point to the suitability of L1CAM as a therapeutic target for an improved therapy of PDAC.

This chapter will describe the current knowledge on the physiological and pathophysiological role of the adhesion molecule L1CAM with an emphasis on its broad pro-tumorigenic functions and, in particular, its role in PDAC. Furthermore, we will figure out the underlying mechanisms and will summarize the preclinical studies on the specific targeting of L1CAM in anti-cancer therapy.

2. L1CAM – structure and physiological function

The adhesion molecule L1CAM (CD171) is a 220 kD transmembrane glyocoprotein and belongs to the immunoglobulin superfamily. The extracellular part of the molecule

comprises six Ig-like domains followed by 5 fibronectin type III repeats. The transmembrane domain is followed by a short cytoplasmic tail of 32kD (Hortsch, 1996; Weidle et al., 2009) (Figure 1). L1CAM can be expressed and mediate its effects as a membrane-bound form but it can also be proteolytically cleaved by different proteases releasing a soluble ectodomain that is likewise functionally active. To date, the metalloproteases ADAM 10 and 17 as well as plasmin have been described to cleave L1CAM generating a soluble 200 kD and 150 kD form, respectively (Maretzky et al., 2005; Mechtersheimer et al., 2001; Weidle et al., 2009) (Figure 1). After ADAM-mediated cleavage, the membrane-bound intracellular C-terminal fragment of L1CAM can be further processed by the presenilin/γ-secretase complex giving rise of a 28 kD fragment (Maretzky et al., 2005). This small intracellular fragment translocates into the nucleus where it is thought to contribute to L1CAM-dependent gene regulation (Maretzky et al., 2005; Riedle et al., 2009).

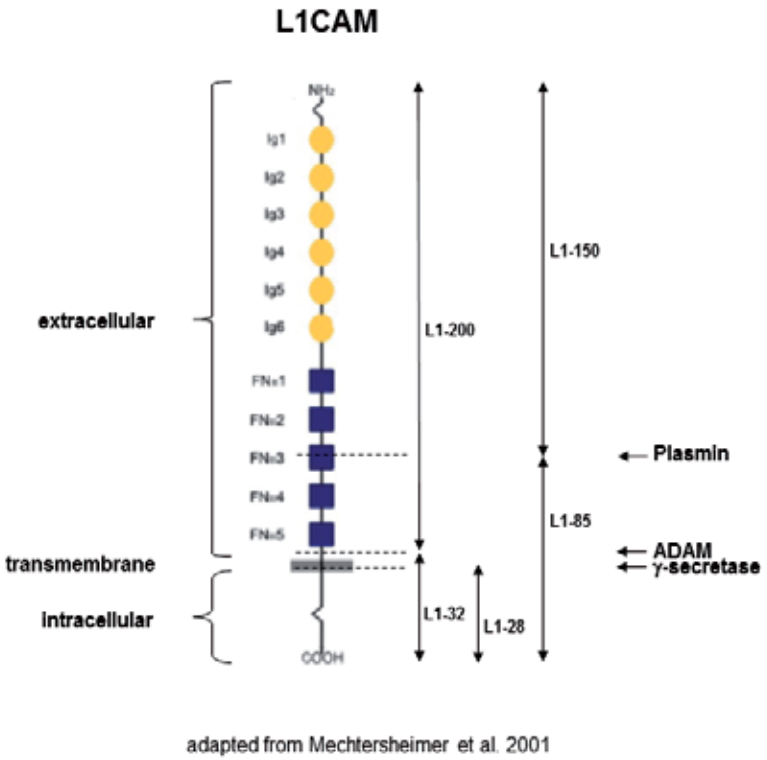

Fig. 1. **Scheme of the L1CAM molecular structure.** The extracellular, transmembrane and intracellular domains are indicated on the left. Cleavage sites for different proteases (plasmin, ADAM, γ-secretase) are indicated by the arrow heads and the dashed lines. The respective cleavage products (L1-200, L1-150, L1-85, L1-32, L1-28) are indicated by the vertical arrowed lines**.**

L1CAM can bind to different substrates/molecules in a cell and context dependent manner. Thus, it can undergo homophilic binding to itself as a membrane-bound or shedded form (de Angelis et al., 1999). In addition, a plethora of other proteins have been described with which L1CAM can interact, e.g. integrins αvβ3, α5β1, αvβ1 or αvβ5 (Ebeling et al., 1996;

Montgomery et al., 1996; Oleszewski et al., 1999) as well as neuropilin-1 (Stoeck et al., 2005), CD24 (Kadmon et al., 1995), neurocan (Oleszewski et al., 1999) and axonin-1/TAX-1 (Kuhn et al., 1991).

L1CAM was originally identified in cells of the nervous system (Rathjen & Schacher, 1984) and is regarded as a major player in its development being involved in neurite outgrowth and fasciculation, synapse formation as well as neuronal cell survival in the developing and adult brain (Hortsch, 1996; Loers et al., 2005; Maness & Schachner, 2007). The pivotal role of this adhesion molecule in the nervous system is underscored by the fact that mutations of the L1CAM gene cause severe neurodevelopmental disorders referred to as L1 syndrome or CRASH syndrome (Fransen et al., 1997; Weller et al., 2001). The different mutations and their resulting malfunctions of L1CAM in the nervous system are outlined in more detail in a recently published review (Schäfer & Altevogt, 2010). The L1CAM gene is located at chromosome Xq28 comprising 28 exons. Two splicing variants of L1CAM have been identified, the full-length form which is predominantly expressed by neuronal cells and a shorter non-neuronal isoform lacking exon 2 and exon 27 which is expressed by most types of cancer (de Angelis et al., 2001; Gast et al., 2005; Geismann et al., 2011; Kallunki et al., 1997; Meli et al., 1999; Shtutman et al., 2006). Besides its expression on neuronal cells, L1CAM expression has been also found in certain populations of hematopoietic cells (Ebeling et al., 1996; Pancook et al., 1997) and recent reports suggested a role for L1CAM in transendothelial migration and trafficking of murine dendritic cells (Maddaluno et al., 2009). Furthermore, L1CAM is expressed by renal tubular epithelial cells under physiological conditions being involved in branching of renal tubes in the kidney (Debiec et al., 1998). However, distribution of L1CAM in adults is quite restricted so that its elevated expression in cancerous tissues which is discussed in the next paragraph favours its suitability as therapeutic target in anti-cancer therapy.

3. L1CAM expression in tumors

To date, two comprehensive analyses on L1CAM tissue expression have been performed using a wide array of different normal and tumor tissues (Huszar et al., 2006; Rawnaq et al.; 2010). Moreover, systematic analyses of L1CAM expression in many types of tissues and tumors provided additional data on L1CAM expression in cancer and cancer related diseases. Elevated expression of L1CAM has meanwhile been detected in a variety of tumors such as neuroblastoma, glioma, melanoma, gynaecological tumors, colon cancer and gastrointestinal stromal tumors (GIST) (reviewed in Raveh et al., 2009). Tumoral L1CAM expression is often found at the invasive front of primary tumors (Gast et al., 2005; Zecchini et al., 2008) strongly supporting a role for L1CAM in metastasis. Hence, elevated tumor associated L1CAM expression correlates with tumor cell dissemination in lymph nodes and the bone marrow indicating micrometastatic spread (Kaifi et al., 200), more advanced tumor stages (Li & Galileo, 2010) and consequently reduced patient´s survival (Fogel et al., 2003; Kaifi et al., 2007; Zecchini et al., 2008). Besides L1CAM expression in tumor tissues, soluble L1CAM was detectable in the serum of 80 % and 90% of ovarian and uterine carcinoma patients at stage III-IV, respectively (Fogel et al., 2003). Zander et al. recently demonstrated that serum concentrations of soluble L1CAM were also elevated in GIST patients compared to healthy controls being particularly enhanced in patients with recurrence and relapse (Zander et al., 2011).

3.1 L1CAM expression in pancreatic tumors

Regarding the expression of L1CAM in PDAC, discrepant data have been published. This might rely on the variable usage of i) the number of analysed tissues, ii) tissue microarrays versus areal sections, iii) detection methods (mostly immunohistochemistry but also western blotting, microarrays), iv) staining protocols (e.g. the pH during antigen retrieval essentially impacts on the staining intensity) and v) scoring systems to determine L1CAM positivity. With respect to the latter, determination of L1CAM positivity can occur e.g. by scoring the staining intensity (weak = < 30% of tumor cells L1CAM+, strong= > 30% of tumor cells L1CAM+) (Rawnaq et al., 2010) or by consideration of tissue samples L1CAM positive when more than 10% of tumor cells exhibited a strong membranous staining (Huszar et al., 2006).

An analysis of a small number of samples by Huszar et al. revealed no L1CAM expression in PDAC and pancreatic neuroendocrine tumors (Huszar et al., 2006). Kaifi et al. demonstrated L1CAM expression in 2 % (2/111 samples) of PDAC and 7,9 % of pancreatic neuroendocrine tumors (5/63 cases). In the latter, L1CAM expression was mostly found in poorly differentiated pancreatic neuroendocrine carcinomas that are described to have the worst prognosis (Kaifi et al., 2006a, 2006b; Rawnaq et al., 2010). A study with 15 tissues of undifferentiated (anaplastic) pancreatic cancer and pancreatic carcinoma with osteoclast-like giant cells revealed L1CAM positivity in 80 % of the analysed samples (Bergmann et al., 2010a). Our group was the first who documented considerable L1CAM expression in a small series of PDAC samples (Sebens Müerköster et al., 2007). An extended analysis with 110 primary PDAC tissues, 15 lymph node and 14 liver metastases revealed tumoral L1CAM expression in 92,7 %, 80 % and 100 % of the samples, respectively (Bergmann et al., 2010b). Another study demonstrated L1CAM expression in 82 % of poorly-differentiated and in 14 % of moderately-differentiated PDAC tumors being absent in well-differentiated PDAC and normal pancreatic tissues (Chen et al., 2011). In line with these data, Ben et al. described L1CAM positivity in PDAC correlating with nodal involvement, vascular and perineural invasion, a higher degree of pain and accordingly with poor survival (Ben et al., 2010). Similarly, Tsutsumi et al. reported L1CAM expression in 23/107 PDAC samples (21,5 %) being predominantly found at the invasive front of the tumors. Again, L1CAM expression was significantly associated with histological grade, lymph node involvement, metastasis and short survival (Tsutsumi et al., 2011). Recently, a functional genome approach analysing PDAC tissues compared to normal pancreatic tissues identified a panel of seven differentially expressed genes including L1CAM which was named "migration signature" according to the functional involvement of the deregulated genes in tumorigenesis (Balasenthil et al., 2011). In search for the mechanisms leading to upregulation of L1CAM in tumors, we analysed pancreatic precursor lesions such as Pancreatic Intraepithelial Neoplasias (PanINs) and tissues of chronic pancreatitis. Indeed, considerable L1CAM expression - albeit weaker than in tumors - was already found in PanINs predominantly in high-grade PanINs 2 and 3 (Bergmann et al., 2010b) as well as in the majority of chronic pancreatitis (Geismann et al., 2009).

In summary, L1CAM expression is found – albeit at varying degree – in PDAC and correlates with advanced tumor stage, poor prognosis and short survival. Notably, L1CAM expression is already present in precursor lesions of PDAC and later on in tumor cells in primary tumors and metastases pointing to a role of this adhesion molecule in pancreatic tumorigenesis. This will be outlined in more detail in the next paragraph.

4. L1CAM function in tumorigenesis

L1CAM expression in tumors can be associated with the activation of several signalling pathways that are known to play a pivotal role in tumor progression e.g. the MAPK/ERK and AKT pathway or FAK-mediated signalling. Current knowledge on the L1CAM-mediated cellular alterations in tumorigenesis largely derived from studies with various types of tumor cells. Nevertheless, these alterations seem to be relevant also in pancreatic tumorigenesis and we will therefore discuss findings on the role of L1CAM in tumor manifestation and progression from studies with PDAC as well as other tumor entities. We also like to refer to the excellent reviews outlining in more detail the current knowledge on L1CAM-mediated signalling (Herron et al., 2009; Kiefel et al., 2011).

4.1 L1CAM and EMT

Epithelial-mesenchymal transition (EMT) represents a key event in the transformation process of an epithelial cell and is characterized by morphological and phenotypical alterations. Through EMT, epithelial/carcinoma cells acquire a motile phenotype so that they become enabled to leave the cellular context and disseminate into distant organs. The first hint that L1CAM might be connected with EMT was provided by the group of Shtutman (Shtutman et al., 2006). In the mamma carcinoma cell line MCF7, L1CAM expression leads to the disruption of E-cadherin- containing adherens junctions and thereby to increased transcriptional activity of β-catenin (Shtutman et al., 2006). Since L1CAM is a target gene of β-catenin signalling (Gavert et al., 2005), activation of β-catenin contributes to sustained L1CAM expression and enhanced cell motility in the cells (Shtutman et al., 2006). In line with these findings, immunohistochemical stainings of endometrial carcinomas revealed L1CAM expression at the leading edge of the tumor while E-cadherin expression was lost which could be linked to an aggressive subtype of this tumor (Huszar et al., 2010). Stimulation of endometrium carcinoma cells with the well-known EMT inducer TGF-β1 led to the upregulation of vimentin and concomitantly to the downregulation of E-cadherin depending on the transcription factor Slug. As a result, stimulated LCAM expressing tumor cells acquired a migratory phenotype (Huszar et al., 2010). In contrast, Gavert et al. recently showed that L1CAM mediated metastasis of colon cancer cells was dispensable of EMT induction and an altered expression of epithelial and mesenchymal marker proteins (Gavert et al., 2011). Thus, the impact of L1CAM on EMT might be either tumor specific and/or tumor stage dependent. However, stimulation of pancreatic ductal epithelial cells with TGF-β1 led to the acquisition of a spindle-shaped cell morphology, upregulation of mesenchymal proteins and L1CAM expression which was dependent on JNK-mediated activation of Slug (Geismann et al., 2009). Accordingly, elevated cell migration and apoptosis resistance could be abolished by interfering with TGF-β1 signalling or by suppression of Slug or L1CAM. Further studies are required to elaborate whether upregulation of L1CAM is part of the EMT or even the inducing event.

4.2 L1CAM and cell motility and migration

On the one hand, the importance of L1CAM for cell migration, invasion and metastasis is based on the detection of L1CAM expressing tumor cells in metastases as well as at the invasive front of the primary tumor (Bergmann et al., 2010b; Chen et al., 2011; Gavert et al.,

2005; Kaifi et al., 2007). On the other hand, numerous in vitro and in vivo studies provide compelling evidence for the role of L1CAM in cell migration of various tumor entities such as ovarian cancer (Arlt et al., 2006; Gast et al., 2005; Zecchini et al., 2008), colon cancer (Gavert et al., 2005; Gavert et al., 2011), melanoma (Meier et al., 2006), glioma (Yang et al., 2009; Yang et al., 2011), glioblastoma stem cells (Cheng et al., 2011), breast cancer (Li & Galileo, 2010) and PDAC (Chen et al., 2010; Geismann et al., 2009). Cleavage of L1CAM seemed to be a prequisite for promoting the adhesion and migration of breast cancer cells (Li & Galileo, 2010) as well as for the motility of glioma (Yang et al., 2009), ovarian cancer (Mechtersheimer et al. 2001) and colon cancer cells (Gavert et al., 2005). In the latter, ADAM 10 has been shown to enhance L1CAM cleavage in L1CAM expressing colon cancer cells and to induce liver metastasis in a mouse model system (Gavert et al., 2007). In addition, ligation of L1CAM to integrins such as αvβ3 (Meier et al., 2006) or αvβ5 (Mechtersheimer et al., 2001) seemed to be pivotal for L1CAM-mediated cell migration leading to the activation of Erk1/2 (Gast et al., 2007) and FAK signalling (Yang et al., 2011). As a result of the L1CAM-mediated Erk1/2 activation genes encoding for pro-migratory proteins such as cathepsin-B or α3-integrins were upregulated (Gast et al., 2007). Besides its ability to induce Erk1/2 and FAK signalling pathways, L1CAM can also lead to the activation of NF-κB, so that inhibition of NF-κB reduced L1CAM-mediated metastasis of colon cancer cells (Gavert et al., 2010).

4.3 L1CAM and angiogenesis

Besides its ability to directly increase motility and migratory behavior of tumor cells, L1CAM might also promote metastasis via its pro-angiogenic properties. Thus, soluble L1CAM was able to stimulate growth and invasion of bovine aortic endothelial cells to a similar extent as the vascular endothelial growth factor VEGF-A_{165} (Friedli et al., 2009). Moreover, stimulation with soluble L1CAM led to tube formation of bovine aortic endothelial cells *in vitro* and increased angiogenesis *in vivo* (Friedli et al., 2009). The pro-angiogenic effect of soluble L1CAM could be abolished by treatment with the L1CAM specific antibody chCE7. Issa et al. showed that endothelial cells in PDAC are characterized by elevated L1CAM expression compared to HUVEC cells where L1CAM expression can be induced by TNF–α, IFN–γ or TGF-β1 (Issa et al., 2009). Antibody-mediated blockade of L1CAM abolished tube formation and tumor endothelial cell transmigration (Issa et al., 2009). Overall, these data point to a role of L1CAM as a pro-angiogenic factor and the potential of an anti-L1CAM antibody therapy in interfering with tumor angiogenesis (see below).

4.4 L1CAM and cell growth

Overexpression of L1CAM has been shown to promote tumor cell proliferation. Accordingly, inhibition of L1CAM expression or function suppresses proliferation of tumor cells, e.g. in cholangiocarcinoma (Min et al. 2010) or ovarian carcinoma (Arlt et al., 2006; Novak-Hofer et al, 2008). Zecchini et al. confirmed the stimulating effect of L1CAM on proliferation of ovarian cancer cells and additionally demonstrated that L1CAM expression does not alter proliferation of non-tumorigenic ovarian epithelial cells (Zecchini et al., 2008). Moreover, inhibition of L1CAM by genetic interference or antibody-mediated blockade impaired growth of tumor cells resulting in a reduced phosphorylation of Erk1/2 (Arlt et al.,

2006; Zecchini et al., 2008). Cotreatment of SKOV3ip ovarian carcinoma cells with anti-L1CAM antibodies and the soy-derived isoflavone Genistein potentiated the anti-proliferative effects of the anti-L1CAM antibody along with reduced activation of Erk1/2, Akt and Src kinase (Novak-Hofer et al., 2008). The growth promoting effect of L1CAM can be attributed to the activation of the Erk1/2 and Akt-pathway of which both are known to accelerate proliferation and growth of tumor cells (Gast et al., 2007, Novak-Hofer et al., 2008). For the induction of these signalling pathways, the interaction with integrins seems to be important as well as the cytoplasmic part of L1CAM because mutations in the RGD binding site which mediates binding to integrins or of the cytoplasmic tail abrogated L1CAM-mediated signalling and cellular responses (Gast et al., 2007; Sebens Müerköster et al., 2009; Kiefel et al., 2011).

4.5 L1CAM and apoptosis resistance

Besides its role in proliferation, several reports show that L1CAM is involved in apoptosis resistance. Loers et al. reported on a role for L1CAM in neuroprotection (Loers et al., 2005) by conferring protection from apoptosis induction in neuronal cells. In their experiments, murine cerebellar neurons grown on L1CAM substrate were protected from apoptosis induced by serum deprivation, oxidative stress and staurosporine treatment. L1CAM-mediated apoptosis resistance was associated with enhanced activation of Erk1/2, Akt and Bad as well as inhibition of caspases (Loers et al., 2005). Our group demonstrated that L1CAM plays a pivotal role in the mediation of chemoresistance of tumor cells which is a hallmark of PDAC. Thus, L1CAM expressing PDAC cell lines such as Colo357 and Panc1 responded much less towards treatment with cytostatic drugs than cells lacking L1CAM expression (Sebens Müerköster et al., 2007) and α5–integrin has been identified as a ligand for L1CAM-mediated chemoresistance (Sebens Müerköster et al., 2009). This chemoresistance was seen in response to drugs exerting different modes of action such as gemcitabine and etoposide indicating a broad protection against drug-induced apoptosis through L1CAM-mediated alterations in cell signalling and gene expression. Thus, interaction of α5-integrin and L1CAM led to an increased activity of the inducible nitric oxide synthase (iNOS) and subsequent increased release of nitric oxide (NO) resulting in the inhibition of caspases. In addition, interaction of α5-integrin and L1CAM led to constitutive activation of NF-κB via an increased production and secretion of IL-1β (Kiefel et al., 2010) which both are likewise important mediators of chemoresistance in PDAC cells (Arlt et al., 2002; Arlt et al., 2003; Müerköster et al., 2003). Stoeck et al. also demonstrated that L1CAM in its membrane-bound as well as in its soluble form confers apoptosis resistance in ovarian carcinoma cells towards C2-ceramide, staurosporine, cisplatin and hypoxia. Long-term treatment with the cytostatic drug cisplatin increased L1CAM expression level in ovarian carcinoma cells m130 (Stoeck et al., 2008) which was similarly observed in PDAC cells after long-term incubation with etoposide indicating a role for L1CAM in the acquired chemoresistance of tumor cells (Sebens Müerköster et al., 2007). Immunohistochemical analyses of pancreatic tissues together with data from coculture experiments indicate that L1CAM is not only upregulated during chemotherapy but also under the influence of the cellular microenvironment. Thus, L1CAM was upregulated in the pancreatic ductal epithelial cell line H6c7 when cultured in the presence of activated fibroblasts/myofibroblasts resembling the situation of a chronic pancreatitis (Geismann et al., 2009). Again elevated L1CAM expression conferred an apoptosis resistant phenotype

even in non-tumorigenic pancreatic epithelial cells. Recent data by Min et al. similarly showed that L1CAM diminished the apoptotic response of cholangiocarcinoma cells towards drug treatment with gemcitabine (Min et al., 2010). In glioblastoma stem cells, a role for L1CAM in the control of DNA damage checkpoint responses and resistance to radiotherapy has been described (Cheng et al., 2011). Radioresistance was mediated by nuclear translocation of L1CAM and subsequent regulation of NBS1 expression which is part of the MRE11-RAD50-NBS1 (MRN) complex and involved in early checkpoint responses. Since L1CAM has been identified as a marker for glioma stem cells being important for tumor formation in vivo (Bao et al., 2008), targeting of L1CAM might be a strategy to eliminate therapy-resistant tumor stem cells that are presumably responsible for therapy relapses.

Overall, all these findings underscore the importance of L1CAM in protection from apoptosis which might i) imply a survival advantage for genetically altered cells during tumorigenesis thereby promoting tumor formation and ii) explain the profound innate and acquired chemo- (radio-)resistance of therapy resistant tumors such as PDAC.

5. L1CAM as target structure in cancer treatment – preclinical results

The soluble form of L1CAM was not only detected in culture medium of several tumor cell lines (Fogel et al., 2003; Gavert et al., 2005; Gavert et al., 2007; Gutwein et al., 2005; Yang et al., 2009) but also in serum and ascites of uterine and ovarian carcinoma patients being associated with poor prognosis (Fogel et al., 2003; Gutwein et al., 2005). In contrast, a first screening of serum samples from patients with chronic pancreatitis or PDAC did not reveal elevated levels of soluble L1CAM (unpublished observations) indicating that soluble L1CAM plays only a minor role in pancreatic tumorigenesis. Accordingly, cell culture experiments revealed no effect of sheddase inhibitors in PDAC cells with regard to chemoresistance and EMT (Sebens Müerköster et al. 2007). However, detection of soluble L1CAM in serum for diagnostic and predictive purposes would allow a viable screening without exposing patients to expensive and troublesome interventions. To validate the specificity of elevated L1CAM ectodomain levels as a tumor serum marker, further screenings of serum samples from patients at earlier tumor stages and also with other diseases (e.g. inflammation) that may lead to upregulation and shedding of L1CAM are required.

The fact that tumoral L1CAM expression is often associated with an advanced tumor stage, metastasis and poor clinical outcome strongly suggests its suitability as a predictive marker in malignancies such as PDAC. In view of its multiple functions in tumor development and progression as well as its prevailing expression in tumors compared to normal tissues, L1CAM represents a promising target structure in anti-cancer therapy. This notion is substantially supported by several preclinical studies using anti-L1CAM antibodies or strategies based on genetic interference. Biweekly treatment with the anti-L1CAM antibody L1-11A dose-dependently inhibited tumor growth of intraperitoneally inoculated SKOV3ip ovarian carcinoma cells and ascites formation by up to 75 % in nude mice (Arlt et al., 2006). In the same tumor model system, therapeutic efficacy of L1CAM antibodies with different isotypes has been evaluated demonstrating that therapy with the L1-9.3/IgG2a antibody results in the best anti-tumor response in terms of reduced tumor burden and prolonged survival (Wolterink et al., 2010). Expression profiling of mRNA isolated from tumors

revealed an altered gene expression after L1CAM antibody treatment including genes involved in apoptosis, chemotaxis, angiogenesis and inflammatory responses. Moreover, antibody-treatment caused a massive infiltration of macrophages into the tumor suggesting that efficacy of anti-L1CAM antibody therapy is based on immunologically and non-immunologically mediated mechanisms (Wolterink et al., 2010). Therapeutic efficacy of radiolabeled anti-L1CAM antibodies has been proven in nude mice orthotopically inoculated with SKOV3ip ovarian carcinoma cells or with neuroblastoma xenografts (Hoefnagel et al. 2001; Knogler et al., 2007). Furthermore, it was demonstrated that mutation of the anti-L1CAM antibody chCE7 led to improved blood clearance and a single 10.5 MBq dose of ^{67}Cu-labeled mutated chCE7 antibody reduced tumor growth and prolonged survival of the mice (Knogler et al., 2007). Min et al. demonstrated in a nude mouse model with subcutaneously inoculated cholangiocarcinoma Choi-CK cells that treatment with an anti-L1CAM antibody three times per week resulted in reduced tumor outgrowth compared to therapy with a control antibody. Similar results were obtained with the cholangiocarcinoma cell line SCK in which L1CAM expression was suppressed by short hairpin RNA (shRNA) (Min et al. 2010). Targeting of L1CAM using lentiviral-mediated shRNA interference in glioma tumor stem cells before injection into nude mice reduced tumor formation and prolonged survival of tumor bearing mice as well (Bao et al., 2008).

Overall, these data highlight the importance of L1CAM in tumor growth and development and the suitability of L1CAM as therapeutic target in anti-cancer therapy. Nevertheless, it has to be critically stated, that in all of the above mentioned studies, antibody treatment started 2 to 3 days after tumor cell inoculation which does not reflect the clinical conditions of a high tumor load in advanced tumor patients but rather the situation of micrometastatic spread.

The fact that even in these models no complete cures were achieved by L1CAM antibody treatment alone points to the need of appropriate combination therapies. In particular for highly malignant tumors such as PDAC, therapeutic targeting of L1CAM alone will not be an effective therapy for cure. Hence, therapeutic strategies combining L1CAM targeting and chemo- or radiotherapy might act synergistically and lead to improved anti-tumor responses. This approach was followed by our group to provide an improved therapy for PDAC. In a SCID mouse model with subcutaneously grown Colo357 tumors, combined treatment with 10 mg/kg anti-L1CAM antibodies (L1-14.10 or L1-9.3/2a) and gemcitabine significantly reduced tumor growth compared to treatment with chemotherapy alone or in combination with control antibodies. This stronger anti-tumor effect could be attributed to an increased number of apoptotic tumor cells along with a reduced tumor vascularization and increased macrophage infiltration (unpublished observation). These data are in line with the findings from Wolterink et al. and suggest that the L1CAM antibody-mediated anti-tumor effect might not only rely on interference with L1CAM-mediated signalling in the tumor cells but also on the induction of anti-tumor immune reactivity. Combined treatment with L1CAM antibodies and chemotherapy has been proven to be an effective anti-tumor therapy also in other tumor models (unpublished observation).

Certainly, these subcutaneous tumor models do not reflect the pathological conditions of PDAC and are therefore limited in their clinical significance. Addressing this issue we used a SCID mouse tumor model with H6c7 cells that were intrapancreatically co-inoculated with pancreatic myofibroblasts (PMFs) (see above). Whilst H6c7 cells inoculated without PMFs

did not produce measurable tumors and metastases, PMF co-inoculated H6c7 cells became highly tumorigenic leading to the formation of primary, stroma enriched tumors in 88% and to liver metastases in 75 % of the inoculated mice, as determined by high-resolution ultrasound. Intriguingly, treatment of these already tumor-bearing mice with 10 mg/kg of the anti-L1CAM antibody L1-9.3/2a resulted in a complete tumor remission and reduced formation of liver metastases in 50 % of the animals (unpublished observation).

6. Conclusions

Originally identified in the nervous system, L1CAM has meanwhile been detected in numerous cancers including PDAC. Aberrant expression of L1CAM in tumors has been identified to be a key player in tumor formation, progression and metastasis. PDAC is also characterized by elevated L1CAM expression in the primary tumor as well as in metastases. The fact that upregulation of L1CAM expression occurs already in PDAC precursor lesions such as PanINs and chronic pancreatitis strongly favours its involvement in pancreatic tumorigenesis. Accordingly, several studies demonstrated a pivotal role of L1CAM in tumor cell migration, survival and chemoresistance of PDAC cells. In view of its broad impact in PDAC progression and its favourably restricted expression in adult tissues, L1CAM represents a promising target to improve treatment of PDAC. Moreover, results from preclinical studies demonstrating that antibody-mediated targeting of L1CAM significantly ameliorated the efficacy of chemotherapy in PDAC cells and resulted in improved anti-tumor responses give rise to optimism. Hence, these findings should be validated in clinical studies and at the same time we have to continue to deepen our understanding on the mechanisms by which L1CAM impacts on tumor cell biology. Targeting of L1CAM alone will definitely not be effective enough in eliminating highly malignant tumors such as PDAC but it rather provides an appropriate therapeutic tool for combined treatment. In this context, more work and efforts are still needed to identify the most effective combination of therapeutic strategies.

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19

p53 Re-Activating Small Molecule Inhibitors for the Treatment of Pancreatic Cancer

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) devours two American lives every 30 minutes (annual death rate >37,000) and is the fourth leading cause of cancer-related deaths in the US (Jemal et al. 2010). Median survival is 4 to 6 months and the 5-year survival is less than 5% (Baxter et al. 2007). The standard chemotherapeutic agent gemcitabine shows dismal response rate and has little impact. Recently, clinicians have incorporated platinum-based genotoxic regimens such as oxaliplatin nevertheless such combinations have little impact on improving the overall survival of PDAC patients (Wang et al. 2011). There are critical unanswered questions regarding the mechanism of drug failure in PDAC and investigations are still a long way from identifying novel drug combination regimens to achieve cure. Therefore, management of PDAC is an ongoing challenge and novel clinically-translatable therapeutic agents that can improve on the dismal survival statistics of PDAC are urgently needed.

To date, PDAC carcinogenesis and drug resistance are only partly understood, due to the heterogeneity of this disease at the cell/tissue level. In general carcinogenesis progresses through the accumulation of genetic alterations resulting in a gain of cell growth and proliferation, and subsequently, in increased dissemination and metastatic potential and PDAC is not an exception (Whitcomb and Greer 2009). Loss or gain of gene function may appear in the form of up-regulation of oncogenes, down-regulation of tumor suppressor genes, and deregulation of genomic maintenance/DNA repair genes, house-keeping genes, and genes that control the apoptosis/cell death/immortalization cascade (Sohn 2002; Hruban et al. 2007; Hruban et al. 1999). PDAC arises from precursor lesions called pancreatic intraepithelial neoplasms (PanINs), which are characterized by the sequential accumulation of alterations in the *K-ras* oncogene and loss of the *CDKN2A*, *TP53 (p53),* and/or *SMAD4* tumor suppressors (Hruban et al. 2000). Although we know the frequencies of such mutations in PDAC, their specific functions during the development of PDAC remain unclear. K-ras and p53 are considered to be among the most critically deregulated genes that participate in cross talk to render PDAC therapy resistance (Hollstein et al. 1998; Hollstein et al. 1994). In the forthcoming passages we will first describe the impact of de-regulated p53 signaling on therapy resistance and then update on the pharmaceutical progress of small molecule inhibitors against p53 and related targets for the treatment of PDAC.

2. Impact of dysfunctional p53 signaling on PDAC therapy resistance

p53 tumor suppressor gene is mutationally inactivated in >50% of PDAC (Hohne et al. 1992; Kalthoff et al. 1993), predominantly through missense mutations (Pellegata et al. 1994). These often result in accumulation of mutant p53 protein, with potentially gain-of-function or dominant-negative properties. The fact that p53 is mutated, rather than deleted, in the majority of PDAC suggests that mutant p53 provides some tumor cell growth advantage. Murine models support this as mice expressing the accumulating p53 mutants $p53^{R172H}$ or $p53^{R270H}$ have increased incidence of osteosarcoma and epithelial carcinomas, some of which spread to distant organs (Lang et al. 2004; Olive et al. 2004). In contrast, mice that harbor a p53 null allele rarely develop metastases. It has also been recognized that mut-p53 and family member p63 protein can inhibit wt-p53 function, indicating that bi-allelic inactivation may not be necessary for loss of function. The third family member, p73, is rarely mutated and has been shown to possess apoptotic function in response to small molecule inhibitors and platinum drugs-induce apoptosis. Furthermore, in the other 50% of PDAC, the p53 gene is normal (wild type wt-) but its function is inhibited by MDM2 (human double minute 2); a protein that is over expressed in PDAC and is the primary focus of this chapter.

The activity of wt-p53 is mainly regulated at the post-translational level through its proteolytic turn over (Brooks and Gu 2006). This is achieved through the interaction with MDM2, which induces wt-p53 degradation by ubiquitin-mediated proteolysis (Bottger et al. 1997). In normal, non-stressed cells, MDM2 induces p53 degradation constantly, making it a short-lived protein. However, in response to DNA damage, MDM2 is auto-poly-ubiquitinated, resulting in its degradation and an associated increase in p53 levels and activity. This regulatory mechanism is subject to a feedback loop since p53 in turn, regulates the level of MDM2 transcription, giving rise to subtle balances between the amounts of p53 and MDM2 (Fig1) (Lahav 2008; Bose and Ghosh 2007). Although other isoforms of MDM2 are also present, i.e. HDM4 (Mancini et al. 2009a; Okamoto et al. 2009; Mancini et al. 2009b), but carry have lesser impact on p53. Despite being an energy consuming process, the MDM2-post-translational regulation of the amount of p53 is advantageous for cells because it is rapid and it increases the odds of having functional p53 following genotoxic stress and is in contrast to transcriptional regulation which is slower and sensitive to DNA damage (Millau et al. 2009). A common *Mdm2* promoter polymorphism is the T→G transformation at nucleotide 309. This *Mdm2 309T/G* promoter polymorphism has been associated with the development of a variety of tumors including PDAC (Grochola et al. 2010b; Bond et al. 2004; Ohmiya et al. 2006b; Galic et al. 2007; Lind et al. 2006). Its significance in clinical outcome is not well outlined, but recent associations with prognosis have been found in lung and gastric cancer (Ohmiya et al. 2006a). The *G* allele is associated with increased affinity for Sp1 binding and higher MDM2 mRNA and protein levels, leading to diminished tumor suppressor activity of the p53 pathway (Iwakuma and Lozano 2003). Over-expression of MDM2 in cancers prevents this normal balance and thus inhibits p53 even though it may not be mutated (bdel-Fattah et al. 2000; Watanabe et al. 1994; Watanabe et al. 1996). Based on functional significance of MDM2 in the biology of p53, one attractive pharmacological approach to wt-p53 activation is to use a small molecule weight inhibitor (SMI) to block the MDM2–p53 interaction (Klein and Vassilev 2004; Secchiero et al. 2008; Vassilev 2004b). Further, it is well recognized that genotoxic drugs such as oxaliplatin work through activation of p53 signaling, however, mutations in the gene or over-expression of MDM2 is

logical to thwart proper p53 response and therefore diminish the therapeutic potential of these drugs. Thus, re-activation of p53 by blocking MDM2 is an attractive pharmacological approach for treatment MDM2 over expressing subtype of PDAC (Azmi 2011; Azmi et al. 2010c). Nevertheless, there are additional de-regulated signaling molecules and p53 masking mechanisms that come into play. It is the lack of understanding of these regulatory control mechanisms that had led to failure in the effective design of drugs. As discussed below, the success of novel drugs is possible if the proper combination is identified that hits each component of the PDAC resistant network to achieve clinically beneficial outcome.

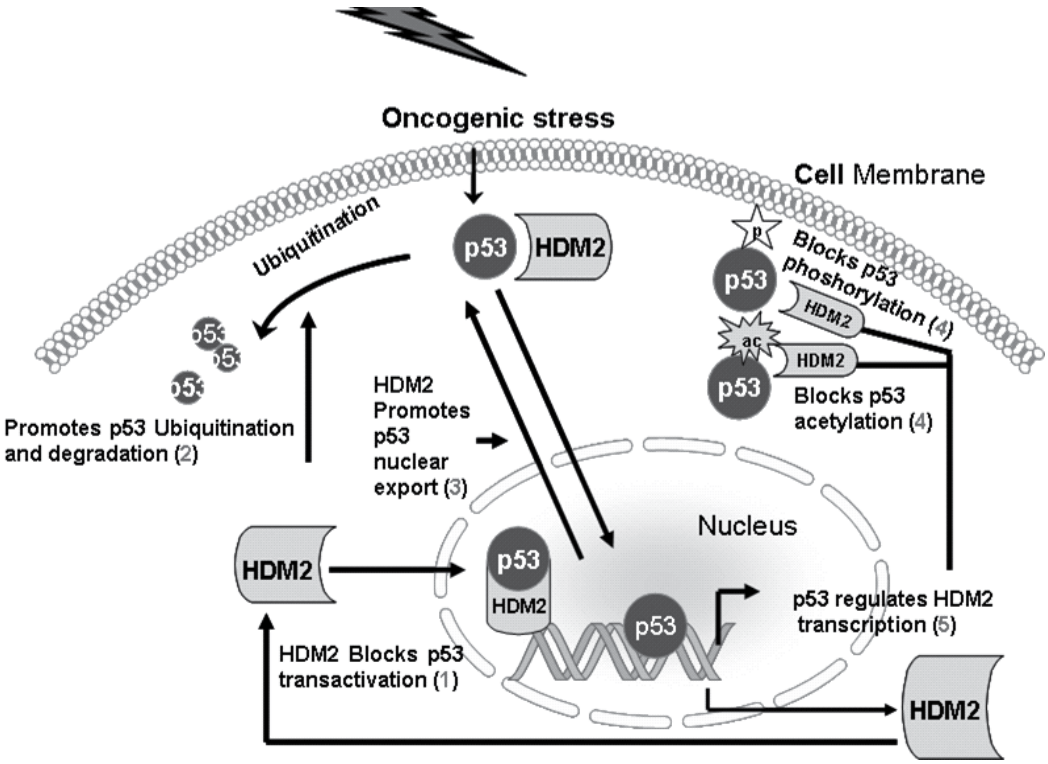

Fig. 1. MDM2 is a Master Regulator of p53: MDM2 blocks p53 activity at multiple levels. (1) MDM2 can bind directly to p53 activation domain and suppress its transcription. (2) MDM2 acts as E3 ligase and promotes p53 proteasomal degradation. (3) MDM2 has a nuclear export signal sequence in its domain structure that is responsible for p53 nuclear export. Cytoplasmic retention of p53 suppresses its nuclear transactivation activity. (4) MDM2 can block post-translational modifications of p53 (acetylation, phosphorylation etc necessary for p53 activity). (5) In turn, the transcription of MDM2 is regulated by p53. (Adopted from Azmi et al. 2011)

2.1 Regulation of p53 by hedgehog driven K-ras-snail axis

PDAC is an oncogenic K-ras driven disease and it is well established that majority of PDAC tumors show alteration in this pathway (Laghi et al. 2002). Although 3 kinds of oncogenic Ras have similar roles in induction of cell proliferation, survival, and invasion through the

stimulation of several pathways (Downward 2003), the mutation rate of K-ras is relatively higher than others, which indicates that the genetic alteration of K-ras is a major driving force for PDAC. Recently, the regulatory network of mutant K-ras signaling on p53 pathway has been established. Studies indicate that in cancer, p53 activity is suppressed via a novel mechanism of K-ras activation and consequent stabilization of snail (Fig2).

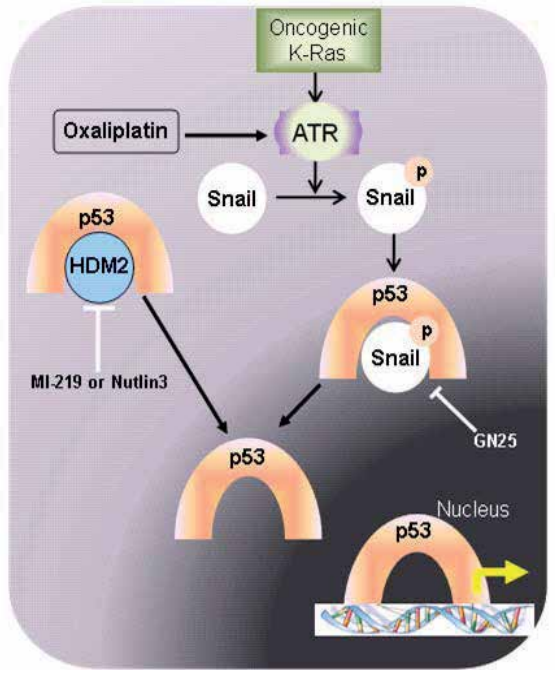

Fig. 2. Schematic diagram showing snail-p53 interaction that suppresses therapeutic response of genotoxic p53 re-activating regimens. Snail is activated through oncogenic K-ras driven ATR pathway while genotoxic regimens additionally induce snail. Novel agents targeting snail can have therapeutic implications against K-ras driven PDAC.

Snail stabilization occurs through activated ATR in K-ras mutated cells that demonstrates a strong direct interaction between snail and DNA-binding domain of p53, resulting in elimination of both protein from the cells (Horiguchi et al. 2009). This novel regulatory network has been shown to be independent of MDM2 or ARF-mediated p53 regulation. In normal cells, activation of oncogenic K-ras has been shown to provoke apoptosis or senescence through p53 activity, indicating that the loss of functions through p53 mutation is inevitable for the tumor progression by oncogenic K-ras (Gorgoulis and Halazonetis 2010). In mouse models, the adenoma is evoked by oncogenic K-ras despite the intact p53 system, which highlights that the additional and unique function of K-ras may enable cancer cells to avoid the tumor suppressive function of p53. Recent studies have also demonstrated the apparent tumorigenic function of oncogenic K-ras in mouse cell transformation (White et al. 2011). While normal mouse fibroblast transfected with N- or H-ras induce apoptosis or senescence, K-ras12V transfected cells become resistant and this is consistent with the multistep carcinogenesis model of colon or PDAC. In fact, genetic mutations of K-ras occur in the early adenoma stage without p53 mutation. However, large portion of adenoma can

be progressed into carcinoma without impaired p53 system or consequent apoptosis or senescence. These observations strongly indicate that oncogenic K-ras-snail axis possesses strong p53 suppressive effects downstream of MDM2 that cannot be ignored. Additionally, hedgehog signaling that is considered driver for PDAC induces Gli that in-turn induces snail adds to the existing ATR-driven suppressive mechanisms (Marigo et al. 1996). Thus, strategies that overcome these inhibitory pathways need to be incorporated in any genotoxic regimen that targets activation of p53 system. In sum, these findings demonstrate that in addition to MDM2 suppression targeting p53-snail binding can bring additional benefit to genotoxic therapy to achieve clinically superior therapeutic effects in PDAC.

Because the interaction between MDM2 and p53 is a primary mechanism for inhibition of the p53 function in cancers retaining wild-type p53, targeting the MDM2-p53 interaction by small molecules to reactivate p53 has emerged as a promising new cancer therapeutic strategy. In this chapter, the emerging SMIs of MDM2-p53 interaction are evaluated with additional discussions on new snail-p53 interaction inhibitors for the treatment of pancreatic cancer.

3. Reactivating the wild-type p53

Inhibition of p53 activity in tumors by the increased expression of MDM2 has been the target of development for many small-molecule-, peptide- and aptamer-based therapies (Lehman et al. 2008). MDM2 is over expressed in many human tumors including PDAC, often owing to an amplification of a chromosome segment that includes *Mdm2*, although over expression of the protein is possible without gene amplification (Do et al. 2009; Assmann et al. 2009; Shinohara et al. 2009; Grochola et al. 2010a; Lang et al. 2009; Economopoulos and Sergentanis 2009; Perfumo et al. 2009). There has been extensive validation of MDM2 as a target, ranging from studies with aptamers and peptides through to antisense approaches and, perhaps most significantly, was described in a path breaking study using a hypomorphic allele of *Mdm2* in the mouse (Mendrysa et al. 2003). In this study, nominal reductions in MDM2 levels were found to be sufficient to trigger a mild p53 response (as shown by increased levels of lymphopenia and apoptosis in intestinal crypts) in response to increased p53 activity. The volume of the thymus is also reduced and there is a small effect on weight gain during development. Gene dosage studies have found levels of MDM2 that selectively inhibit the development of colon carcinoma induced by the absence of adenomatous polyposis coli (*APDAC*) without adverse affects on normal tissues. These powerful studies provided proof of a therapeutic index for MDM2 inhibition that has now been confirmed by the first small molecule candidates, including Nutlin (Vassilev et al. 2004), MI-219 (Shangary and Wang 2009) and reactivation of p53 and induction of tumor cell apoptosis (RITA) also known as NSC 652287 (Hedstrom et al. 2009), which produce tumor regression *in vivo* in human tumor xenografts in nude mice. These initial studies paved way for large scale investigations on different types of MDM2 inhibitors and are discussed below.

3.1 Non-peptidic small-molecule inhibitors of MDM2-p53 interaction

The progress in the design of nonpeptidic, small-molecule inhibitors of the MDM2-p53 interaction (mentioned herein as MDM2 inhibitors; sometimes also called as MDM2i inhibitors) proceeded very slowly for almost a decade after the publication of the crystal

structures. The very first class of bona fide, potent, nonpeptidic, small-molecule MDM2 inhibitors, known as Nutlins, was reported in 2004 (Vassilev 2007; Vassilev 2005; Vassilev 2004a). The Nutlins contain a cis-amidazole core structure and one analogue, Nutlin-3, has potent in vivo antitumor activity in xenograft models of human cancer-retaining wild-type p53. The discovery of the Nutlins provided the important proof-of-concept and fueled enthusiasm for the design and development of small-molecule MDM2 inhibitors. In the last 4 years, several new classes of small-molecule MDM2 inhibitors have been discovered using different approaches (Canner et al. 2009; Shangary et al. 2008a; Fotouhi and Graves 2005). Using a computational structure-based de novo design strategy, a new class of spiro-oxindoles that are potent inhibitors of MDM2 (Ding et al. 2006), as exemplified by MI-63 and MI-219 were designed. In this regard Nutlin-3 a cis-imidazole has been well studied in different cancers. Our MI series of MDM2 inhibitors belong to different class (spiro-oxindole) and have a slightly higher affinity towards MDM2 when compared to Nutlins. Using a structure-based de novo design strategy it was shown that the interaction between p53 and MDM2 is primarily mediated by four key hydrophobic residues (Phe 19, Leu 22, Trp 23 and Leu 26) of p53 and a small but deep hydrophobic cleft in MDM2. Nutlin-3 mimics the interactions of the p53 peptide to a high degree, with one bromophenyl moiety sitting deeply in the Trp pocket, the other bromophenyl group occupying the Leu pocket, and the ethyl ether side chain directed toward the Phe pocket. In essence, the imidazoline scaffold replaces the helical backbone of the peptide and is able to direct, in a fairly rigid fashion, the projection of three groups into the pockets normally occupied by Phe19, Trp23, and Leu26 of p53. However, unlike Nutlin-3 in case of our inhibitors (MI series), computational modeling predicted that MI-219 mimics the four (instead of three in case of Nutlin-3) key binding residues in p53 (Phe-19, Leu-22, Trp-23 and Leu-26) resulting in optimal hydrogen bonding and hydrophobic interactions with MDM2. Both Nutlins and MI-219 enter many types of cultured cells and inhibit the p53–MDM2 interaction with a high degree of specificity, leading to the stabilization of p53 and the activation of the p53 pathway. Proliferating cancer cells that express wild-type p53 are effectively arrested in the G1 and G2 phases of the cell cycle or can undergo apoptosis when treated with micro molar concentrations of Nutlins (Shangary et al. 2008b). This indicates that some cells are more susceptible to Nutlin-induced apoptosis than other cells in which a reversible cell cycle arrest is observed. The key cellular characteristics that underlie this difference in response are the subject of intense investigation. This differential response may occur owing to abnormalities further downstream in the p53 pathway. Other small molecules that have been developed to target the p53–MDM2 interaction include benzodiazepenes (Patel and Player 2008). The benzodiazepene-based derivatives disrupt the MDM2–p53 interaction *in vitro* with IC_{50} values of 0.5–2 μM and have also been shown to suppress the growth of cell lines containing wild-type p53. Administration of the benzodiazepene derivative TDP665759 to normal mice led to an increase in p21 (also known as WAF1 and CIP1) levels in liver samples (Koblish et al. 2006). Finally, TDP665759 synergizes with doxorubicin both in culture and in xenografts of A375 melanoma cells to decrease tumor growth.

With so many targets identified against the MDM2-p53 interaction and supporting preclinical laboratory evidence it is imperative that effective MDM2 inhibitors will become a major form of therapy in the coming years. However, several potential drawbacks to targeting the MDM2–P53 interaction can be envisioned. First, MDM2 is induced by p53 activation as part of an inducible feedback loop that negatively regulates the p53 response.

Therefore, the drugs would induce their target, limiting their potential efficacy. Second, the current molecules fail to effectively target MDM4. The binding pocket of the N terminus of MDM2 has shown itself to be eminently druggable, and a future challenge is whether or not these drugs can proceed to the clinic and whether they can also be refined to target other MDM2 family members such as MDM4. Apart from this avenue of research, other target sites have been identified in this p53 regulatory pathway that show the potential for drug development, and it remains to be seen if they generate therapeutic leads that have low toxicity in normal tissues.

3.2 Compounds that target p53 regulators

Activated p53 is under multiple post-translational control that includes acetylation, methylation, phosphorylation, neddylation and sumoylation (Lee and Gu 2010; Kruse and Gu 2009; Halaby and Yang 2007; Schumacher and Gartner 2006; Chuikov et al. 2004; Haupt 2004; Brooks and Gu 2003; Wiederschain et al. 2001; Somasundaram 2000; Craig et al. 1999). Activating p53 using small may not be sufficient for proper p53 function and combinations with agents that suppress post-translational p53 blockers such as acetylation would benefit the overall outcome of such therapy. An example of this type of agent which was identified through a p53-based phenotypic screen are tenovin-1 and its more water-soluble derivative tenovin-6 (Lain et al. 2008; Brooks and Gu 2008). Tenovins rapidly increase p53 levels in cells treated with low micro molar concentrations, and daily intraperitoneal injection of tenovin-6 at 50 mg per kg delays xenograft tumor growth in mouse models. Through a yeast genetic screen and subsequent enzymatic assays tenovins were shown to inhibit the NAD^+-dependent deacetylase activity of SIRT1 and SIRT2 (Canto and Auwerx 2009; Campisi and Yaswen 2009), two members of the sirtuin family of class III histone deacetylases. p53 deacetylation by SIRT1 impairs p53 stability and transcriptional activity. Therefore, inhibiting the sirtuins should lead to increased p53 stability. Indeed, treatment of MCF-7 cells with tenovins led to the accumulation of acetylated p53 and acetylated tubulin, which are established substrates of SIRT1 and SIRT2, respectively. Further chemical optimization of the potency of the tenovins is now possible owing to the elucidation of SIRT1 and SIRT2 as the cellular targets. The discovery and characterization of the tenovins is an example of how current technological advances in target identification and p53 basic research contribute to the understanding of the mechanism of action of bioactive small molecules.

3.3 Snail-p53 interaction inhibitors

Rapid progress has been made in the development of novel chemicals which can block the K-Ras-mediated p53 suppression through the screening of the chemical library. Using ELISA-based chemical screening for discovering potent chemical inhibitors that are effective to prevent the binding were discovered. Interestingly, one group of the chemical library particularly showed high effects in blocking the interaction of Snail and p53 (Lee et al. 2010b). Since this chemical library was arranged in accordance with its chemical structural similarity, it strongly implies that the similar structural motif of chemicals can impact p53-snail interaction. Among the compounds of this group, B3 and C2 were selected as candidates that could effectively provoke p53 and its target genes, such as p21 and PUMA, in K-Ras mutated cancer cell lines (Lee et al. 2009a). GN25 and GN29 has been revealed to show substantial results that induce p53 and p21 expression, as much as Nutlin-3 (small

molecule inhibitor that specifically blocks p53-MDM2 binding). Strong candidates (GN25 and GN29) can activate the p53 in K-Ras mutation dependent manner (Lee et al. 2010a) (Fig 2). GN chemicals show similar activity on p53 activation only in K-Ras mutated cell. Moreover, GN25 shows no cytotoxic effect in normal or K-Ras wild type cancer cells.

Of importance is the observation that GN chemicals can induce p53 and p21 occurred as strong as in the treatment of DNA damaging agents such as adriamycin or etoposide, and the anti-proliferating effect of GN chemicals was even stronger than Nutlin-3. In addition, in normal fibroblast, any significant differences not only on the p53 expression level, but also on its proliferation and viability was not detectable by GN chemicals, but by Nutlin-3, which reveals that GN chemicals possess the powerful and specific property unique to other chemicals. The extensive duration of the p53 activity response to low concentration of GN25 and GN29 was shown for over 24 hours, which indicate that these chemicals successfully remedy problem of very short re-activation of p53. One crucial fact is that another additional derivative, GN25-1 containing the same side chain but a modified nuclear structure of GN25, has no effect on the induction of p53, which suggest that the conserved nuclear structure of the GN chemical is critical in increasing p53 activity.

In the condition of disrupted Snail, p53 induction could be shown in K-Ras mutated cancer cells, but there is no synergic and additional effect by GN25 and 29, which clearly indicate that p53 induction by GN25 and 29 can occur through interfering Snail-p53 binding. To identify the specificity of these chemicals on Snail-p53 binding, several surrounding factors involved in the p53 function should be checked. Since the middle region of Snail is normally targeted for CK1/GSK3β-dependent phosphorylation (Cano et al. 2000). In addition, although p53 has no influence on β-Catenin phosphorylation by CK1/GSK3β, recombinant p53 sufficiently interferes with CK1/GSK3β-mediated Snail phosphorylation (Yook et al. 2006). Indeed, quercetin (inhibitor of Snail-p53 binding) and GN25 could recover CK1/GSK-3β-mediated Snail phosphorylation even in the presence of p53 (Lee et al. 2009b). In contrast, GN25 did not affect the physical interaction of p53 with other proteins such as WRN making it a p53 specific targeted SMI (Blander et al. 1999).

One of very exciting feature is that GN25 and GN29 block the interaction between Snail with wild type p53 but not with mutant type p53. This is resulted from the property of chemicals, which can bind to wild type p53. Indeed, GN chemical can rescue the only wild type p53 in wild and mutant p53 expressed cells from K-Ras or Snail-mediated p53 suppression. Thus, GN chemical can work in p53 mutated cancer cells, if it possesses single copy of p53. In fact, although GN25 and 29 decrease total p53 expression level in p53 WT/MT cells (no effect on the transcriptional level), it can induce p21 and PUMA and reduce the proliferation and viability of cells. This result was not accomplished by other DNA-damaging agents including Adriamycin or Nutlin-3. By the use of GN chemicals, all of the p53 proteins might be protected from Snail-mediated elimination in p53 WT/WT status. These features of GN chemical can extend the application range from p53 wild type cancer to p53 mutated cancer and from early cancer to late stage cancers.

4. MDM2 inhibitor investigations in PDAC

Testing of MDM2 inhibitors have been restricted in PDAC and this may be due to lack of suitable cell lines (Capan-2 is the only wt-p53 PDAC cell line). To this end our laboratory is

the first to show the growth inhibitory and apoptotic potential of MDM2 inhibitors MI-319, MI-219 and Nutlin-3 in PDAC (Azmi et al. 2010b). These inhibitors specifically induced cell growth inhibition and apoptosis in wt-p53 PDAC cells. Growth inhibition and apoptosis by MDM2 inhibitors was accompanied by increase in levels of p53 along with $p21^{WAF1}$ and the proapoptotic Puma. In these studies immunoprecipitation-western blot analysis revealed reduced association of MDM2-p53 interaction in drug exposed PDAC cells. Further, using wt-p53 xenograft of Capan-2, we found that oral administration of MI-319 at 300 mg/kg for 14 days resulted in significant tumor growth inhibition without any observed toxicity to the animals. No tumor inhibition was found in mut-p53 BxPC-3 xenografts. We also explored combination treatments of these inhibitors with chemotherapeutics such as gemcitabine and cisplatin. Interestingly MI-319, MI-219 combination specifically enhanced cell growth inhibition and apoptosis in PDAC cells with wt-p53 suggesting that this potent combination can be used in the clinic for wt-p53 population in this dreadful disease. We also tested the combination of MI-319 with cisplatin which although less popular for PDAC yet is a potent p53 inducer. Apart from being a potent combination in wt-p53 PDAC tumors most interestingly MDM2 inhibitor-cisplatin combination drastically induced growth inhibition apoptosis and tumor growth inhibition in mut-p53 PDAC cell lines and xenografts respectively (Azmi et al. 2010a). Mechanistically using siRNA silencing it was proven that the p53 family member p73 (which is rarely mutated in PDAC and other cancers) was found to be responsible for the observed anticancer/anti-tumor effects. That the combination worked in a p53 null system ($HCT116^{-/-}$) further reiterated that MI-319-cisplatin combination could effectively induce apoptosis through a p73 dependent mechanism. However, further in-depth mechanistic studies are warranted specifically in PDAC.

The availability of potent and specific MDM2 inhibitors, such as Nutlin-3 and MI-219, has provided the opportunity to examine in detail the molecular mechanism of p53 activation. In competition with a p53-based peptide, Nutlin-3 and MI-219 bind with high affinity to MDM2 (K_i = 36 and 5 nmol/L, respectively). They block the intracellular MDM2-p53 interaction and induce the accumulation of p53 and the activation of the p53 pathway in tumor and normal cells. Conventional genotoxic anticancer agents and radiation also induce the accumulation and activation of p53, but they do so by posttranslational modifications of p53, such as phosphorylation. In contrast, Nutlin-3 induces neither DNA damage nor p53 phosphorylation in cells. Hence, small-molecule MDM2 inhibitors represent a new class of non-genotoxic agents that can reactivate the p53 function. Apart from regulating p53 recent studies have shown p53 independent functions of MDM2. In this regard Zhang and co-workers have extensively reviewed a whole range on targets governed by MDM2 (Zhang and Zhang 2005). Targeting MDM2 by agents such as MI-219 or Nutlin may affect a myriad of other key cellular molecules that play significant role in cell growth and apoptosis. In-depth mechanistic studies on the mode of action of inhibitors on MDM2 and the consequent p53 reactivation are lacking and it was of interest to us to explore the roles of crucial proteins that are involved in the regulation of p53. Activated p53 is known to be influenced by multiple post-translational control processes such as phosphorylation and acetylation that positively regulate p53 function (Stommel and Wahl 2005). Acetylation is an important epigenetic phenomenon in the biology of p53 (Gu et al. 2004; Ito et al. 2002). Upon stress, p53 is acetylated at Lys382 which enhances its DNA binding activity (Hasegawa and Yoshikawa 2008). Moreover, deacetylation of p53 by SIRT1 has been shown to repress p53 mediated cell cycle regulation and apoptosis. SIRT1 is also known to deacetylate another

protein Ku70 which, in turn, interacts with Bax and is responsible for blocking Bax entry into mitochondria. Therefore, we sought to determine whether acetylation of p53 could be influenced by our inhibitors in PDAC cells. Indeed our results showed that MI-219 treatment suppresses SIRT1 protein and simultaneously enhances acetylation of p53. Using state of the art Surface plasmon resonance techniques we studied the binding between MI-319 or Nutlin-3 and Ku70 and our results confirm high affinity association between the two. Interestingly MI-219 treatment resulted in the suppression of Ku70 expression along with disruption of Ku70-Bax interaction. This observation is of great importance because it proves that MDM2 inhibitor not only blocks MDM2 which is its primary target but also suppresses two secondary targets the negative regulator 'SIRT1', which is a molecule that regulates p53 function and Ku70. Although it is too preliminary to confirm the true binding/interaction site of Ku70 or SIRT1 to MI-319, yet is can be speculated that MI-319 or Nutlin-3 may interact with peptide sequence (LSQETFSDLWKLL) similar to p53 transactivation domain towards which both Nutlin-3 or MI series of inhibitors were built.

As MI-219 does not alter MDM2 expression yet Ku70 and SIRT1 are suppressed suggesting that these drugs may have a MDM2 independent role in the biology of cells. However, compelling evidence in literature supports to a MDM2 dependent mechanism of action of these drugs on Ku70 and SIRT1. Our cell free FRET based SIRT1 activity assay showed inhibition of SIRT1 activity by MDM2 inhibitors. Yet in a cellular system the dynamics of SIRT1 is complex. Studies so far suggest that only wt-p53 can inhibit SIRT1 while cells that have lost or have mutations in p53 have over expressed SIRT1 and cannot repress it. This certainly points out that the suppression of SIRT1 in our system is p53 dependent however elucidation of the exact mechanism of action requires further work. As far as Ku70 is concerned, very recently Nutlin, a drug with similar mode of action as MI-219 was shown to disrupt MDM2-Ku70 interaction. Based on our results and those of others we propose multiple mode of action MI-219 on SIRT1 and Ku70. MDM2 inhibitors down regulate SIRT1 that in principle may prevent Ku70 and p53 deacetylation. Surface plasmon resonance and Co-IP results confirm that MI drugs directly bind to Ku70 as well as disrupt Ku70-Bax interaction. Although yet to be proved, it is suggested that such a direct binding may induce conformational changes in Ku70 rendering it ineffective in binding to Bax and therefore allowing the latter to induce apoptosis. MI drugs also directly suppress Ku70 mRNA and protein expression which in turn allows p53 induced free Bax to mediate apoptotic events.

Additionally, we have utilized a systems biology and network modeling approach to investigate in mechanistic detail the mechanism of action of MDM2 inhibitor and its oxaliplatin combination in PDAC (Azmi et al. 2010d; Azmi et al. 2011). Microarray profiling of a wt-p53–containing PDAC cell line (Capan-2) treated with either MI-219, oxaliplatin, or their combination, revealed some very interesting results that may have clinical implications. Global analysis of genes showed that MI-219 treatment resulted in the alteration of only 48 genes, which highlights the targeted nature of MDM2 inhibitor MI-219. On the other hand, oxaliplatin is a cytotoxic agent and caused alteration of 761 genes. The combination of MI-219 with oxaliplatin resulted in 767 genes being altered. The most important aspect of this finding is the emergence of 286 synergy-specific unique genes that were not found in the MI-219 alone or in the oxaliplatin-treated group. This finding confirms that the synergy between MI-219 and oxaliplatin is at the gene level. Principle component analysis showed that the global gene signatures between single treatments

versus combination treatments were non-overlapping and could be differentiated at different time points. Molecular network modeling of a total of 767 gene-associated pathways revealed a total of 22 statistically enriched functional groups that were linked to biologically distinct functional pathways. Interestingly, network modeling of the 286 synergy-unique genes showed statistical enrichment of 14 disease (cancer) relevant pathways. This finding suggests that these pathways are relevant to cancer, further indicating that the combination synergy between MI-219 and oxaliplatin is at the gene level, comprising distinct biologically meaningful processes. Further analysis of the combination treatment network revealed the presence of several local networks, or hubs, rather than a single hub of activity interconnecting MDM2-p53. Central players such as the CREB binding protein (CREBBP; i.e., ubiquitously expressed gene) that is involved in the transcriptional coactivation of many different transcription factors, including p53, collaborates/cooperates with ARF (CARF) that is responsible for p53 stability, and NF-κB and early growth response protein (EGR1) tumor suppressor module, all of which are known to positively affect p53 reactivation, which in principle would drive cells toward increased apoptosis. Most importantly, these observed gene changes could also be validated at the mRNA and protein level.

Other investigators have also performed expression signature analysis of Nutlin-3. For example gene expression profiling of Nutlin-3 has been done by Zauli and group in B-cell lymphocytic leukemia (B-CLL) (Zauli et al. 2009). In their study B-CLL patient samples were exposed to Nultin-3 and cDNA expression profiling was performed. With the exception of a few cases, the authors noted induction of a characteristic gene expression profile (GEP) signature that was similar in the majority of B-CLL patient samples. Most significantly, lack of characteristic signatures correlated with poor response to Nutlin-3. However, partial lack of response in these wt-p53 B-CLL samples was not due to defects in the ability of Nutlin-3 to promote p53 induction, but suggested the involvement of secondary masking mechanisms. Nutlin-3 gene signatures were all related to the p53 network and included downstream affector genes such as Fas and Bax and activation of auto-regulatory MDM2. Such type of biological analysis, if coupled with network modeling may provide further information on the entire set of genes modulated by Nutlin-3 in B-CLL patients. Results of these studies will significantly aid in the design of clinically successful drug combinations for other malignancies, which will ultimately benefit the overall survival of patients irrespective of the mutational and functional status of p53. In another study, a large-scale RNA interference–based short hairpin RNA (shRNA) barcode screen was applied to gain insight in the mechanism of action of Nutlin-3 (Brummelkamp et al. 2006). In this study it was shown that aside from p53, 53BP1 was critical mediator of Nutlin-3–induced cytotoxicity. 53BP1 is part of a signaling network induced by DNA damage that is frequently activated in cancer but not in healthy tissues (DiTullio, Jr. et al. 2002). These results suggest that tumor specificity of Nutlin-3 may result from its ability to turn a cancer cell–specific property (activated DNA damage signaling) into a weakness that can be exploited therapeutically.

5. Current status of MDM2 Inhibitors in the clinic

Although proven to be successful in the laboratory in multiple cancer models, MDM2 inhibitors or approaches that utilize reactivation of p53 have a long way to go before they

are acceptable in the clinic for PDAC. Currently some SMIs that reactivate the mut-p53 through protein conformational changes are currently in Phase I clinical trials (Brown et al. 2009). In addition to the potential clinical applications, discovery of the first MDM2 antagonist, Nutlin-3a provided the initial proof of concept that inhibition of protein-protein interactions was a feasible approach to pharmaceutical design. Since that time, a deluge of patents and reports have disclosed a high number of diverse molecules showing potency and selectivity toward MDM2 at the same time lacking in solubility. Careful attention to medical chemistry was employed to improve bioavailability of these scaffolds leading to compounds with optimized PK properties. The intense labor of research has finally begun to bear fruit as demonstrated by the advancement of JNJ-26854165 and RG7112, into early phase clinical trials. Preliminary data from trials of both compounds suggest potential for advancement to Phase II trials and beyond. A few examples are PRIMA (Phase I APR-246), CP-31398 (Phase I) and PhiKan-08 (Phase I). SMIs that activate p53 through disruption of MDM2-p53 binding such as MI-219, Nutlin-3 are in phase I. RITA a p53 binding targeted agent, tenovin (SIRT1 inhibitor are still in a pre-clinical testing phase. Leptomycin B (a CRM1 (Exportin 1) binding agent that mediates p53 reactivation) is in Phase I while Actinomycin D (an RPL11 and RPL5 (Ribosomal protein L) releasing agent) has been approved for Phase I (Choong et al. 2009). Certain combinations such as nutlin with mitotic inhibitors for example BI-2536 (PLK1 (Polo-Like Kinase) inhibitor) or with VX680 (Aurora kinase inhibitor) are also in Phase I.

6. Conclusion

In PDAC, multiple de-regulated signaling especially the MDM2 over-expression and hyper activated K-ras driven pathways hone in on p53 and suppress its proper function in controlling various cellular processes. Studies indicate that these suppressive mechanisms render p53 re-activating genotoxic therapies ineffective. The well-studied role of p53 in coordinating cellular response to stress, aberrant growth signals and genomic instability has established a solid rationale for the targeting of MDM2 and K-ras driven snail to restore therapeutic response to treatment of PDAC. The entry of orally-administered MDM2 antagonists into clinical trials represents a significant advancement for the field of small molecule drug discovery in PDAC.

7. Acknowledgment

The authors acknowledge the grant support from National Cancer Institute (NIH) R01CA109389 to R.M. Mohammad)

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20

Toll-Like Receptors as Novel Therapeutic Targets for the Treatment of Pancreatic Cancer

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1. Introduction

Toll-Like Receptors (TLRs) are critical mediators of the innate immune response and subsequent activation of adaptive immune responses to pathogens that invade the body. TLRs on immune cells are the basis of our multigenic, innate immune, inflammatory response to pathogenic signature molecules that cause tissue damage. Functional TLRs are expressed not only on immune cells, but also on non-immune cells including cancer cells. TLRs are critical mediators of cellular transformation, tumor progression, and metastasis.

2. Toll-Like Receptors

TLRs are pattern recognition receptors that were originally identified in immune cells and described to recognize pathogen-associated molecular patterns (PAMPs) and initiate an innate immune response and subsequent adaptive immune responses against infection or tissue damage. They protect mammals from pathogenic organisms, such as viruses, by generating an "innate immune" response to products of the pathogenic organism (Takeda et al., 2003). This response results in increases in genes for several inflammatory cytokines and chemokines, major histocompatibility (MHC) genes I and II, as well as co-stimulatory molecules, and is critical for the development of antigen-specific adaptive immunity (Takeda et al., 2003). In addition to their presence on immune cells, TLRs are also found to be expressed normally on multiple non-immune cell types including, but not limited to epithelial cells that line the digestive system, lungs, and female reproductive tract, as well as on pancreatic beta cells, and keratinocytes in the skin where they function to regulate cellular proliferation and apoptosis in response to infection and/or other damaging environmental insults (radiation, chemicals, etc.)(Andonegui et al., 2003; Ortega-Cava et al., 2003; Giarratana et al., 2004; Schroder & Maurer, 2007; Nasu & Narahara, 2010; Yamasaki et al., 2010; Ayari et al., 2011). Regardless of cell type, TLRs recognize not only exogenous PAMPs, but also endogenous damage-associated molecular patterns (DAMPs) (Table 1). Eleven TLRs have been described in humans to date (TLR1 to TLR11) (Sato et al., 2009).

TLRs are located both on the cell surface and in the cytoplasm, are differentially expressed in different cell types under a variety of conditions and disease states, and recognize different PAMPs and DAMPs (Table 1).

Perhaps two of the most well studied TLRs are TLR3 and TLR4. In non-immune cells, as is the case for immune cells, dsRNA can activate two distinct anti-viral response pathways by activating TLR3 signaling. One, coupled via the adapter molecule, Toll-IL-1 receptor (TIR) domain-containing adapter inducing IFN-β (TRIF) (also called TIR domain-containing adaptor molecule-1 [TICAM-1]), i.e. (TRIF/TICAM)-1, activates IFN regulatory factor (IRF)-3 and the production of type 1 interferons (IFN-α or IFN-β) via TRAF-3 (Oganesyan et al., 2006; Schneider et al., 2006). The type 1 IFNs, acting as autocrine/paracrine ligands increase STAT-1 and IRF-1 activation, critical factors in expression of chemokines such as CXCL10 and genes such as VCAM-1. Specifically, TLR3 is expressed in the endosomal membrane and recognizes extracellular viral dsRNA and/or its synthetic analog poly I:C. Upon binding to dsRNA, TLR3 becomes dimerized, and two specific tyrosine residues (Tyr^{759} and Tyr^{858}) in the TIR domain of TLR3 become phosphorylated and are essential for dsRNA-induced recruitment of the adaptor protein TRIF/TICAM-1 (Sarkar et al., 2004). Phosphatidylinositol 3-kinase (PI3K) is then recruited to the two phosphorylated tyrosine residues and is required for phosphorylation and activation of IRF-3. Additionally, it has been shown that TLR3 associates with c-Src in response to dsRNA and that c-Src is necessary for PI3K-dependent activation of IRF-3, although the precise role of c-Src in this process is currently not well understood (Johnsen et al., 2006). TRIF/TICAM-1 dissociates from TLR3 and forms a complex with receptor interacting protein 1 (RIP1), TRAF-3 and NF-κB activating kinase (NAK)-associated protein 1 (NAP1). The TRIF/TICAM-1/TRAF-3/RIP-1/NAP-1 complex participates in the recruitment and activation of TBK-1 and IKKε which phosphorylate and activate IRF-3 (Sasai et al., 2005; Hacker et al., 2006; Oganesyan et al., 2006). Once phosphorylated IRF-3 translocates into the nucleus and together with nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and AP-1 induces IFN-β gene transcription (Sato et al., 2000). The second pathway is coupled to a different site on TRIF/TICAM-1 via TRAF-6 and activates NF-κB and MAP Kinase pathways important in the production of pro-inflammatory and inflammatory cytokines, e.g. TNF-α, IL-1β, and IL-6, as well as chemokines e.g. MCP-1.

TLR4 signaling has both a MyD88-dependent and MyD88–independent mechanism when activated [reviewed in (Lu et al., 2008)]. There is significant homology between TLR3 and TLR4 IRF-3/IFN signaling (the MyD88-independent TLR4 pathway); TRIF/TICAM-1 is a common signaling intermediate in both signaling pathways. As is the case with dsRNA-induced TLR3 signaling, activation of TLR4 signaling leads to TRIF/TICAM-1 recruiting TRAF-3 and RIP-1 which in turn leads to the recruitment and activation of TBK-1 and IKKε (Hacker et al., 2006; Oganesyan et al., 2006; Guo & Cheng, 2007). The TRIF/TICAM-1/TRAF-3/RIP-1/TBK-1/IKKε complex phosphorylates and activates IRF-3 (Fitzgerald et al., 2003; Hemmi et al., 2004). Once phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription (Sato et al., 2000). The MyD88-dependent pathway activates IRAK-4, IRAK-1, TRAF-6, and others to lead to the activation of transcription factors NF-κB, AP-1, and IRF-5, which induce the expression of pro-inflammatory cytokines.

Toll-Like Receptor	**Predominant Cellular Localization**	**PAMPs/DAMPs/Other Ligands**	**Disease Associations**
TLR1	Plasma Membrane	triacyl lipopeptides, modulin (phenol-solube)	cancer, psoriasis, sepsis, leprosy
TLR2	Plasma Membrane	glycolipids, triacyl lipopeptides, heat shock proteins, high mobility group box 1 protein, rare LPS species (*P. gingivalis*), lipopeptides, lipoteichoic acid, measles haemagglutinin, mannuronic acids, neisseria porins, peptidoglycan, zymosan (Beta-glucan), bacterial fimbriae, *Yersinia* virulence factors, CMV virions, saturated fatty acids	cancer, type 2 diabetes, psoriasis, pre-eclampsia, alzheimer's, herpes simplex encephalitis, rheumatoid arthritis, acne vulgaris, acute rheumatic fever, asthma, atherosclerosis, chronic obstructive pulmonary disease, diabetic nephropathy, subhorrheic dermatitis
TLR3	Endosomal Membrane	dsRNA (self and viral), poly I:C	cancer, type 1 diabetes, herpes simplex encephalitis, Hashimoto's thyroiditis, virus associated autoimmune disease
TLR4	Plasma Membrane	lipopolysaccherides, saturated free fatty acids, fibrinogen, fibronectin, heat shock proteins, flavolipins, *S. pneumoniae* pneumolysin, heparan sulfate, hyaluronic acid, high mobility group box 1 protein, MMTV envelope proteins, nickel, paclitaxel, RSV fusion protein, respiratory syncytial virus coat protein, mannuronic acid polymers, teichuronic acids, bacterial fimbriae, surfactant protein A, β-defensin 2	cancer, type 2 diabetes, toxic shock, ulcerative colitis, atherosclerosis, pre-eclampsia, alzheimer's, stroke, rheumatoid arthritis, chronic obstructive pulmonary disorder, Crohn's disease, periodontitis, peripheral arterial disease,

Toll-Like Receptor	Predominant Cellular Localization	PAMPs/DAMPs/Other Ligands	Disease Associations
TLR5	Plasma Membrane	flagellin	cancer, psoriasis, systemic lupus erthyematosus
TLR6	Plasma Membrane	diacyl lipopeptides, bacterial cell wall components, modulin (phenol-soluble)	cancer, leprosy (Hansen's disease), filarisis, asthma, hypersensitivity pneumonitis
TLR7	Endosomal Membrane	self ssRNA, ssRNA, bropirimine, loxoribine, imidazoquinoline	cancer, systemic lupus erythematosus, rheumatoid arthritis, arthritis
TLR8	Endosomal Membrane	self ssRNA, small synthetic compounds, imidazoquinoline	cancer, rheumatoid arthritis, viral keratitis, crimean-congohemorrhagic fever
TLR9	Endosomal Membrane	self DNA, unmethylated CpG DNA	allergies, asthma, cancer, multiple sclerosis, systemic lupus erythematosus, Graves' ophthalmopathy, crimean-congohemorrhagic fever, arthritis
TLR10	Plasma Membrane	Unknown	cancer, asthma, nephropathy, viral keratitis

Table 1. Toll-Like Receptors; Cellular Localization, Agonists, and Disease Associations

3. TLRs, chronic inflammation, and cancer

Overwhelming evidence suggests that chronic inflammation is crucial to the onset and progression of a multiplicity of human cancers, including pancreatic cancer (Kuper et al., 2000; Garcia et al., 2004; von Hafe et al., 2004; Berstein, 2005; Otake et al., 2005; Lu et al., 2006). The exact link between chronic inflammation and carcinogenesis is unclear, however many studies have shown that pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, etc.) are important for the proliferation, survival, metastasis, and escape from immune surveillance of many of these cancers (Garcea et al., 2005; Lu et al., 2006), and have identified nuclear factor-κB (NF-κB) as a key modulator of inflammation-induced carcinogenesis (Chen et al., 1995; Guttridge et al., 1999; Hinz et al., 1999; Perkins, 2000; Coussens & Werb, 2002; Esposito

et al., 2002; Ghosh & Karin, 2002; Karin et al., 2002; Karin & Lin, 2002; Li & Verma, 2002; O'Hanlon et al., 2002; Alexiou et al., 2003; Chen et al., 2003; Furbert-Harris et al., 2003; Lin & Karin, 2003; Greten et al., 2004; Huber et al., 2004; Luo et al., 2004; Niu et al., 2004; Pikarsky et al., 2004; Stoffel et al., 2004; Heyninck & Beyaert, 2005; Preciado et al., 2005; Yang et al., 2005a). Chronic inflammation as a result of disease, microbial infection, and/or obesity is an important risk factor for the development of a variety of cancers (Khatami; Kuper et al., 2000; Calle & Kaaks, 2004; Khatami, 2011) (Table 2). Chronic inflammation is thought to induce malignant transformation via activation of oncogenes, induction of immunosuppression, and inhibition of tumor suppressors.

It is now recognized that TLRs are important in development of carcinogenesis and tumor progression. The chronic inflammatory state observed in autoimmune disease, microbial infection, and obesity is mediated via activation of TLR signaling on both immune and non-immune cells. TLRs on immune cells recognize PAMPs and DAMPs and initiate an innate immune response and subsequent adaptive immune responses against infection or tissue damage. As part of this acute, innate immune response cytokines and chemokines are produced and released by the immune cells, which will subsequently upregulate TLR expression on non-immune cells. When this occurs, the TLRs on the non-immune cells can become stimulated by the same PAMPs and DAMPs that activated the TLRs on the immune cells, leading to very high levels of disease-causing inflammatory proteins. In a prolonged state of infection and/or tissue damage, sustained high levels of inflammatory proteins can lead to autoimmune, inflammatory diseases, and cancer in individuals with certain genetic and/or environmental susceptibilities (Table 1).

Multiple TLRs have been implicated in a variety of cancers including pancreatic cancer, melanoma, breast cancer, prostate cancer, colorectal cancer, lung cancer, cervical cancer, liver cancer, etc. (McCall et al., 2007; Sato et al., 2009; Schwartz et al., 2009) (Table 3). Activation of TLRs on cancer cells promotes chronic inflammation which stimulates cancer cell proliferation, migration, tumor angiogenesis, and creates a tumor microenvironment which impairs the anti-tumor function of the immune system allowing tumors to develop and survive.

4. Mechanisms of TLR regulation of carcinogenesis

4.1 Activation of TLR signaling leads to the production of cytokines that control growth

As previously described, chronic TLR activation and signaling in both immune and non-immune cells by environmental antigens are now linked to oncogenesis, tumor growth, and invasive spread (Schmausser et al., 2005; Kelly et al., 2006; Fukata et al., 2007; He et al., 2007; Ilvesaro et al., 2007; Goto et al., 2008; Kim et al., 2008; Yoneda et al., 2008; Curtin et al., 2009; Xie et al., 2009; Zhou et al., 2009). Activation of TLR signaling results in the activation of transcription factors NF-κB and AP-1, as well as Type I Interferon (IFN) signaling pathways with subsequent production of "oncogenic" cytokines, and the activation of MAPK and AKT signaling pathways (Figure 1). Multiple TLR-induced cytokines including TNF-α, IL-1, IL-6, IL-8, IL-10, IL-23, etc. have been linked to oncogenesis. TLR activation also upregulates many growth factors such as TGF-β, VEGF, CXCR4, and adhesion molecules such as ICAM-1 (Kelly et al., 2006; He et al., 2007; Ren et al., 2007; Zhou et al., 2009). These TLR-mediated processes have been linked to various cancers including colon, pancreas, melanoma, breast,

Type of Cancer	**Disease/Infection Association**
Thyroid Cancer	Hashimoto's Thyroiditis
	Obesity
Colorectal Cancer	Inflammatory Bowel Disease
	Colitis
	Crohn's Disease
	Obesity
Cervical Cancer	Human Papilloma Virus
Liver Cancer	Hepatitis Virus B and C
Pancreatic Cancer	*Helobacter pylori*
	Obesity
Prostate Cancer	Obesity
Hematologic Malignancies	Epstein-Barr Virus
	Cytomegalovirus
Esophageal Adenocarcinoma	Obesity
Renal Cancer	Obesity
Endometrial Cancer	Obesity
Gallbladder Cancer	Obesity
Breast Cancer	Obesity
Gastric Cancer	Epstein-Barr Virus
	Helobacter pylori
	Obesity
Non-Hodgkin's Lymphoma	Epstein-Barr Virus
	Human Herpes Virus 8
	Human Immunodeficiency Virus
Hodgkin's Disease	Epstein-Barr Virus
Nasopharyngeal Carcinoma	Epstein-Barr Virus
Burkitt's Lymphoma	Epstein-Barr Virus
Vulvar Cancer	Human Papilloma Virus
Anus Cancer	Human Papilloma Virus

Type of Cancer	Disease/Infection Association
Penis Cancer	Human Papilloma Virus
Head and Neck Cancer	Human Papilloma Virus
Kaposi's Sarcoma	Human Herpes Virus 8
	Human Immunodeficiency Virus
Casteleman's Disease	Human Herpes Virus 8
Adult T-cell Leukaemia	Human Thymus-Derived-Cell Leukaemia/Lymphoma Virus-1
Bladder Cancer	Schistosomes (*S. haematobium*)

Table 2. Associations Between Disease, Infection, Obesity, and Cancer.

prostate and many others (Sato et al., 2009). In addition, these cytokines also activate transcription factors that induce the expression of several tumor promoting and anti-apoptotic genes which will be discussed in Section 4.2 below.

Toll-Like Receptor	Type of Cancer
TLR1	Colon, Prostate
TLR2	Brain, Breast, Colorectal, Gastric, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian
TLR3	Breast, Colorectal, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian, Pancreatic
TLR4	Bladder, Brain, Breast, Cervical, Colorectal, Gastric, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian, Pancreatic, Prostate
TLR5	Cervical, Colorectal, Gastric, Ovarian
TLR6	Hepatocellular Carcinoma, Prostate
TLR7	Chronic Lymphocytic Leukemia, Lung
TLR8	Lung
TLR9	Breast, Cervical, Colorectal, Gastric, Glioma, Hepatocellular Carcinoma, Lung, Pancreatic, Prostate
TLR10	Nasopharyngeal, Prostate
TLR11	None

Table 3. TLRs are Associated with Human Cancers

These immune-response and tumor-associated cytokines have complex and often contradictory effects depending on the specific tumor, the specific TLRs activated, and the innate immune response to the malignancy. Indeed, the tumor microenvironment which includes tumor cells, tumor-derived fibroblasts, as, well as macrophages, T cells, and APCs each produce inflammatory cytokines forming a "milieu" which on one hand facilitates the differentiation and expansion of tumors and on the other tries to suppress this process.

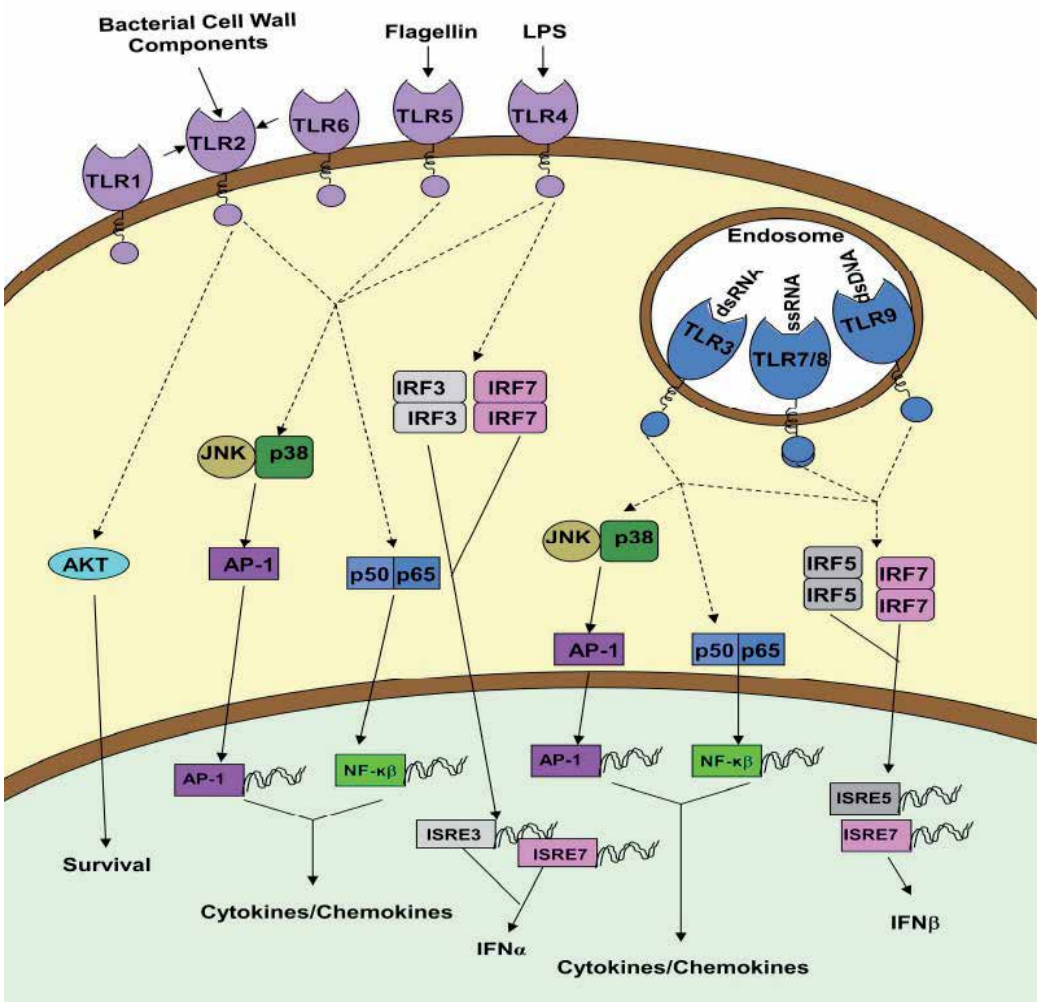

Fig. 1. TLR Signaling

The most important TLR-mediated cytokines involved in initiation and growth of malignant tumors appear to be tumor-derived IL-1, TNF-α and IL-6. TNF-α and IL-6 are potent activators of NF-κB pathways and JAK-STAT signaling pathways which also directly contribute to tumor induction and growth (See Section 4.2). TNF-α and IL-6 also induce anti-aptotic genes such as Mcl-1, Bcl-x, cell-cycle regulators (cyclins D1/D2, c-Myc), and inducers of angiogenesis (VEGF) (Calo et al., 2003). TNF-α in the tumor microenvironment also increases myeloid cell recruitment via IL-17. Importantly, IL-6 is one of the most potent activators of the oncogenic transcription factor STAT3 (Calo et al., 2003).

TLR mediated IL-1 expression within tumors directly stimulates tumor growth as well as stimulating angiogenesis which enhances a tumor's capacity to spread or metastasize (Gemma et al., 2001; Elaraj et al., 2011). Expression of IL-1 within tumors, the surrounding tissues, or endothelial cells is associated with aggressive growth characteristics (Elaraj et al., 2006; Sawai et al., 2006).

In contrast, IL-10 is normally an inhibitory cytokine which blocks NF-κB activity and the JAK-STAT signaling pathway but has been shown to help certain tumors escape normal immune surveillance (Linehan & Goedegebuure, 2005; Perrone et al., 2008; Strauss et al., 2009). IL-10 can induce CD4+CD25+Foxp3+ regulatory T cells (Tregs) in the tumor microenvironment which secrete additional IL-10 and TGFβ, which can then suppress the anti-tumor function of non-Treg T cells (Linehan & Goedegebuure, 2005; Perrone et al., 2008; Strauss et al., 2009).

4.2 TLR signaling activates transcription factors important for tumorigenesis

Two of the most notorious and well-studied oncogenic transcription factors are nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3). Both NF-κB and STAT3 are activated by a variety of stimuli (stressors, cytokines, etc.), and while they are regulated by entirely different signaling mechanisms, they both control the expression of proliferation-enhancing, anti-apoptotic, angiogenic, and immune-modulating genes. NF-kB and STAT3 also interact and mediate crosstalk between tumor cells and inflammatory cells within the tumor microenvironment to promote the development and progression of multiple types of human cancers including but not limited to pancreatic, colon, gastric, skin, head and neck, and liver cancers (Grivennikov & Karin; Lin et al.; Bromberg et al., 1999; Greten et al., 2004; Yu & Jove, 2004; Yu et al., 2009).

NF-κB, which is directly activated via the MyD88-dependent branch of TLR signaling, is one of the most studied transcription factors and arguably the most important for tumor promotion (Chaturvedi et al., 2011). NF-κB is constitutively activated in most cancers typically due to stimulation of TLRs, pro-inflammatory cytokine receptors (such as TNF-α and IL-1), and antigen receptors (Dinarello, 1994; Kruglov et al., 2008; Bezbradica & Medzhitov, 2009; Karin & Gallagher, 2009). This constitutive activation of NF-κB has been linked to inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance and radioresistance (Beg & Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996; Huang & Miyamoto, 2001; Joyce et al., 2001; Luo et al., 2004; Mantovani et al., 2008; Chaturvedi et al., 2011). Activation of NF-κB results in the upregulation of several genes and cytokines which are associated with cell growth and proliferation. These include critical cellular progression genes and anti-apoptotic genes including specific inhibitor of caspase 8, c-FLIP29, the caspase inhibitors cIAP1 & cIAP2, the anti-apoptotic member of the B-cell leukaemia/lymphoma 2 (Bcl2) family, Bcl-XL BCL2 and BCL-XL, c-myc, c-myb. Cyclin D1/2 stimulation by NF-kB activation has also been shown to stimulate cell-cycle progression. Many of these together upregulate JNK activity and subsequent activation of the AP-1 transcription factor which also enhances cell survival (Karin, 2006). Inhibitors of NF-κB have been shown to decrease tumor cell proliferation and aid in the potency of chemotherapeutics (Luo et al., 2004; Chaturvedi et al., 2011).

In non-stimulated cells, STAT3 is kept in an inactive form in the cytoplasm (Darnell et al., 1994; Yu & Jove, 2004). Upon stimulation STAT3 is phosphorylated at two critical residues; tyrosine 705 (Tyr^{705}) and serine 727 (Ser^{727}) (Wen et al., 1995; Barboza et al., 2004; Gartsbein et al., 2006; Yeh et al., 2006), dimerizes (Yoshimura et al., 2007), and translocates to the nucleus where it activates a wide array of genes critical for tumor development and

progression. STAT3 is activated by many cytokines, including those cytokines that are products of the TLR signaling pathway, including cytokines of the IL-6 family that signal through gp130 (IL-6, IL-11, IL-27, etc.), the IL-10 family (IL-10, IL-22, IL-19, IL-20), and the epidermal growth factor (EGF) family (VEGF, IL-21, IL-23, HGF). Like NF-κB, STAT3 also activates anti-apoptotic genes such as Bcl-xL, Bcl-2, and c-IAP2 (Yu & Jove, 2004; Chang et al., 2006; Rebouissou et al., 2009), cell cycle and proliferation genes such as Cyclin D1 and c-Myc (Levy & Darnell, 2002; Naugler & Karin, 2008; Bollrath et al., 2009), and members of the AP-1 family such as c-Jun and c-Fos (Hirano et al., 2000; Yang et al., 2005b; Yang et al., 2007), whereas others such as Mcl-1 and Survivin are STAT-3-dependent (Yu & Jove, 2004). Thus, STAT3 is an attractive target for anti-cancer therapy and numerous strategies have been employed to inhibit constitutive STAT3 signaling in cancer cells (Meydan et al., 1996; Turkson et al., 2001; Coleman et al., 2005; Song et al., 2005; Duan et al., 2006; Schust et al., 2006; Iwamaru et al., 2007; Siddiquee et al., 2007; Goel et al., 2008; Hatcher et al., 2008; Lin et al., 2010a; Lin et al., 2010b).

Activator Protein 1 (AP-1) transcriptional complexes also play a pivitol role in malignant cellular transformation (Lopez-Bergami et al., 2009). AP-1 can be activated through the TLR Myd88-dependent pathway by a variety of growth factors and cytokines (Akira, 2006). Increased exposure to environmental carcinogens such as tobacco, nicotine and asbestos have been shown to increase AP-1 activity and correlate with tumorgenesis, tumor invasion and metastasis (Lopez-Bergami et al., 2009). AP-1 can induce the activation of well-known oncogenes including FOS and Jun which are observed in many cancers (Lopez-Bergami et al., 2009). The expression of Jun can further activate other oncogenes including Ras, BRAF and EGFR. Increased FOS expression has been associated with poor prognosis and progression (Lopez-Bergami et al., 2009). Inhibition of AP-1 complexes as well as Jun and FOS have been shown to inhibit tumor formation suggesting that AP-1 may be a viable target for therapeutic intervention (Lopez-Bergami et al., 2009).

4.3 Activation of TLR signaling leads to the production of cytokines and upregulation of other proteins that control metastasis

An important effect of inflammation on cancer is the ability to stimulate tumor invasiveness and metastasis. This ability depends in part, on activation of TLRs on immune cells and cancer cells. The pro-inflammatory microenvironment contributes to shaping the gene expression profile that is required for metastatic behavior of cancer cells.

4.3.1 NF-κB

TLR activation of MyD88, TRAF6, and NF-κB in inflammatory cells and tumor cells within the tumor microenvironment play a key role not only in cancer development but also in tumor progression (Gohda et al., 2004; Greten et al., 2004; Pikarsky et al., 2004; Kaisho & Akira, 2006; Inoue et al., 2007). A correlation was found between the amount of tumor associated macrophages (TAMs) present in the tumor and prognosis; the higher the density of TAMs, the poorer the prognosis (Duncan et al., 1998). TLR-mediated pro-inflammatory cytokine production from tumor associated macrophages (TAMs) play a key role in tumor progression and metastasis (Gohda et al., 2004; Inoue et al., 2007). For example, activation of the TLR-TRAF6- NF-κB pathway in tumor associated inflammatory cells, and the

subsequent release of pro-inflammatory cytokines from those cells has been shown to result in the activation of NF-κB in precancerous cells which drives the growth and malignant transformation of those cells (Gohda et al., 2004; Inoue et al., 2007). In addition, TLR-mediated activation of NF-κB is necessary for the differentiation and maturation of osteoclasts, which drive the resorption of bone and generate a microenvironment ideal for tumor cells to proliferate and colonize, thus enhancing bone metastasis of certain types of cancer cells, particularly breast cancer cells (Coleman & Rubens, 1987; Sasaki et al., 1995; Lomaga et al., 1999; Naito et al., 1999; Kobayashi et al., 2001). Several genes regulated by NF-κB encode adhesion molecules, MMPs, serine proteases, heparanase, and chemokines that have been shown to be important for tumor invasion and metastasis (Karin & Greten, 2005). Blockade of NF-κB activation reduces proliferation, metastatic ability, and enhances apoptosis of cancer cells (Nakanishi & Toi, 2005; Inoue et al., 2007).

4.3.2 TLR4

TLR4 signaling has been directly implicated in the regulation of cancer cell metastasis. Activation of TLR4 signaling in cancer cells by lipopolysaccharide (LPS), an exogenous ligand for TLR4, induces synthesis of IL-6, inducible nitric oxide synthase (iNOS), and IL12 p40. This effect on cancer cells is similar to what is observed during the activation of macrophages. Blockade of the TLR4 pathway has been found to delay tumor growth and prolong survival in mice (Huang et al., 2005a). Recently it was demonstrated that LPS increases the invasiveness of pancreatic cancer cells and that increases in invasiveness by LPS was hampered by blocking the NF-κB signaling pathway. More specifically, the LPS-dependent invasiveness was decreased by blocking the TLR4/MyD88/NF-κB signaling pathway, once again connecting TLR-mediated inflammation with cancer invasion and progression (Ikebe et al., 2009). In fact, a clinical study of 30 cases of pancreatic ductal adenocarcinoma found that TLR4, NF-κB and hypoxia-inducible factor 1-alpha (HIF1α) were over-expressed compared to surrounding tissues. Survival of patients with an absence of TLR4 expression in tumor tissues was significantly longer than those with TLR4 expression (Zhang et al.).

4.3.3 Wnt5a

Wnt proteins are a family of secreted glyocoproteins involved in critical cellular processes during embryogenesis. Wnt5a is a member of the Wnt family that has been implicated in carcinogenesis and inflammation. Non-canonical Wnt5a activates B-catenin-independent pathways important for cell migration and polarity.

Wnt5a has been implicated in pancreatic cancer for many years. A decade ago the gene expression of Wnt5a signaling members were found in tissue samples of pancreatic adenocarcinomas (Crnogorac-Jurcevic et al., 2001). Wnt5a expression was found to be gradually increased in pancreatic intraepithelial lesions and highly expressed in advanced pancreatic cancer (Ripka et al., 2007). Despite these associations, the role of Wnt5a in cancer remains controversial as some studies show that Wnt5a may work as a tumor suppressor while others show an oncogenic effect (McDonald & Silver, 2009). Although the exact role of Wnt5a in cancer remains unclear, the expression of Wnt5a in tumor samples has been correlated with advanced stages and poor prognosis in gastric, colon, prostate, lung, and malignant melanoma (Iozzo et al., 1995; Lejeune et al., 1995; Saitoh et al., 2002; Weeraratna et al., 2002; Huang et al., 2005b).

In vitro studies have shown that Wnt5a induces cell migration, proliferation and invasion in a variety of cancer cell lines (Weeraratna et al., 2002; Kurayoshi et al., 2006; McDonald & Silver, 2009), lending support to the hypothesis that Wnt5a may be involved in invasion or metastasis of several different cancers. At present the precise mechanisms linking Wnt5a with cancer invasion and metastatis are still largely unknown, however recent studies have hinted at possible molecular mechanisms. The Ca2+ signaling and subsequent protein kinase C (PKC) activation was suggested as the mechanism for enhanced motility and invasiveness for malignant melanoma (Weeraratna et al., 2002). In addition, a recent study associated polarized cell migration with the Wnt5a-ROR2 signaling pathway (Nishita et al.). Wnt5a involvement during EMT in pancreatic cancer was also suggested (Ripka et al., 2007). Wnt5a was described as a target of the homeobox transcription factor CULT1, enhancing migration, proliferation and invasiveness during pancreatic tumorigenensis (Ripka et al., 2007).

An IL-6 / STAT3 / Wnt5a signaling loop has been described by different groups (Katoh, 2007; McCall et al., 2007). Our group demonstrated that IL-6, a TLR signaling product, can activate STAT3 with resulting overexpression of Wnt5a in papillary thyroid carcinoma cells and that phenylmethimazole (C10), a derivative of the anti-thyroidal medication methimazole, has the ability to block TLR3 signaling, IL-6 production, as well as decrease growth and migration of papillary thyroid carcinoma cells (McCall et al., 2007). We hypothesized that the C10 effect on growth and migration of the papillary thyroid cancer cells was related to its suppressive effect on TLR3 signaling which led to the downregulation of TLR-mediated STAT3 and Wnt5a signaling (McCall et al., 2007). This was the first study that linked TLR signaling with Wnt5a and cancer cell growth and migration.

More recently our group has shown that TLR3 and Wnt5a RNA are constitutively expressed in human pancreatic cancer and malignant melanoma cell lines in culture. In similar findings to what we reported in human papillary thyroid cells, C10 inhibits TLR3 expression and signaling in addition to growth and migration of these human pancreatic cancer and malignant melanoma cells. Moreover, in this report we established *in vivo* efficacy by showing that C10 delays tumor growth in mouse models of human pancreatic cancer and malignant melanoma. These studies showed that this phenomenon was also associated with inhibition of STAT3 activation (Schwartz et al., 2009). Since STAT3 activation is a strong regulator of Wnt5a expression, and since C10 can block migration of these pancreatic and malignant melanoma cells *in vitro* as well as Wnt5a expression and signaling, we suspect that C10 may also act to prevent metastasis in *vivo*.

4.3.4 Other TLR-related molecules

Tumor necrosis factor alpha (TNF-α), a product of TLR signaling, is a critical cytokine that induces expression of other inflammatory mediators and proteases important for tumor invasiveness and metastasis. Although at high doses extrinsic TNF-α cause hemorrhagic necrosis, at low concentrations it acts as an endogenous tumor promoter. It can be produced by malignant epithelial cells or stromal cells (Balkwill, 2002). The tumor promotion capacity depends on activation of NF-κB (Luo et al., 2004; Pikarsky et al., 2004). TNF-α expression/production is associated with poor prognosis, loss of hormone responsiveness

and cachexia (Luo et al., 2004). TNF-α increases vascular permeability, can stimulate the migration and extravasation or intravasation of cancer cells or can acts as a growth factor (Luo et al., 2004).

TLR signaling products IL-1α and IL-1β in the tumor microenvironment both contribute to increased invasiveness and metastasis (Gemma et al., 2001; Elaraj et al., 2011). IL-1 can promote metastasis by different mechanisms; first by increasing the adhesiveness of the endothelium via VCAM-1 or mannose receptor expression in endothelial cells. A second mechanism may involve induction of MMPs, cytokines and chemokines in tumor or stromal cells (Anasagasti et al., 1997; Song et al., 2003). A third mechanism is via the induction of angiogenic factors such as VEGF and IL-8 (Lewis et al., 2006). In 2003 two groups independently established that IL-1α and IL-1β were critical for the invasiveness and metastasis of pancreatic cancer and melanoma tumor cells (Sawai et al., 2003; Voronov et al., 2003). IL-1β is mainly produced by myeloid cells, with intricate transcriptional and post-transcriptional control. IL-1β increases tumor invasiveness and metastasis by promoting the production of angiogenic factors by stromal mononuclear cells (Saijo et al., 2002). IL-1α is secreted mainly by epithelial cells undergoing necrosis (Sakurai et al., 2008). In liver it was found that IL-1α released by necrotic hepatocytes induces IL-6 synthesis by Kupffer cells which activates pro-oncogenic transcription factor STAT3 (Naugler et al., 2007). IL-1 receptor activation by either form of IL-1 can lead to induction of IL-6. In multiple myeloma IL-6 promotes survival and proliferation of cancer cells via activation of STAT3 and extracellular signal – regulated kinase ERK signaling (Honemann et al., 2001). IL-6 - STAT3 signaling was also found in chemically induced liver carcinogenesis as well as many other types of cancers (Calo et al., 2003; McCall et al., 2007; Naugler et al., 2007; Schwartz et al., 2009).

COX-2, a TLR4 signaling product (Fukata et al., 2006), is highly expressed in a variety of cancers such as colorectal, gastric, esophageal, breast and prostate carcinomas. COX-2-produced prostaglandin E2 (PGE2) increases tumor invasiveness and metastasis and enhances production of IL-6, IL-8, VEGF, iNOS, MMP2 and MMP9 among others (Gasparini et al., 2003). COX-2 inhibition shows chemopreventive and antimetastatic activity in a variety of human cancers through disruption of the inflammatory microenvironment (Baek & Eling, 2006).

TLR-induced TGF-β is produce by myeloid cells, mesenchymal cells and cancer cells in hypoxic and inflammatory conditions (He et al., 2007; Ren et al., 2007; Zhou et al., 2009). It is one of the most highly expressed cytokines in the tumor microenvironment and has a large influence on tumor cell invasiveness and metastasis (Yang et al., 2010). The induction of angiopoietin-like 4 (ANGPTL4) in cancer cells by TGF-β disrupts vascular endothelial cell cell to cell junctions, increases the permeability of lung capillaries, and facilitates the trans-endothelial passage of tumor cells (Padua et al., 2008).

Versican is an aggregating chondroitin sulfate proteoglycan highly expressed in several cancers (Pirinen et al., 2005). Versican enhances tumor cell migration, growth and angiogenesis (Zheng et al., 2004). Versican has pro-inflammatory activity, it induces macrophages activation and stimulates the secretion of TNF-α and other cytokines (Wight, 2002; Kim & Karin, 2011). In addition, versican interacts with several adhesion molecules

expressed by inflammatory cells (Wight, 2002) and activates endothelial cells, fibroblasts and macrophages in the tumor microenviroment (Wang et al., 2009). Importantly, versican activates TLR2 on macrophages to induce NF-κB and MAP kinase (MAPK) signaling with the subsequent production of pro-inflammatory cytokines such as IL-6 and TNF-α (Wang et al., 2009). The inhibition of versican expression in LCC cells eliminates their metastatic behavior (Kim et al., 2009). In addition, a related proteoglycan, biglycan was found to activate macrophages through TLR2 and TLR4 (Schaefer et al., 2005).

Helix-loop-helix protein Twist, is a key transcription factor that regulates cell movement and tissue reorganization during early embryogenesis with a role in the epithelial-mesenchymal transition (EMT) process during normal development. Suppression of Twist in metastatic 4T1 mammary carcinoma cells specifically inhibits their ability to metastasize to lung but not their ability to form primary tumors (Yang et al., 2004) and loss of Twist expression prevents the entry of metastatic cells into the circulation (Yang et al., 2004). Together, these findings suggest that Twist can contribute to invasion and metastasis by promoting the EMT developmental program in cancer. Interestingly, Twist expression can be induced in response to NF-κB activation and is therefore upregulated in response to inflammation (Pham et al., 2007). This could be a mechanism through which tumor-associated inflammation may stimulate metastatic progression through induction of Twist-dependent EMT (Yang et al., 2004; Pham et al., 2007).

Another mechanism of cancer invasiveness and metastasis was identified in a metastatic prostate cancer model that involves the repression of Maspin. Maspin is a member of the serpin family with well-established anti-metastatic activity in breast and prostate cancers. It was found that the metastatic behavior of isolated cells was dependent on the activation and nuclear accumulation of IKKα (Preciado et al., 2005). Repression of Maspin transcription required nuclear translocation of catalytically active IKKα, which occur only in advanced prostate tumors that contain inflammatory infiltrates and cells that express receptor activator of nuclear factor kappa-B ligand (RANKL) and LTα:β (Preciado et al., 2005). RANKL can lead to repression of maspin-expression in an IKKα-dependent manner (Zou et al., 1994; Luo et al., 2007). Thus, IKKα infers its pro-mestastatic effect by repressing transcription of the maspin gene.

Although the molecular mechanisms involved in these metastatic processes seem to be complex and not well understood, TLR signaling and related proteins appear to play an important role.

4.4 TLRs have an important role in tumor angiogenesis

In 1995, Judah Folkman wrote (Folkman, 1995) that "recent discoveries of endogenous negative regulators of angiogenesis, thrombospondin, angiostatin, and glioma-derived angiogenesis inhibitory factor, all associated with neovascularized tumors, suggest a new paradigm of tumorigenesis. It is now helpful to think of the switch to the angiogenic phenotype as a net balance of positive and negative regulators of blood vessel growth. The extent to which the negative regulators are decreased during this switch may dictate whether a primary tumor grows rapidly or slowly and whether metastasis grows at all." Folkman recognized that cancer has the ability to spread to adjacent or distant organs; he recognized that tumor cells could penetrate blood or lymphatic vessel walls, spread through

the blood or lymphatic vessels to another site, where they could proliferate; but he further recognized that the key to the metastatic or tumor growth process was angiogenesis. At that time he did not envision the importance of the Toll-like receptor and signal system in regulating this switch from normal to pathologically driven angiogenesis.

Cell growth, cell development, and cell migration, independent of whether immune or non-immune cell in origin, require blood vessel formation to feed the inflamed areas with nutrients and oxygen, particularly chronically inflamed and growing tissues. This is reviewed in Grote, et al, whose work can be construed not only as an important component of the following discussion but the "bible" on which it is based (Grote et al., 2011). Vascular growth is termed angiogenesis or vasculogenesis. Angiogenesis is the formation of new blood vessels by sprouting or by intussusception of preexisting vessels; vasculogenesis defines a process whereby progenitor cells differentiate into endothelial cells (Ribatti, 2010). Both processes often occur together and are often termed neovascularization, as for example in atherosclerotic lesions.

Tumor angiogenesis is a multistep process (Nishida et al., 2006). Simplistically, first the basement membrane in tissues is injured locally with resultant tissue destruction and hypoxia. Second, endothelial cells activated by angiogenic factors migrate into the damaged area. Third, endothelial cells stimulated by angiogenic factors proliferate and stabilize. Then, angiogenic factors continue to influence tissue nutrient supply and waste removal. It is intuitive that this is not a simple process and must be highly controlled (Carmeliet, 2000).

One example of this is the role of hypoxia in tumor angiogenesis. The irregular pattern and organization of the tumor vasculature result in some cells being more than 100 μm from a blood vessel, the accepted diffusion limit for oxygen. Progressive hypoxia with distance from the oxygen source results in induction in hypoxia-inducer factor 1 alpha (HIF-1α) and 1 beta (HIF-1β) and, upregulated gene expression (VEGF, Ang2, iNOS, PDGF-B), increased glycolysis, and stimulation of angiogenesis.

Secondly, blood vessels have endothelial cell walls, a media composed of fibroblasts, fat cells, collagen molecules, and mesenchymal tissue, and a surrounding smooth muscle and epithelial cell layer in arteries or just an epithelial cell layer in non arterial vasculature. Blood vessels form in a regular pattern as part of a normal vascular network but in a disordered array in chronic inflammatory states or cancer. In examining angiogenesis, one must thus consider multiple cell types, multiple coordinated interactions, and complex regulatory networks.

The complexity is evidenced molecularly (Carmeliet, 2000; Olsson et al., 2006). VEGF (vascular endothelial growth factor) is now recognized to be 5 VEGF ligands (A-D) in different spliced or processed variants, yet each is a dimeric glycoprotein of about 40K. Placenta growth factor (PLGF) is also a family member. They bind to 3 receptor tyrosine kinases (VEGFR 1-3) which can have an overlapping functional pattern, and can have multiple co-receptors, including neuropilins, proteoglycans, and heparin-sulfate. Each VGFR has a different function: VEGFR1 is important in hematopoetic progenitor cell recruitment; it also regulates monocyte migration; VEGFR2 and 3 control endothelial cell function during angiogenesis. Lest this complexity overwhelm us, Tie receptors and their angiopoitin (Ang) ligands are a second endothelial cell-specific receptor tyrosine kinase

system which interacts with the VEGF-VEGFR kinase system during angiogenesis (Partanen et al., 1992; Augustin et al., 2009). Ang-Tie interactions normally control signals leading to vessel quiescence and the last steps of vessel maturation.

Capillaries develop and grow with a VEGF gradient. Endothelial cells at the leading edge of the capillary tube, tip cells, have filopodia and express multiple VEGFR family members. "Behind them" in the advancing gradient of growth and development are highly proliferative, differentiating "stalk" cells and resting cells expressing components of the Ang-Tie system. A key component regulating the "sprouting" tip cells vs stalk cells is the Delta/Notch Signaling system (Gridley, 2010). Again complexity exists. There are 4 different Notch Receptors, Notch 1-4. Notch receptors have a single transmembrane domain binding to membrane ligands Delta-like (Dll) 1-4 and Jagged. Notch signaling in stalk cells induces a quiescent endothelial cell phenotype whereas TIP cells enriched in DLL4 promote sprouting activity and capillary growth (Gridley, 2010). Other factors in associated cells, such as basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) control "endothelial cell coverage" by pericytes and smooth muscle cells to establish vasculature stability and maturation (Distler et al., 2003).

In sum, the regulated action of a multiplicity of angiogenic factors and receptor kinases control capillary sprouting, growth, differentiation (e.g., tip vs stalk) and endothelial cell "coverage" and "stabilization." Moreover this list does not even consider a multiplicity of cytokines, chemokines, and growth factors with pro-angiogenic importance (Bussolino et al., 1991). Together, these, plus controlling inhibitory factors such as angiostatin, define a balanced pro- or anti-angiogenic system in normal tissues. It is now accepted that the "angiogenic switch" is "off" when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules and is "on" when the net balance is tipped in favor of angiogenesis. A list of angiogenic stimulators and angiogenic inhibitors was summarized in "Angiogenesis in cancer and other disease" an Insight Review Article by Peter Carmeliet and Rakesh Jain (Carmeliet & Jain, 2000). This multiplicity is much expanded now, and offers an array of targets to stimulate angiogenesis, for example after a myocardial infarction (Vandervelde et al., 2005), or to inhibit angiogenesis to control dysregulated cell growth precipitated by chronic inflammation, for example in cancer, atherosclerosis, or obesity-induced diabetes (Cao, 2009). A VEGF-neutralizing monoclonal, anti-TNF-α, is one of many therapies targeting single gene products in this complex cascade. Of interest, numerous individual specific antibodies and inhibitors of specific tyrosine kinases have been evaluated in the past 10-15 years; however, not surprisingly, this approach has had limited success in a complex, interrelated, redundant signal system/pathway.

4.4.1 Inflammation and angiogenesis

Accumulating evidence supports a link between inflammation and angiogenesis. The two processes are intimately intertwined in inflammation-associated wound healing and tissue regeneration. Thus, the acute gene response set off by the innate immune process results in cytokines, chemokines, and growth factors produced at the site of the injury, which not only induce inflammation but also successive tissue repair. Angiogenesis is an important component of this tissue repair as evidenced by increased expression of VEGF. The inflammation-induced wound healing includes, in addition, pro-angiogenic factors such as basic fibroblast growth factor (bFGF), TGF-β, TNF-α, insulin-like growth factor-1 (IGF-1),

monocyte chemotactic protein-1 (MCP-1), IL-6 and IL-8, PDGF, to name but a few, which attract endothelial cells, smooth muscle cells, and epithelial cells needed for vessel growth and maturation.

Chronic inflammation leading to different pathological states, such as obesity-induced diabetes, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and cancer, nevertheless have a common gene activation process and angionesis component. That chronic inflammation leads to diverse pathologic states involving the same molecular events should not be a surprise if one recognizes the commonality of inflammation and wound repair in all tissues, despite their varied differentiated states. Organ specific diseases do not negate common mechanisms of wound repair and angiogenesis in each.

4.4.2 Toll-like receptor signaling in inflammation and angiogenesis

To date, most studies linking TLR signaling to angiogenesis are *in vitro* and are largely descriptive. Thus, there is literature describing the use of several TLR agonists to induce expression and secretion of multiple angiogenic factors in an array of different cell types *in vitro.* The majority, however use LPS to stimulate TLR4 signaling with increased VEGF as a marker of angiogenesis.

1. LPS activation of TLR4 signaling *in vitro* increases adenosine promotion of angiogenesis through the A_{2A} Receptor system to increase VEGF (Hara et al., 2009). This cardio-protective nucleoside stimulates angiogenesis by increasing VEGF in macrophages, thereby likely offering tissue protection after ischemic injury. Moreover, there is a synergistic down-regulation of TNF-α (Leibovich et al., 2002). This angiogenic "switch" was noted also with TLR2, TLR7, and TLR9 agonists (Pinhal-Enfield et al., 2003). There is an *in vivo* correlate, since MyD88-deficient mice had decreased generation of new capillaries in response to an A_{2A}R agonist (Macedo et al., 2007).
2. LPS-increased TLR4 signaling was shown to increase endothelial sprouting *in vitro* via a TRAF6, NF-κB, JNK stimulatory process (Pollet et al., 2003). The requirement for TRAF6 *in vitro* and *in vivo* was established using a retrovirally expressed dominant negative TRAF6 in endothelial cells (Pollet et al., 2003). Moreover, inhibition of c-Jun N-terminal kinase (JNK) activity or NF-κB activity downstream of TRAF6 inhibited LPS-induced endothelial sprouting. Inhibition of only NF-κB but not JNK activity blocked bFGF, not TRAF6 induced angiogenesis. In sum, a direct endothelial role of TLR4 activation via TRAF6 is important in inducing angiogenesis in endothelial cells (Pollet et al., 2003). This has a pathologic counterpart in pathological corneal neovascularization that can cause impaired vision when induced by infections wherein TLR4 and VEGF are increased (Rodriguez-Martinez et al., 2006).
3. *Bactonella henselae* infections increase MCP-1 in endothelial cells, which chemotactically attracts monocytes to produce VEGF and increase angiogenesis (McCord et al., 2005). This is an NF-κB dependent process independent of TLR4 or LPS.
4. The TLR4/MyD88 signal system is important in VEGF production and angiogenesis in liver endothelial cells stimulated with LPS (Jagavelu et al., 2010).
5. *Mycoplasma* infections of the pulmonary tract causing a chronic inflammatory process increase angiogenesis and vascular remodeling (McDonald, 2001). This appears to be associated with a TLR2/6-dependent induction of NF-κB and a MAPK cascade by a 2 kDa macrophage activating diacylated lipopeptide (MALP-2) present in the

mycoplasma and secretion of GM-CSF from endothelial cells and monocytes. MALP-2 induced angiogenesis *in vitro* and *in vivo* is suppressed by GM-CSF, explaining both angiogenesis and remodeling in the same tissues.

6. Increased VEGF in chondrocytes and VEGF and IL-8 in fibroblasts are associated with inflammatory cell induced angiogenesis in chronically inflamed joints associated with arthritis. This progressive self-destructive angiogenic process involves a peptidoglycan (PGN) TLR2 ligand from Gram-positive bacteria (Cho et al., 2007).

4.4.3 Toll-like receptor signaling in tumor angiogenesis

Cancer is associated with, or induced, by chronic inflammation. Nevertheless, a clear definition of TLR induced angiogenesis varies because of the diversity of cancers and incomplete studies of all.

In gastric cancer linked to chronic *H. pylori* infections, cyclooxygenase 2 (COX-2) plays a critical role. Thus, *H. Pylori* activation of TLR2 and TLR9 signals activate the MAPK cascade leading to increased COX-2 and COX-2-dependent prostaglandin E_2 (PGE_2) release. This contributes to cancer cell invasion and angiogenesis (Chang et al., 2005). The COX-2 increase can be attenuated by the specific COX-2 inhibitor, NS398 or celecoxib. The cAMP response element (CRE) and AP-1 sites, but not NF-κB on the COX-2 promoter, are involved in MAPK-regulated COX-2 expression. Differential binding of CREB-1, ATF-2, and c-jun to the CRE site and c-fos, c-jun, and ATF-2 to the AP1 site were demonstrated and attenuated by different MAPK inhibitors as well as mutants of TLR2 and TLR9. In sum, these results showed that *H. pylori* activated TLR2 and TLR9 to activate MAKs, particularly p38, and downstream transcription factors (CREB-1, ATF-2, c-jun, and c-fos) resulting in activations of CRE and AP-1 on the COX-2 promoter (Chang et al., 2005).

Sustained pro-inflammatory processes in cancer, as well as diabetes, atherosclerosis, and rheumatoid disease are associated with increased angiogenesis and disease progression. Necrotic cells release high mobility group B1 (HMGB1), a pro-inflammatory cytokine, which signals TLR2 and TLR4 and the receptor for advanced glycation end products (RAGE) to increase angiogenesis by up-regulating NF-κB and VEGF in hematopoietic cells and endothelial cells (van Beijnum et al., 2008). HMGB1 seems to be involved in a positive feedback mechanism that sustains inflammation and angiogenesis contributing to disease progression. Endothelial cells express HMGB1 as well as RAGE receptors, TLR2 and TLR4. The HMGB1 can increase NF-κB activity, which can, in turn, increase HMGB1 receptors.

Inflammation-induced oxidative stress and angiogenesis are a common theme in tissue regeneration and remodeling in cancers. End products of lipid oxidation, such as ω-(2-carboxyethyl)pyrrole (CEP), are generated, accumulate, and are recognized by TLR2 in endothelial cells, leading to a MyD88-dependent angiogenic responses independent of VEGF (West et al., 2010). These endogenous ligands accumulating during cancer-induced tissue disruption promote angiogenesis via a TLR-dependent pathway. Grote, et. al., summarize this visually in Figure 4 of their review (Grote et al., 2011).

Nevertheless, a counter-regulatory TLR path can be activated as well, which inhibits angiogenesis and cancer progression. For example, the immune-modulatory TLR9 agonist, IMO, inhibits micro-vessel formation and cancer growth (Damiano et al., 2006). Similarly the

TLR7 agonist imidazoquinoline and the TLR9 agonist un-methylated cytosine- phosphate-guanosine (CpG) oligonucleotide exhibit strong local activity against leukemia in Phase I/II trials at different centers (Spaner & Masellis, 2007). Their importance appears, however, to be to sensitize CLL cells to other cytotoxic agents so any future lies with combined chemotherapy, radiotherapy, or other more "toxic" agents.

Resistance to anti-HER2 monoclonal antibody, trastuzumab is an issue in breast cancer patients. The novel Toll-like receptor 9 (TLR9) agonist termed IMO, immune modulatory oligonucleotide, potentiates the anti-EGFR/HER2 signaling of monoclonal trastuzumab. It modulates a functional interaction between TLR9 and HER receptors at a membrane level, producing a cooperative antiangiogenic effect (Damiano et al., 2009).

In skin cancer, strategies to inhibit neovascularization and angiogenesis include blockade of COX-2, m-TOR, sonic hedgehog, growth factor receptor activation, and activation of TLR by imiquimod (Li & Li, 2008). Separately, Myricetin, a phytochemical from onions, berries and red wine, suppresses ultraviolet (UV) B-induced angiogenesis by inhibiting PI-3 kinase activity *in vivo* in mouse skin. The chronic UVB exposure induced neovascularization that is associated with increased VEGF, matrix metalloproteinase (MMP)-9 and MMP-13 expression. The myricetin inhibited UVB-induced hypoxia inducible factor-1α expression. The myricetin effect was associated with attenuation of UVB-induced PI-3kinase activity and phosphorylation of Akt/p-70(S6K) (Jung et al., 2010).

The complexity of TLR3 action in individual tumors with both increased and decreased angiogenic activity was suggested *in vitro* by Paone, et al. (Paone et al., 2010). These authors had shown TLR3 activation in LNCaP and PC3 lines, with more efficiency in the former cells from a less aggressive tumor. They subsequently describe novel pro-tumor machinery. Triggered by TLR3 activation by polyI:C in PC3 cells, they show increased expression of the specific 1.3 isoform of HIF-1 alpha and nuclear accumulation of this complex in PC3 cells with decreased apoptosis and in secretion of functional VEGF. This is not the case in less aggressive LNCaP cells. However, in both cell lines, transfection of the 1.3 isoform of HIF-1 alpha causes decreased apoptosis and increased secretion of functional VEGF. They suggest basal levels of the 1.3 isoform of HIF-1 alpha distinguish differential responses to TLR activation.

In sum, the role of TLR in inducing signaling to increase tumor growth and angiogenesis is clear but largely poorly defined. Nevertheless, Grote et al. and others (Chang et al., 2005; Spaner & Masellis, 2007; Damiano et al., 2009) suggest that future modulation of TLR signaling could be the basis for a therapeutic approach to cancer and inhibition or control of abnormal angiogenesis to limit tumor growth. However, this is potentially difficult because not only are there TLR-induced pro-angiogenic signals but also anti-angiogenic signals. Moreover, the different cells interacting in the process present a complex network to control. Trials of anti-VEGF monotherapy provide a caution as well (Freedman et al., 2002; Henry et al., 2003) since they have not yielded consistent beneficial results. It is now recognized that not one (VEGF) but a multiplicity of potent angiogenic factors act in concert with VEGF for proper vessel formation and maturation (Augustin et al., 2009; Gridley, 2010). Rather than a unique anti-angiogenic "bullet," what may be needed is a broadly acting agent acting on a multiplicity of cells and a multiplicity of steps in the TLR stimulated cascade as in the case of diabetes.

5. TLR Involvement in pancreatic cancer

Toll-like receptors were first implicated in the pathogenesis of pancreatic cancer in 2009 in two separate reports. First, TLR3 was first described by our laboratory to play a role in the regulation of pancreatic cancer growth and migration. In this report we demonstrated that TLR3 and Wnt5a were coordinately constitutively expressed in a human pancreatic cell line (PANC-1) derived from a human pancreatic ductal adenocarcinoma, and that phenylmethimazole (a TLR signaling inhibitor) inhibits growth and migration of these pancreatic cancer cells in cell culture and inhibits pancreatic cancer tumor growth *in vivo* in a *nude* (*nu/nu* mice, which lack T cells) mouse model of human pancreatic cancer (Schwartz et al., 2009). In a separate 2009 report, Ikebe et. al., showed that LPS activation of the TLR4/MyD88 signaling pathway increases the invasive ability of PANC-1 and Aspc-1 (another pancreatic cancer cell line derived from a human pancreatic ductal adenocarcinoma) cells, while blockade of TLR4, MyD88, or NF-kB signaling decreases the LPS-dependent increased invasive ability (Ikebe et al., 2009). Together, these studies were the first to implicate TLR expression and signaling in pancreatic cancer cells as playing a role(s) in pancreatic tumor growth and migration. These studies helped establish that TLR expression and signaling in the pancreatic cancer cells (i.e. non-immune cells) themselves may be an important contributor to disease development, an idea that is now widely accepted for a multitude of autoimmune/inflammatory diseases including cancer. As briefly mentioned earlier, clinical relevance of these findings has recently been noted in a study that investigated the expression and clinical relevance of TLR4, NF-κB, and hypoxia-inducible transcription factor-1α (HIF-1α) in pancreatic adenocarcinoma (Zhang et al., 2010). In this study, TLR4 and HIF-1α expression was measured via real time polymerase chain reaction (PCR) in 30 cases of pancreatic ductal adenocarcinoma and its adjacent tissues, and TLR4, NF−κB, p65, and HIF-1α protein expression was measured by immunohistochemistry in 65 cases of pancreatic ductal adenocarcinoma and 38 cases of corresponding adjacent tissues. In addition, the relationship between TLR4 or HIF-1α and pathologic features, and the association between TLR4 and HIF-1α, were also analyzed. The Kaplan-Meier method was used to assess the impact of expression of TLR4 and HIF-1α on survival of the patients with pancreatic cancer. Results of these analysis revealed that TLR4, NF-κB, and HIF-1α are all overexpressed in pancreatic adenocarcinoma, that TLR4 may regulate HIF-1α expression, and that TLR4 and HIF-1α act synergistically to promote the development of pancreatic adenocarcinoma.

6. TLRs as potential therapeutic targets for pancreatic and other cancers

The therapeutic use of TLR agonists has been investigated in several cancer models. The rationale for inducing TLR signaling in a tumor setting is that: (*a*) TLR signaling will target tumor cells, inducing apoptosis or inhibiting the generation of factors that augment tumor growth or, (*b*) TLR signaling will enhance a resident or therapy-driven antitumor immune response that will eventually lead to tumor cell destruction. At present, mixed results have been obtained using TLR agonists against different TLRs. For example, with respect to the effect of TLR agonists on tumor cells, it has been shown in mouse breast xenograft cancer models that the antitumor effect of TLR3 agonists was dependent on the expression of TLR3 receptors in tumor cells, and that dsRNA treatment improved outcomes in patients harboring TLR3-positive breast tumors (Salaun et al., 2011). Similarly, CpG treatment was

able to trigger tumor cell death in human neuroblastoma cells, and tumor-targeted delivery of this TLR9 agonist increased survival in a xenograft model of mouse neuroblastoma (Brignole et al., 2010). In human patients harboring low-grade B-cell lymphoma, when CpG was delivered intratumorally in combination with radiotherapy almost 50% of the patients showed complete regression (Brody et al., 2010). CpG molecules were able to interact with the transformed B cells that express TLR9 receptors.

Treatment with TLR agonists has also shown to induce an antitumor response by either enhancing dendritic cell (DC) vaccination or T cell adoptive therapies. For example, tumor-localized delivery TLR agonists such as poly(I:C) or CpG combined with adoptive transfer immunotherapy was effective to control tumor growth in an established model of aggressive murine B16F10 melanoma(Amos et al., 2011). In particular, it is proposed that TLR agonists enhance T cell adoptive therapy by inducing a better interaction of these cells with activated resident DCs and by augmenting the activity of these T cells through IFNγ induction (Amos et al., 2011). TLR agonists have also been proposed as adjuvants for DC antitumor vaccination. In particular, TLR agonists have been shown to enhance the efficacy of DC vaccines in mouse models of melanoma and brain tumors (TLR7/8 agonist) (Prins et al., 2006; Ma et al., 2010), sarcoma (TLR3/9 agonists) (Zheng et al., 2008); or lung tumors (TLR9)(Cho et al., 2009) among others.

On the other hand, it has also been shown that TLR agonists can promote cancer cell survival and migration, and tumor progression. For example, TLR agonists have been shown to increase tumor viability and metastasis of human lung cancer cells (TLR7/8) (Cherfils-Vicini et al., 2010); proliferation of human myeloma cells (TLR3) (Chiron et al., 2009); adhesion and metastasis of human colorectal cancer cells (TLR4) (Hsu et al., 2011); and migration of human glioblastoma (TLR4) or human breast cancer cells (TLR2) (Thuringer et al., 2010).

We considered that these contradictory results are due to the complex nature of the tumor microenvironment. Tumors are more than cancer cells; they are also composed of non-tumor cells and the extracellular matrix. In particular, in pancreatic cancer the tumor microenvironment is composed of endothelial cells, leukocytes (lymphocytes, macrophages, dendritic cells, mast cells and neutrophils), mesenchymal cells (stellate cells and fibroblasts), neural cells and an extracellular matrix rich in fibronectin, collagen and periostin (Farrow et al., 2008; Erkan et al., 2011). These components of the tumor microenvironment often support tumor cell growth, or suppress immune responses against tumor cells. For example, stellate cells mediate fibrosis by generating high amounts of extracellular matrix components (Masamune et al., 2009), while fibroblasts release hepatocyte growth factor (Xu et al., 2010) facilitating tumor cell proliferation. Similarly, macrophages generate cytokines and growth factors (Pinhal-Enfield et al., 2003) that stimulate both tumor cell proliferation and angiogenesis. However the tumor microenvironment can also hamper therapeutic efforts. In addition, the nature of the tumor endothelium can prevent the delivery of antitumor factors within the tumor as has been previously shown for ovarian cancer (Buckanovich et al., 2008). In the same way, the presence of tumor-associated regulatory T cells or myeloid derived suppressor cells can render attempts to generate a powerful antitumor immune response by therapeutic vaccination ineffective (Whiteside, 2008).

Interestingly, although both tumor cells and tumor-associated leukocytes can express TLR, their signaling can induce the generation of different molecules (Palha De Sousa et al., 2010).

Thus, TLRs are present both in tumor cells and leukocytes, but may have different activities. Further, the signaling pathway may be not the same among different leukocyte populations such as T cell or antigen presenting cells. Indeed, TLR signaling on mast cells (Oldford et al., 2010) or Tregs cells (Zhang et al., 2011) may contribute to tumor inhibition, while TLR signaling on macrophages can contribute to tumor progression (Pinhal-Enfield et al., 2003). Thus there are diverse effects that TLR signaling can induce on different cells within the tumor microenvironment. Together, this data argues for specific targeting of tumor microenvironment components when applying TLR agonist therapies for cancer. For example, TLR agonists can be prepared for their delivery to particular cells within the tumor microenvironment (Bourquin et al., 2010). This type of strategy was successfully used to activate tumor-associated DCs in ovarian cancer, promoting antitumor immune response *in vivo* (Cubillos-Ruiz et al., 2009; Scarlett et al., 2009).

On the other hand, the use of TLR antagonists may prove beneficial for those tumors in which the tumor microenvironment promotes tumor cell survival and metastasis upon TLR signaling. TLR antagonists might also decrease the levels of activation of stromal cells, such as tumor-associated macrophages. Macrophages express an array of TLRs and are able to produce several growth factors via TLR signaling (Pinhal-Enfield et al., 2003). Moreover, abrogation of TLR-4 signaling in tumor-associated macrophages was able to decrease tumor growth (Lee et al., 2009). In particular for pancreatic cancer, it has been shown that TLR3 and TLR4 signaling promotes the invasiveness of pancreatic tumor cells (Ikebe et al., 2009). In this context, our recently published manuscript showing that C10 inhibited tumor growth in an *in vivo* model of pancreatic cancer highlights the relevance of using TLR antagonists for tumor therapies (Schwartz et al., 2009). Since C10 abrogates both TLR3 and TLR4 signaling (Schwartz et al., 2009; McCall et al., 2010), this molecule will be extremely relevant as a novel therapeutic agent for the treatment of those cancers whose microenvironment induces tumor progression via TLR signaling.

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Grb7 – A Newly Emerging Target in Pancreatic Cancer

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1. Introduction

Growth factor receptors are transmembrane glycoproteins involved in many aspects of cell biology ranging from protein and nucleic acid synthesis, cell growth, differentiation and migration to ultimate death of cells (Kiel et al., 2010; Lemmon et al., 2010). On binding by growth factors, the receptors undergo dimerization and autophosphorylation (Burz et al., 2009; Prenzel et al., 2001). The receptor phosphorylation is in turn responsible for recruiting intracellular molecules so as to form a network of signalling complexes critical for the transfer of the signal to downstream events. One class of cytoplasmic proteins recruited in such a way is the growth factor receptor binding (Grb) proteins. As the name implies, Grb proteins were originally identified because of their ability to associate with growth factor receptors (Margolis et al., 1994). Characteristically, Grb proteins form supramolecular complexes with growth factor receptors essential for growth factor mediated signal transduction (Songyang et al., 1993, 1994), though interactions with non-growth factor receptors is also well documented (Margolis et al., 1994; Songyang et al., 1994). Currently 14 Grb proteins are identified, with several implicated in the genesis and development of human cancers (Margolis et al., 1994).

Growth factor receptor bound protein 7 (Grb7) belongs to a subfamily of Grb proteins comprising Grb7, growth factor receptor bound protein 10 (Grb10) (Frantz et al., 1997; Lim et al., 2004) and growth factor receptor bound protein 14 (Grb14) (Cariou et al., 2004; Holt et al 2005). The Grb7 family of adaptor proteins share high sequence and functional homology (Songyang et al., 1993; Holt et al 2005). The group was discovered using a technique dubbed CORT (cloning of receptor targets), an expression/cloning system that uses a tyrosine phosphorylated receptor as a probe to screen protein libraries (Margolis et al., 1992). Specifically, Grb7 was identified using CORT screening of a mouse cDNA expression library with tyrosine phosphorylated C-terminus of the epidermal growth factor receptor (Skolnik et al., 1991; Lowenstein et al.,1992). In common with other adaptor proteins, Grb7 facilitates the coupling of multiple transmembrane and cytoplasmic receptors to downstream effectors molecules (Margolis et al., 1994; Yokote et al., 1994). Grb7 has attracted particular attention since it was noticed to be massively overexpressed, along with EGFR2, in a number of cancers including pancreatic cancer (Stein et al., 1994; Tanaka et al., 1997).

1.1 Expression of Grb7

The human Grb7 gene is located on the positive strand of chromosome 17. Cytogenetic analysis shows that the gene is mapped to the 17q12-q21 loci, as documented by the National Centre for Biotechnology Information(NCBI) (http://www.ncbi.nlm.nih.gov/mapview/). The human Grb7 gene is 9, 352 nucleotides in length and known to encode a primary Grb7 RNA transcript of 4,596 nucleotides in size with the mature Grb7 mRNA known to be composed of 14 exons comprising 1,599 nucleotides. The chromosomal location of Grb7 is found within the erbB2 amplicon (Lucas-Fernández et al.,2008; Kauraniemi et al., 2007), a region known to comprise genes frequently over amplified in cancers (Mano et al.,2006). As will be described later, the localization of Grb7 on this amplicon appears to explain the occurrence of Grb7 over-expression. Fig. 1 shows the overall organization of Grb7 gene and its products.

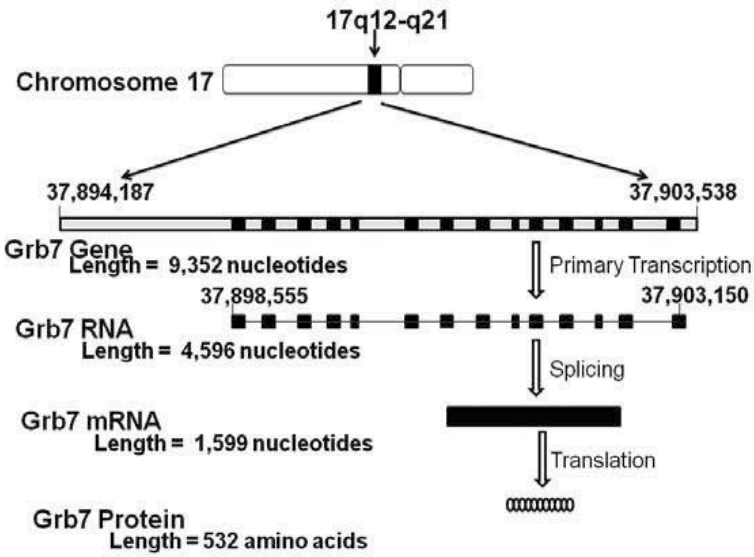

Fig. 1. The overall organization of Grb7 and its products. Grb7 is localized on the long arm of chromosome 17, at 17q12-q21. It is comprised of 14 exons, indicated by filled boxes. In Grb7 mRNA all the exons are merged and represented by single full filled rectangle. The numbers on top of each structure represent the start and end of a given nucleotide sequence. Grb7 amino acids are represented by a string of ellipses.

Grb7 protein displays a distinct expression profile across species, tissues and organs (Margolis et al., 1992). Under normal conditions it is expressed in human tissues including placenta, intestine, brain, lung, kidney, esophagus, mouth, prostate, mammary gland, uterus, ovary, cervix, liver, pancreas, testis, embryonic tissue, lymph node, trachea, larynx, bladder, thymus, skin, eye, ascites, stomach, pharynx and connective tissue [Unigene, www.ncbi.org]. Grb7 is an intracellular protein primarily found in the cytosol though it is localised to focal contacts, mitochondria and cell membrane under certain circumstances (Shen et al., 2004). In addition, Grb7 is found to be localized as an integral component of stress granules (Tsai et al., 2008). It is found to be conserved amongst mammals as the gene and its protein product are found in a number of mammalian species with high sequence

homology. Nonetheless, it is the over expression of Grb7 that is associated with a number of human maladies such as pancreatic and other cancers.

1.2 Grb7 as a mediator of multiple signalling pathways

Grb7 was initially identified as a binding partner of growth factor receptor (Margolis et al., 1992; Han et al., 2001). It has been shown in numerous studies to interact with a diverse spectrum of biomolecules since its initial identification. These include the various growth factor receptors, transmembrane receptor tyrosine kinases, cytoplasmic protein kinases (in particular focal adhesion kinase (FAK)), phosphatases, GTPases, ligases, adaptor proteins, caveolins, phosphoinositides and other biomolecules (Shen et al., 2004; Han et al., 2001; Daly et al., 1998; Holt et al., 2005). These binding partners are known to participate in a myriad of biochemical signalling in their own right. Noteably, while a large number of binding partners functioning upstream of Grb7 have been identified, the precise downstream events leading to Grb7 effects are not yet elucidated. The most recent data suggest that Grb7 is able to recruit RasGTPases leading to phosphorylation of ERK1/2 and cell proliferation (Chu *et al.*, 2010). Another set of studies have identified interactions between Grb7, RNA and the RNA-binding protein HuR leading to the proposal that Grb7 can act at the level of translational regulation (Tsai *et al.*, 2007, 2008).

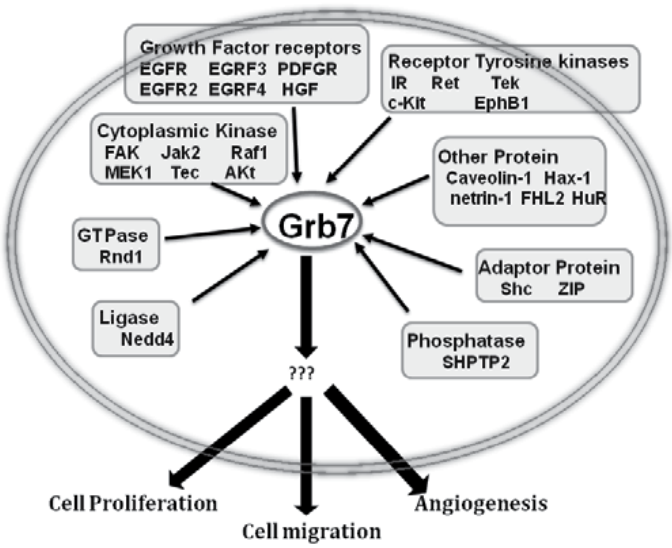

Fig. 2. Grb7 as a mediator of several signalling pathways.

Fig. 2 displays various identified binding partners of Grb7. Importantly, many upstream binding partners of Grb7 are connected with cancer cell properties (Pero et al., 2003; Golubovskaya et al., 2009). For example, the integrin pathway via FAK is important for cell migration (Golubovskaya et al., 2009 ; Zhao et al., 2009). In addition, various growth factor receptors are frequently implicated in growth and proliferation of cancer cells (Witsch et al,. 2010). Indeed there are clinically available anticancer drugs in use that act on EGFR2 such as Trastuzumab (Herceptin®)(Roy et al., 2009), Erlotinib(Tarceva®)(Kim et al., 2002), and Gefitinib(Iressa®)(Velcheti et al., 2010). Trastuzumab is a humanized monoclonal antibody

that binds to the extracellular domain of EGFR2 whereas Gefitinib and Erlotinib are a small molecule drugs binding to the ATP binding site of the intracellular kinase domain of the receptor. Apart from the above shown interaction partners of Grb7, a number of membrane bound macromolecules such as phosphoinositides are reported as important partners of Grb7 mediated signalling (Reiske et al., 2000).

2. Grb7 in pancreatic cancer

Grb7's overexpression in cancer cells has prompted investigation of its role in different properties of cancer cells such as migration, proliferation, invasion and metastasis. Other disease states related with cell development have also been at the centre of investigation. Pancreatic cancer is among the most aggressive and leading causes of cancer deaths worldwide (Bardeesy et al., 2002). The clinical relevance of Grb7 expression in pancreatic cancer was studied by Tanaka and co-workers with the application of immunohistochemical analysis (Tanaka et al., 2006). Through a comparative study of Grb7 overexpression in normal vs maliginant cells, they showed that Grb7 was expressed in 61% of pancreatic cancer cell lines as compared to non-cancerous samples. Furthermore, the study reported Grb7 and erbB2 genes co-amplification as high as 59% in the pancreatic tumour cells that overexpress Grb7 but not in those cells that did not over express Grb7. Furthermore, upregulation of Grb7 has also been separately reported in pancreatic cancer cell lines (Jonson et al., 2003).

Interestingly, Grb7 overexpression was noted to contribute to the migratory, proliferative, metastatic and invasive properties of pancreatic cancer cells. For example, in a study of patients with lymph node metastasis, Grb7 overexpression was noted in 67% of the studied cases, indicating a relationship between Grb7 level and metastatic potential of pancreatic tumours (Tanaka et al., 2006). In a cell motility assay, Grb7 over-expression was shown to directly correlate with the migratory potential of NIH 3T3 cell lines, particularly when it is phosphorylated by FAK (Han et al., 1999). In addition, the association of Grb7 with FAK is reportedly an important factor in the regulation of cell proliferation, and cancer cell growth indicating the crucial role of Grb7 in tumourigenesis. To further evaluate the role of Grb7 in mediating tumourigenesis, BrdU incorporation assay was conducted on A431 carcinoma cells to find out that the knockdown of Grb7 resulted in an evident inhibition of cell proliferation (Chu et al., 2009). A similar conclusion was reached with the use of siRNA to knockout Grb7 and assess its impact on pancreatic cancer cell migration by Tanaka et al. They showed that the use of siRNA to knock down Grb7 in pancreatic cell was associated with reduction of migratory potential of pancreatic cancer cell lines (Tanaka et al., 2006). In a recent experiment, Furuyuma and co-workers have examined the significance of FAK in pancreatic cancer formation to discover that FAK was expressed in up to 48 % of the studied cases and, importantly, its expression was found to relate to tumour size (Furuyama, et al., 2006). Since Grb7 is a binding partner of FAK and Grb7 over expression has been implicated in tumour size of other cancers, it might be the case that Grb7 has been co-implicated in the pancreatic tumour size. Genes on 17q12-q22 chromosomal region, which also includes the Grb7 locus, are noted to be amplified in some pancreatic tumours (Bashyam et al., 2005).

Finally, the druggability of Grb7 protein has been investigated by using a specific peptide inhibitor on different properties of cancer cells. With the use of cell migration experiments using a modified Boyden assay, a Grb7 peptide inhibitor was found to have a reduced the

migratory potential of a pancreatic cell line. This was specifically noted in pancreatic cancer cell lines that over expressed Grb7 (such as MiaPaca2 and PK8 cells), but was not found to reduce migration of other human pancreatic cancer cell lines that did not over express Grb7 (such as KLM1 cells) (Tanaka et al., 2006). Likewise, in an attempt to determine whether the Grb7 peptide inhibitor could arrest the metastasis of pancreatic cancer cells, Tanaka et al performed a peritoneal metastasis experiment to find out that treatment by the peptide resulted in a fewer peritoneal metastases of pancreas cancer cells as compared to the control. In addition, they report that the number as well as the total weight of tumour nodules per mouse was significantly reduced on treatment by the Grb7 selective peptide inhibitor.

3. Grb7 in other cancers

Apart from its role in pancreatic cancer, Grb7 has been extensively investigated as a target in a number of other human cancers including breast, gastric, hepatic, blood and testicular cancers. In breast cancer, for example, it is found to be over-expressed in a number of breast cancer cell lines. In particular, its co-over expression and co-amplification with ErbB2 is widely investigated (Shen et al., 2004). This might emanate from the fact that ErbB2 and Grb7 are found on the same chromosomal region at 17q12-q21, termed the erbB2 amplicon (Kauraniemi et al. 2006; Glynn et al., 2020). Moreover, Grb7 and ErbB2 form a functional association in growth factor dependent signalling (Holt et al., 2005) and are shown to synergistically enhance tumour formation. The mechanism of Grb7 dependent tumour formation as described in a recent paper (Chu et al., 2010) is proposed to involve Ras-GTPases which in turn promote phosphorylation of ERK1/2, thereby stimulating tumour growth. Moreover, co-overexpression of Grb7 and ErbB2 have also been associated with worse outcomes in some breast cancer subjects (Nadler et al., 2010). The fact that Grb7 is found within the core of the ErbB2 amplicon at 17q12 is what explains most of the co-implications in breast cancer. However, a study conducted to identify the contribution of co-amplified genes has demonstrated that Grb7 alone may be a factor in breast cancer carcinogenesis. With the use of RNA interference technology, it is has been shown that Grb7 knockout SKBR3 and BT474 breast cancer cell lines possessed decreased cell proliferation and cell-cycle progression (Kao et al., 2006). Furthermore, recent experiments involving siRNA have shown that removal of Grb7 by RNA-interference reduced the viability of BT474 xenograft cancer cells and increased the activity of the antitumour drug lapatinib (Nencioni et al., 2010).

Grb7 has also been identified as a culprit in other cancers. For example, in gastric cancer, over expression of Grb7 was found in up to 31% of esophageal carcinomas and that the over expression was shown to strongly correlate with extra mucosal invasive potential of gastric tumours (Tanaka et al., 1997). In a related study, more than 8-fold amplification and over expression of ErbB2 and Grb7 in primary gastric cancer cells was reported and the over expression was associated with the development of more aggressive gastric cancer phenotypes (Kishi et al., 1997). Similarly, up to 45% Grb7 overexpression was noted in some esophageal carcinomas as compared to normal mucosa (Tanaka et al., 2000) which was directly related with the development of lymph node metastases (Tanaka et al., 1997) suggesting that Grb7 overexpression is a major risk factor in such cancer populations. Itoh et al. showed that Grb7 overexpression was correlated with the level of FAK in Hep3B cells and that such over expression was a cause for invasive and metastatic potential exhibited by

the hepatocellular carcinoma (HCC) cell lines and that suppression of Grb7 expression delayed the onset of HCC tumour formation in mice (Itoh et al., 2007). Studies by Haran et al showed that Grb7 was not only over expressed but its expression correlated with the severity of the Chronic lymphocytic leukemia. They showed that up to 88 % of Grb7 expression was detected in Stage IV as compared to 18 % in the Stage I of leukemia (Haran et al., 2004). In other studies, mutations, copy number and expression levels of genes have shown Grb7 to be involved in the development of testicular germ cell tumours with up to a 63 % increase in Grb7 expression in primary tumour samples (McIntyre et al., 2005). These studies indicate the emergence of Grb7 as promising therapeutic target in a number of malignancies. The developments efforts made against Grb7 will be discussed below.

4. The molecular architecture of Grb7 protein

Human Grb7 protein is comprised of 532 amino acids. Its constituent residues are organized into a number of protein domains that serve different but complementary functions to the overall signalling role of Grb7 (Margolis et al., 1994). Grb7 domain components are well conserved across the species, and serve similar roles in different proteins (Han et al., 2001; Margolis et al., 1994). The modular structure of Grb7 is composed of a proline rich domain, a Ras-associating domain, a pleckstrin homology (PH) domain, Src homology 2 (SH2) domain and a BPS domain (between the PH and SH2 domains) (Filippakopoulos et al., 2009; Pawson, 1994). Fig. 3 illustrates the various domains of Grb7 together with the approximate amino acid residue bounds. The amino acid composition and the specific roles of each domain will be described in greater detail under each heading.

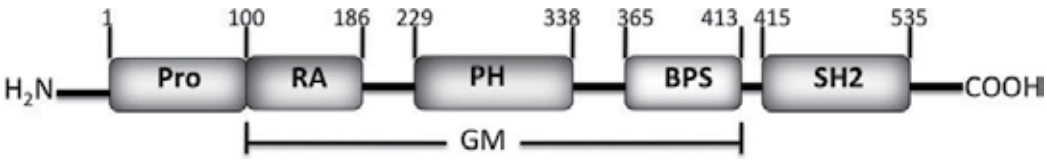

Fig. 3. The modular organization of Grb7 protein. Numbers indicate the residue number of the amino acids.

4.1 The N-terminal domain

The N-terminal domain of Grb7 comprises the first 100 residues of Grb7. It is a proline rich motif comprising a conserved sequence made of residues PS/AIPNPFPEL and is likely to exist as an unstructured domain in the absence of a binding partner. Up until now little has been known as to the binding partners of Grb7 via its N-terminal domain. Recently, however, experiments have indicated novel potential binding partners. For instance, mouse Grb7 has been shown to interact with HuR (Tsai et al., 2008), an RNA-binding protein important in regulation of nuclear-to–cytoplasmic shuttling of mRNA (Doller et al., 2008). The Grb7-HuR interaction is found to be mediated by the N-terminal domain of Grb7 (Tsai et al., 2008). Moreover, mouse Grb7 is also found to bind RNA via its proline rich N-terminal domain (Doller et al., 2008). These interactions have yet to be verified for human Grb7 and their physiological function remain to be elucidated.

4.2 The central GM region

The region of Grb7 bounded by the N- and C-terminal domains is what is referred to as the central GM, for Grb and Mig domain (Han et al., 2001). It consists of the longest stretch of Grb7 protein comprising about 300 amino acid residues. Its role in Grb7 signalling is much more studied and better known compared to the N-terminal domain. It characteristically displays more than 50% sequence similarity with the *Caenorhabditis elegans* protein Mig-10 (Manser et al., 1997; Ooi et al., 1995) from which it derives part of its name. Mig-10 (Migratory-10) is established to be critical for cell migration during embryogenesis (Manser et al., 1990). The presence of such a conserved sequence with known function lured researchers to investigate the role of Grb7 in cell migration, which predictably was proven to be the case. It is postulated that it is this domain that makes Grb7 an important cell migratory protein (Siamakpour-Reihani et al., 2009; Shen et al., 2002). The GM region is known to comprise three well conserved but non-contiguous domains: Pleckstrin homology (PH) domain, RA (Ras-associating) domain and a BPS (between PH and SH2) domain (Stein et al, 1994; Margolis et al., 1994).

The PH domain contains is a 110 amino acid long domain corresponding to residues 229-338 of Grb7. It is suggested to bind membrane bound phosphoinositides, thereby assisting Grb7's association to these molecules. Moreover, it is found to interact with FHL2, a signalling protein important in transcription regulation and cytoskeletal re-arrangement (Siamakpour-Reihani et al., 2009). Recently it is also reported to interact with Hax-1 (Hs-1 Associated protein X-1), another protein important in cell migration and apoptosis, and to explain a role for Grb7 dimerization in a head to tail manner (Siamakpour-Reihani et al., 2009, 2010). As a part of the GM region, it is proposed to play a role in cell migration

The RA domain of Grb7 is 87 amino acids long stretching from residues 100 to 186. Along with the PH domain, it is found to be important in the intramolecular dimerization of Grb7 by interacting with the SH2 domain, where the interaction is found to occur with micromolar affinity (Siamakpour-Reihani et al., 2010; Depetris et al., 2009). The phenomenon of dimerization is an important mechanism for the functioning of Grb7 (Porter et al., 2005). The RA domain is found in a number of proteins. It is also suggested to have a role in the involvement of Grb7 in Ras signalling pathway and for cell proliferation (Stein, et al, 2001). Together with the PH domain, the RA domain is reported to interact with the Hax-1 protein (Siamakpour-Reihani et al., 2010).

The BPS domain is a functional region of about 65 residues corresponding to residues 365-413 of Grb7. It is found between the PH and SH2 domain. The BPS region is thought to facilitate the interactions of SH2 domain to upstream partners of Grb7 (Stein, et al, 2001). Moreover, it is suggested the BPS domain could contribute to the specificity of Grb7 binding to its partners (Stein et al., 2003; Scharf et al., 2004). In the other Grb7 families such as Grb10 and Grb14, the BPS domain is found to interact with the activated IR and IGFR (He et al., 1998). It displays up to 60% sequence similarity among the Grb7 family members, The BPS domain is found to be intrinsically unstructured (Moncoq et al., 2003), though a very short structured stretch of about 9 residues was identified for Grb14 protein (Moncoq et al., 2004).

4.3 The C-terminal domain

By far the most widely investigated and thoroughly characterized region of Grb7 is the C-terminal Src homology 2 (SH2) domain (Pawson, 1994; Daly, 1997). It corresponds to residues 415-535 of the Grb7 protein. SH2 domains are phosphotyrosine peptide binding modules that are also found in a number of related proteins (Janes et al., 1997). Indeed, the discovery of Grb7 as an adaptor protein was dependent on this property of the SH2 domain. It is known to mediate the physical association Grb7 with a diverse array of membrane bound and cytoplasmic binding partners of Grb7 (Daly et al., 1998; Margolis et al., 1992). In particular, the SH2 domain is responsible for the recognition of specific phosphotyrosines (pTyr) residues via a well-described cationic pocket and surrounding peptide-binding cleft (Janes et al., 1997). The SH2 mediated association of Grb7 with its binding partners commences the first step in Grb7 dependent signal transduction. As such it forms an essential module for the variety of Grb7 mediated oncogenic transformations (Daly et al., 1998; Pero et al., 2003).

The binding specificity of the SH2 domain to upstream partners of Grb7 has been studied and characterized at length (Margolis et al., 1992;Daly et al., 1998). These studies have deciphered the sequence around the phosphorylated tyrosine recognized by Grb7 to be more or less conserved, from which a recognition motif of the sequence pYXN has been established. In other words, the presence of asparagine at a +2 position relative to phosphorylated tyrosine (pY) is what the SH2 domain of Grb7 specifically demands of its binding partners (Margolis et al., 1992; Daly et al., 1998). The position at +1 to the pY residue, indicated as X, is where any amino acid would be tolerated. This condition for recognition by SH2 domain is found in the great majority of established Grb7 binding partners including erbB2 (Stein et al., 1994) [60], Tek(Jones et al., 1999), c-Kit (Thömmes et al., 1999), SHPTP (Keegan et al., 1996), Shc(Frantz et al., 1997), PDGFR (Yokote et al., 1994). However, some exceptions has been noted where the +2 Asparagine is not required as in FAK (pYAE) (Han et al., 2001), EphB1(pYRD) (Han et al., 2002), cavoelin (pYRD)

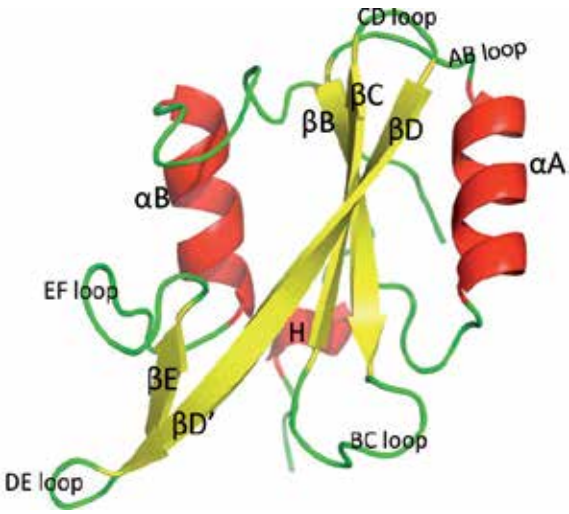

Fig. 4. Domain fold of Grb7 SH2 protein. Cartoon representation of the Grb7 SH2 domain shown as helix (red); B-sheet (yellow) and loops (green) and the structural motif labeling.

(Lee et al., 2000) or where the tyrosine does not have to be phosphorylated as in RndI(YDN)(Vayssière et al., 2000). Typically, the YXN recognition stretch is known to bind in a turn conformation as is established for a number of Grb2 antagonist peptides reported.

The experimental structure of Grb7 SH2 domain has been solved both by NMR and X-ray crystallography (Porter et al., 2007; Ivancic et al., 2003). The crystal structure is solved to 2.1 Å resolution with an overall tetrameric assembly by our group (PDB ID: 2QMS). As shown in Fig. 4, the Grb7 SH2 domain comprises two pairs of anti-parallel β-sheets flanked by a pair of α-helices (Porter et al., 2007). Such a structure is a general feature of SH2 domain proteins (Pawson, 1994; Margolis et al., 1994). According to the accepted nomenclature (Margolis et al., 1994), the central anti-parallel β-sheet is formed by the βB, βC and βD loops where as the two α-helices are labeled αA and αB (see Fig. 4 for details) which implies that the domain fold of Grb7 could be described as αAβBβCβDαB.

5. The development of Grb7 antagonists

Grb7 has become a promising target in pancreatic and other human cancers. Though Grb7 is a multidomain protein, most of the inhibitor development efforts are focused on the identification of agents that interact with its SH2 domain. This is because the SH2 domain commences the first and hence the fate determining step in the entire process of Grb7 dependent signalling (Holt et al., 2005). Moreover, the SH2 domain possesses a well defined and characterized binding pocket amenable to a variety of ligand design efforts (Porter et al., 2007). In addition, the requirement of the SH2 domain to bind to Grb7's myriad of upstream partners is generally conserved (Margolis et al., 1994; Han et al., 2001) where a minimal recognition motif is put forth. These factors endow the SH2 domain as an attractive module to target in the development of Grb7 based therapeutic agents. Hence all the Grb7 antagonists identified are specifically designed to act on the SH2 domain of the protein.

5.1 Polypeptide antagonists of Grb7

Inspired by the conservative motif requirement of Grb7 SH2 domain to bind its upstream binding partners, Pero et al conducted a PHAGE display experiment to identify the first polypeptide antagonist of Grb7 (Pero et al., 2002). The peptide was initially discovered as a 19 residue polypeptide flanked by Cys residues at positions 1 and 11. Interestingly, it was shown that the peptide was inactive in its open form and cyclization via disulphide formation was necessary for activity against Grb7. The original 19 residue structure was then simplified by removing residues outside the two Cys residues and the disulfide linkage was replaced with a thioether moiety to effect the ring closure. This 11 residue cyclic polypeptide, named G7-18NATE (sequence: WFEGYDNTFPC), was tested and proved to posses the same affinity as the larger disulfide containing form (Pero et al., 2002). An important attribute of this lead peptide is its selectivity for Grb7 and the fact that it is not phosphorylated. The chemical structure of G7-18NATE is displayed in Fig. 5.

The binding affinity of the G7-18NATE prototype peptide has been characterized extensively by isothermal titration calorimetry (Porter et al., 2007; Spuches et al., 2007; Ambaye et al., 2011a), surface plasmon resonance (Gunzburg et al., 2010) and ELISA assays (Luzy et al., 2008). Such investigations provide invaluable information that should guide the

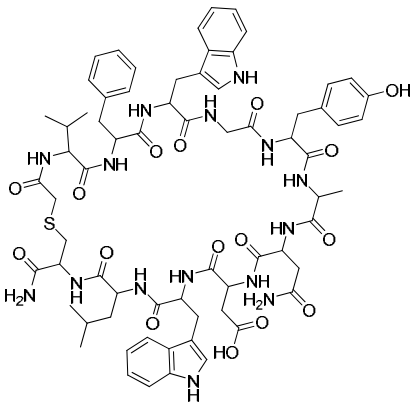

Fig. 5. Chemical structures of G7-18NATE lead polypeptide Grb7 antagonist.

further optimization of this lead polypeptide. The three ITC experiments concur on the affinity of the peptide for Grb7 SH2 domain (34.5 μM, 13.5 μM, 35.7 μM) indicating moderate affinity binding. Though this represents a breakthrough in Grb7 antagonist development, the affinity is not sufficient for animal experimentation. In other words, further optimization is necessary to transform the peptide into a clinical candidate. An important clue in this regard is afforded by isothermal titration calorimetry where deconvolution of the binding affinity into its components shows that the binding of G7-18NATE is enthalpically driven and entropically forbidden. This appears in line with the observation that the open form of G7-18NATE is devoid of any antagonistic activity (Pero et al., 2002). This knowledge could help in optimizing the lead peptide structure so as to improve the affinity.

Grb7 is an intracellular protein. Since G7-18NATE is a polypeptide, the plasma membrane represent a potential obstacle for it use in cellular systems. However, this has been overcome by the use of other peptides known to assist in crossing biological membranes. For this purpose, G7-18NATE was synthesized with a 19 residue long cell-penetrating sequence termed Penetratin for in vivo studies (see Fig. 6). The cell proliferation and migration inhibition assay conducted with this cell permeable derivative (G7-18NATE-Penetratin) demonstrate the combined effect of membrane crossing (Penetratin) and Grb7 inhibition (G7-18NATE) (Tanaka et al., 2006; Pero et al., 2007). Another cell-penetrating peptide with an 11 residue arginine rich sequence was also investigated for the cell permeablising effect (Pero et al., 2007). Both peptides were shown to have a synergistic effect with Doxorubicin in decreasing cancer cell proliferation. A related experiment conducted on pancreatic cancer cell migration effect clearly established the potential of G7-18NATE in diverse cancer cell lines (Tanaka et al., 2006). Our experience with G7-18NATE is that the length of the Penetratin tail can be cut short and still enter cells (Ambaye et al., 2011a). A Penetratin sequence consisting of only the last 8 residues was sufficient for membrane translocation of G7-18NATE. The cytoplasmic localization of G7-18NATE with this short penetratin was also confirmed (Ambaye et al., 2011a). Moreover, the possible interference on binding of G7-18NATE by the short Penetratin sequence was investigated by ITC and shown not to impede G7-18NATE binding. These experiments demonstrate that comparable in vitro-in vivo correlations can be achieved with the use of a shorter Penetratin.

Most recently the structure of the G7-18NATE peptide was determined in complex with the Grb7-SH2 domain using X-ray crystallography (Ambaye et al., 2011c). This revealed the

critical residues involved in binding the Grb7-SH2 domain, and their conformational arrangement. The same study also reported other phage-display derived Grb7-SH2 binding peptides with similar binding affinities for Grb7-SH2 domain as the lead G7-18NATE. These peptides all possessed the amino acid residues shown by the structure to be critical for binding by the structural study. This information will help to guide the design of future peptides with improved affinity and maintained specificity for Grb7.

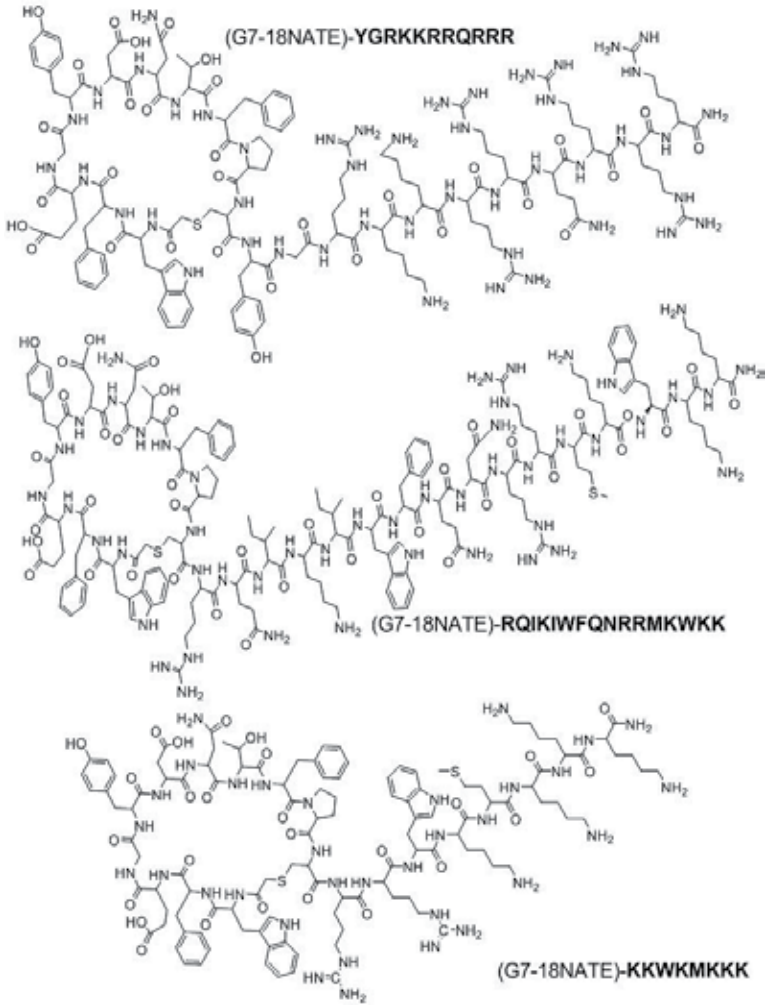

Fig. 6. Sequence and Chemical Structures of cell permeable G7-18NATE derivatives (Pero et al., 2007; Tanaka et al., 2007). One letter amino acid symbols in the sequence is indicated in bold fonts.

5.2 Short peptide antagonists of Grb7

Other short phosphorylated peptides based on the consensus recognition motif have also been developed and tested [Howl et al., 2007), see Fig.7. This includes peptides based on the sequence around the phosphotyrosine residue of erbB1, erbB2, erbB3 and ephB1 on Grb7

upstream binding partners. The phosphorylated peptides range from 6 to 11 residues with a dissociation equilibrium constant varying from 0.6 μM to 366 μM. Finally, peptides that were previously reported as Grb2 antagonists were tested for their inhibitory effect on Grb7. Interestingly, the results show that not only do the peptides retain the Grb7 inhibitory effect, but that the activity rank is maintained on both Grb2 and Grb7 antagonism, though a quantitative difference is observed (Spuches et al., 2007). These peptides, unlike G7-18NATE, do not show selectivity for Grb7.

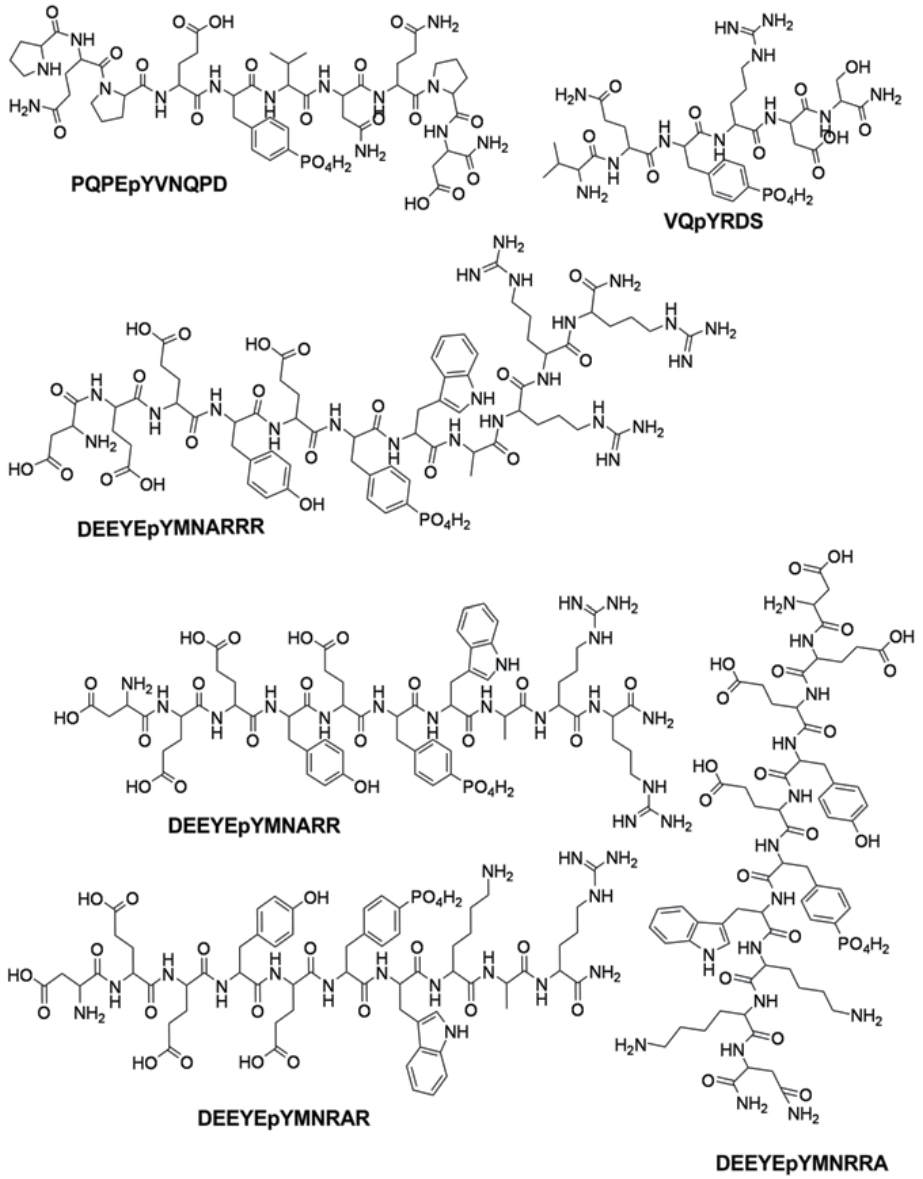

Fig. 7. Sequence and chemical structure of phosphorylated peptide antagonists of Grb7 (Howl et al., 2007). One letter amino acid symbols in the sequence is indicated in bold fonts.

5.3 Small molecule antagonists of Grb7

Recently, we embarked upon identifying small molecule antagonists of Grb7 (Ambaye et al., 2011b). In particular, the availability of the structure of peptides bound to SH2 domains has allowed us to apply series of computational chemistry approaches to idenitify potential antagonists of Grb7. Fig. 8 shows the structures of the most potent antagonists. The binding activity was examined first with ThermoFluor based denaturation followed by full thermodynamic characterization by isothermal titration calorimetry. This correlated with growth inhibition of Grb7 oversexpressing cancer cells. The result indicates near equivalent micromolar affinity values indicating the potential of non-peptide structures in cell based experiments. Fig. 8 show the benzopyrazine based antagonists of Grb7.

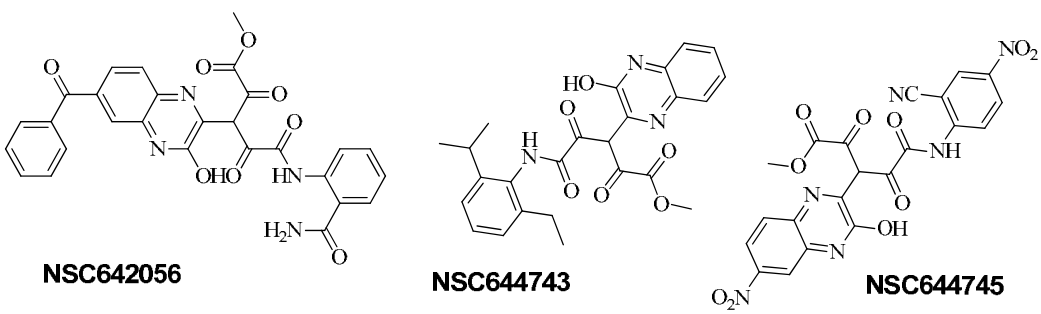

Fig. 8. Small molecule inhibitors of Grb7 (Ambaye et al., 2011b).

6. Conclusion and future outlook

Pancreatic cancer remains one of the leading causes of morbidity and mortality with the impact expected to rise in our aging societies. The limited efficacy and intolerable toxicity of available treatments means novel drugs with a novel mechanism of action are always sought. From the clinical standpoint, new targets provide novel drugs, novel mechanistic bases and potentially more efficacious means to treat diseases. Given its established role in malignancies such as pancreatic cancer and the fact that there is no drug that acts on it, Grb7 based drug development is likely to be a promising endeavour in the foreseeable future.

Grb7 is found a diverse array of signalling events critical for carcinogenetic transformation of human cells. Its druggablity is proven with the use of synthetic peptides, and an excellent start is made with the discovery of a peptide with specificity for Grb7. The major challenge for the use of peptide based drugs, however, comes from its intracellular localization. The recent development of cell permeable Grb7 antagonists is encouraging, and suggests that the permeability issue could be surmounted with little extra effort. Remaining issues to be

solved, however, include the need for a higher affinity peptide and the generally poor stability and metabolism of peptide-based drugs. Though several challenges are still ahead, the data obtained so far seem strongly encouraging to pursue Grb7 based anti-tumour drug development.

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22

Human Telomerase Reverse Transcriptase Gene Antisense Oligonucleotide Increases the Sensitivity of Pancreatic Cancer Cells to Gemcitabine *In Vitro*

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1. Introduction

Resistance to gemcitabine is the major problem in pancreatic cancer chemotherapy, and recent evidence suggests that down-regulation of hTERT mRNA could enhance the antitumor efficacy of other well known chemotherapy agents targeting DNA. The aim of this study was to evaluate the combined antitumor efficacy of antisense oligonucleotides (AS-ODN) targeting hTERT mRNA and gemcitabine in human pancreatic cancer cells. Our results showed that transient transfection in clones of the human pancreatic cancer cell lines BxPC-3 and Panc-1 with 0.2μM hTERT AS-ODN for 24 h diminished the abundance of hTERT mRNA and inhibited telomerase activity, but only resulted in a slightly attenuated ability of proliferation. While pretreatment with 0.2μM AS-ODN for 24 h followed by gemcitabine in BxPC-3 or Panc-1 cells led to tumor cell growth suppression more significantly than gemcitabine alone in MTT, and the IC50 of gemcitabine was reduced to about 8.7 times in Panc-1 cells, and 4.2 times in BxPC-3 cells. Likewise, after treatment with gemcitabine for 48 h, the AS-ODN-transfected cells exhibited significantly decreased colony formation ability relative to the parental cells. Apoptosis analysis indicated that hTERT AS-ODN increased the gemcitabine-induced apoptosis in both cell lines. All together, these findings implied that hTERT AS-ODN could increase the chemosensitivity of gemcitabine through down-regulation of hTERT mRNA expression and inhibition of telomerase activity, which may make it an attractive agent for the sensitization of pancreatic cancer cells to gemcitabine.

Pancreatic cancer is one of the most common causes of cancer death in the world. Surgery is the only chance for cure, unfortunately, late diagnosis often results in less than 20% of patients for tumor resection [1,2]. Gemcitabine, a novel pyrimidine nucleoside analogue, has become the standard first-line chemotherapeutic agent used in patients with pancreatic cancer [1]. It is, however, moderately effective, showing a tumor response rate of only 12% [3] and a median survival time of 5 months [4]. Increasing the susceptibility of pancreatic

cancer cells to gemcitabine, therefore, is of importance to the outcome of therapy. In order to investigate the mechanisms of gemcitabine -resistance, multiple mechanisms have been proposed, including enhanced NF-kB activation [5], increased activity of Src tyrosine kinase and expression of the M2 subunit of ribonucleotide reductase (RRM2) [6], deficiency in deoxycytidine kinase (dCK) [7], and altered transport over the cell membrane [8]. Recent studies indicate that acquired gemcitabine resistance in pancreatic cancer cells may be mainly attributed to an altered apoptotic threshold [9].

Telomerase is an RNA-dependent DNA polymerase that is rarely present in normal somatic cells but is observed in 85% of all cancer cells tested, making the telomerase enzyme an attractive target for anticancer therapeutics [10,11]. The human telomerase is composed of a constitutively expressed RNA subunit (hTR), human telomerase-associated protein (TEP1) and a catalytic protein subunit (hTERT). The protein subunit hTERT is a reverse transcriptase, and hTERT expression is the rate-limiting component of the telomerase complex and therefore determines telomerase activity [12]. The main function of the telomerase is the lengthening and capping of the ends of linear chromosomes, the telomeres [13-15].Uncapped or critically shortened telomeres cause cell apoptosis (15). Many labs have reported that telomerase may play an active role in the response to DNA damaging agents [16-18], and could been implicated in suppression of apoptosis [19]. It has also been demonstrated recently that antisense-mediated down-regulation of hTERT quickly induced programmed cell death in human tumour cells [20-23] and sensitized cancer cells to DNA damaging agents through the activation of the apoptotic program [24-26]. For pancreatic cancer, it was not known so far whether hTERT mRNA silencing leads to sensitization to gemcitabine as the standard of care for pancreatic cancer.

In this study, sequence-specific antisense oligonucleotides targeting the coding region of the protein component of human telomerase were designed to examine whether hTERT mRNA and telomerase activity could be inhibited and chemosensitivity to gemcitabine could be increased in pancreatic cancer cells.

2. Materials and methods

2.1 Oligonucleotides and drug

Based on the hTERT gene cDNA sequence (4015 nt; accession no. AF015950), the antisense oligonucleotide was designed to be complementary to the translation initiation region of hTERT mRNA; the antisense oligodeoxynucleotide sequence (AS-ODN) is 5′-GGAGCGCGCGGCATCGCGGG-3′; Non-specific oligodeoxynucleotide sequence (NS-ODN) is 5′-CATTTCTTGCTCTCCACGCG-3′as a control, having the same base number as the antisense oligonucleotide but with different sequence. All oligodeoxynucleotides were fully phosphorothioate, and were synthesized by Invotrogen (Carlsbad, CA, USA). Their lack of significant interfering homology was validated using BLAST analysis. Gemcitabine was obtained from Eli Lilly, and the dilutions of gemcitabine were freshly prepared before each experiment.

2.2 Cell culture and transfection

Pancreatic cancer cell lines BxPC-3 and Panc-1 were kindly provided by the center laboratory of the Second Hospital of ChangZhou in China, and were routinely incubated in

DMEM (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37℃ in a humidified chamber of 95% air containing 5% CO2. The transfection procedure of oligonucleotides(ODN) was performed according to the user manual of Oligofectamine TM Reagent(Invitrogen, Carlsbad, CA, USA). Briefly, The cells were seeded the day before the experiment in different culture plates at different density per well at 30% to 50% confluence on the day of the experiment, and then were transfected with 0.2μM of Oligofectamine and 0.2μM of oligonucleotides (ODN) in the serum-free DMEM, incubated at 37℃ for 4 hr, and then added different volume of growth medium containing 3× the normal concentration of serum according to the different culture plates without removing the transfection mixture. To assess ODN uptake, pancreatic cancer cells were transfected with the FITC-labeled ODN, and then Flow cytofluorometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) was used to quantify FITC -positive cells at defined times after transfection.

2.3 Quantitative RT-PCR

Pancreatic cancer cell lines BxPC-3 and Panc-1 were harvested with trypsin, washed with PBS, and collected by centrifugation at 1,000 rpm for 5 min. Total RNA was extracted using SV Total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's protocol. And its purity and quality were measured by Bio-visible spectrophotometer (Eppendorf, Germany); 1% agarose gel electrophoresis was used to assess the integrity of the obtained RNA. cDNA with a total volume of 20μl was synthesized using the reverse transcription system containing reverse transcriptase (Promega, Madison, WI, USA) according to the recommended protocol by the manufacturer. Real-time quantitative PCR of the target hTERT gene and β-actin as internal control was carried out with icycler iQ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The 20 μl PCR reaction mixture contained 1× primers and probe mixture [Applied Biosystems, Foster city , CA. Assay IDs: Hs99999022_m1 (hTERT); Hs99999903 _m1 (β-actin)], 1× Absolute QPCR Mix (ABgene, Surrey, UK). The PCR conditions were 50°C for 2 min, 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Relative gene expression quantifications were calculated according to the comparative Ct method using β-actin as an endogenous control and commercial human total RNA (BD Clontech, CA, USA) as calibrators. Final results were determined by the formula $2^{-\Delta\Delta CT}$ method[27].

2.4 Telomerase activity assay

A commercial telomerase PCR ELISA kit (Roche Diagnostics. Scandinavia AB, Stockholm, Sweden) was used to determine telomerase activity in cells according to the manufacturer's instructions. Briefly, 5μl amplification product which had been denaturated at room temperature for 10 min with 20μl denaturation reagent was hybridized with a digoxigenin-labeled probe specific for human telomeric repeats. The probe bound to the strand with the labeled biotin at the 5' end. The hybrid was immobilized to a streptavidin-coated microtiter plate via the biotin-labeled primer at 37 ºC on a shaker for 2 h, and washed 3 times. The reaction product was detected with 100μl anti-digoxigeninperoxidase and 100μl peroxidase substrate TMB. Color intensities were measured with a model 450 microplate reader (BIO-RAD) at 450 nm.

2.5 Cell viability assay

Cytotoxicity was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, ODN-transfected and Oligofactamine- transfected cancer cells growing in log-phase were trypsinized and seed at 2×10^3 cells per well into 96-well plates and allowed to attach overnight. Medium in each well was replaced with fresh medium or medium with various concentrations of drug in at least 6 replicate wells and left contact for 48 h. One-fifth volume of CellTiter 96 AQueous One Solution was added to each well and incubated for an additional 3 h, Absorbance was determined with a microplate reader (BIO-RAD) at 490 nm. The blank control wells were used for zeroing absorbance. Each experiment was allocated ten wells containing drug-free medium for the control. The inhibition rate (I %) was calculated using the background-corrected absorbance by the following equation: $I\% = 100\times (A_{\text{untreated control well}} - A_{\text{experimental well}}) / A_{\text{untreated control well}}$. The IC50 was defined as the concentration required for 50% inhibition of cell growth. Each experiment was performed in triplicate, with representative data presented.

2.6 Colony-forming cell assay

Pancreatic tumor cells were transfected with 0.2μM AS-ODN or NS-ODN for 24 h, and then the transfected cells were treated with gemcitabine at 0.05μM in BxPC-3 cells and 0.8μM in Panc-1cells for 48 h. Subsequently, gemcitabine-treated cells and parental cells (300 cells /well) were plated in triplicate in 60-mm Petri dishes. On day 7, the plates were fixed in 70% methanol and treated with Giemsa stain. Clonogenic survival was determined by counting the macroscopically visible colonies.

2.7 Apoptosis assay

Cells quantification of apoptosis cells was performed using an Annexin-V- FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were plateded in a 60-mm Petri disk and treated with drugs for 48h .Then cells were collected and resuspended in 500μl of binding buffer, and 5μl of Annexin- V-fluorescein isothiocyanate (FITC) and 5μl of propidium iodide (PI) were added. Analyses were performed with a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.8 Statistical methods

Values were expressed as means ±standard deviations. Statistical comparison was performed using Student's ***t***-test, and a ***p*** value of less than 0.05 was considered statistically significant.

3. Results

3.1 Assessment of AS-ODN uptake by pancreatic cancer cells

The use of 0.2μM Oligofectamine allowed a very efficient internalization of ODNs already after 4 h of transfection (> 20% FITC-positive cells), and at 24 h, the fluorescence intensity in both cell lines reached the strongest (> 30% FITC-positive cells), and then gradually decreased (data not shown). In contrast, transfection without Oligofectamine resulted in

<10% FITC-positive cells. After a 24-h transfection, the percentages of FITC-positive cells could attain to 33.6% in BxPC-3 cells and 41.8% in Panc-1 cells.

3.2 hTERT antisense oligodeoxynucleotide(AS-ODN) down-regulates hTERT mRNA expression and telomerase activity of pancreatic cancer cells

We first examined the mRNA expression of hTERT mRNA in BxPC-3 and Panc-1 using quantitative RT-PCR. The expression levels of hTERT mRNA in Panc-1 cells was higher than that in BxPC-3 cells ($p < 0.001$) (Fig. 1). We further examined whether hTERT AS-ODN could downregulate the expression levels of hTERT mRNA in both cell lines. As shown in Fig. 1, treatment with 0.2μM hTERT AS-ODN for 24 h down-regulated the levels of hTERT mRNA in BxPC-3 to 29 % and in Panc-1 cells to 35 %, relative to the Oligofectamine- treated control. While the same concentration of NS-ODN sequence did not down-regulate the levels of hTERT mRNA expression in both cell lines. We also examined the effects of gemcitabine on the levels of hTERT mRNA expression, and the results showed that gemcitabine alone at IC50 for 24 h only led to moderate down-regulation of hTERT mRNA in BxPC-3 cell lines and slight up-regulation of that in Panc-1 cell lines. Additionally, we examined the effect of suppressing hTERT mRNA on telomerase activity. We found that NS-ODN control clones showed significant telomerase activity, equal to parental cells, whereas 0.2μM AS-ODN

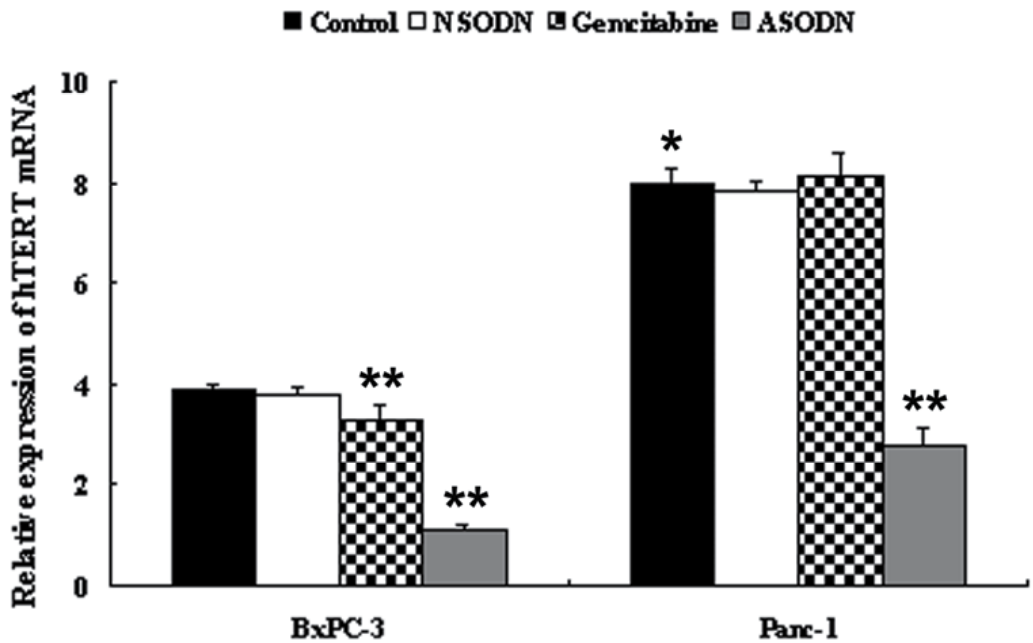

Fig. 1. Sequence-specific suppression of hTERT mRNA by hTERT antisense oligonucleotide in BxPC-3 cells (A) and Panc-1 cells (B). Both cell lines were treated with 0.2μM ODN or gemcitabine at IC50 for 24 h. Cells were harvested for RNA analysis after 24 h incubation. Relative gene expression quantifications were calculated according to the comparative Ct method. Final results were determined by the formula $2^{-\Delta\Delta CT}$ method. Values represent means ± SD, from three independent experiments; *$p < 0.001$ vs oligofectamine transfected BxPC-3 control cells. **$p < 0.05$ vs oligofectamine transfected control group.

clones expressed significantly decreased levels of telomerase activity in both cell lines (Fig. 2) at 24 h as assessed by TRAP-ELISA Assay. At the same time, we found the level of telomerase activity in Panc-1 parental cells was higher than that in BxPC-3 parental cells (p=0.003), and gemcitabine at IC50 for 24 h moderately down-regulated the telomerase activity in both cell lines(data not shown).

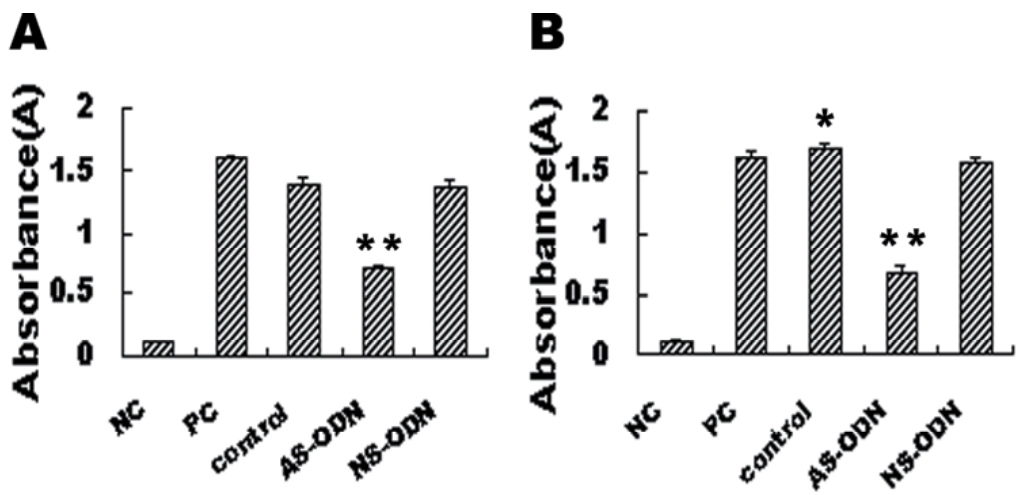

Fig. 2. Telomerase activity in transfected cells of BxPC-3 (A) and Panc-1 (B). Telomerase PCR ELISA was performed 24 h after transfection in triplicates. Values represent the mean Absorbance ± SD, from three independent experiments; *p < 0.001 vs oligofectamine transfected BxPC-3 control cells. **p < 0.001 vs oligofectamine transfected control group.

3.3 hTERT antisense oligodeoxynucleotide (AS-ODN) increases gemcitabine-induced cytotoxicity in pancreatic cancer cells

We next investigated whether the addition of hTERT AS-ODN could indeed increase gemcitabine sensitivity. BxPC-3 and Panc-1 cells were treated with gemcitabine in the presence of 0.2μM hTERT AS-ODN at different concentrations. Fig. 3 shows the IC_{50} value of gemcitabine in BxPC-3 and Panc-1 cells were 0.23μM and 7.13μM, respectively. That is to say Panc-1 cells were 31-fold more resistant to gemcitabine than BxPC-3 cells, suggesting that the more higher expression of hTERT mRNA or telomerase activity, the more resistant of cancer cells to gemcitabine. hTERT AS-ODN was able to reduce the IC50 of gemcitabine to about 8.7 times in Panc-1 cells, and only about 4.2 times in BxPC-3 cells, suggesting hTERT antisense oligodeoxynucleotide could increase gemcitabine- induced cytotoxicity in both cell lines, and sensitize the gemcitabine -resistant cells. But the same concentration NS-ODN control sequence could not increase gemcitabine-induced cytotoxicity in both cells (data not shown). At the same time, we found AS-ODN treatment at 0.2μM for 24 h resulted in a slightly attenuated ability of proliferation in both cell lines (data not shown), suggesting that a lag phase between telomerase inhibition and growth inhibition and/or cell death may limited the application of telomerase inhibition therapy alone in solid cancer treatment.

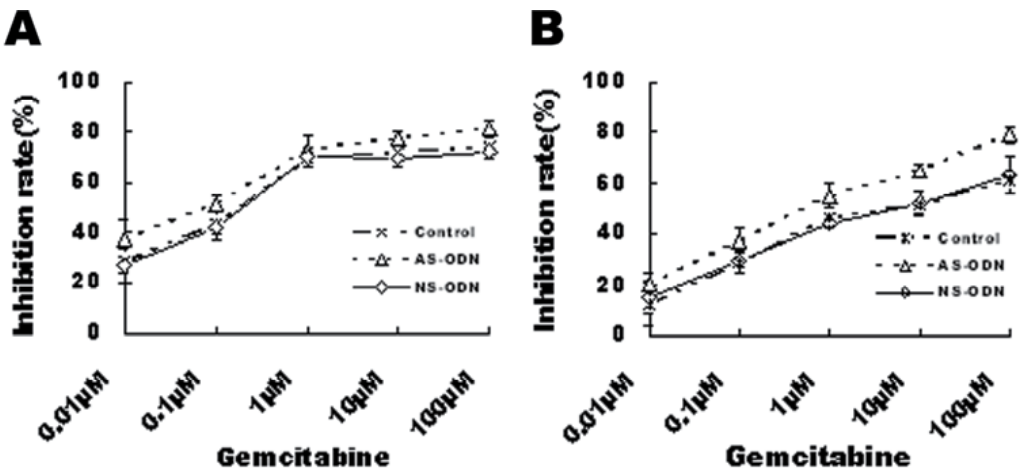

Fig. 3. hTERT antisense oligodeoxynucleotide(AS-ODN) increases gemcitabine-induced cytotoxicity in BxPC-3(A) and Panc-1(B) cells. Briefly, ODN transfected cells and parental cells were treated with gemcitabine at different concentrations. The inhibition rate (I %) was calculated using the background-corrected absorbance by the following equation: I% = 100× ($A_{\text{untreated control well}} - A_{\text{experimental well}}$) / $A_{\text{untreated control well}}$. Values represent the mean inhibition rates ± SD, from three independent experiments, compared to an untreated control cells.

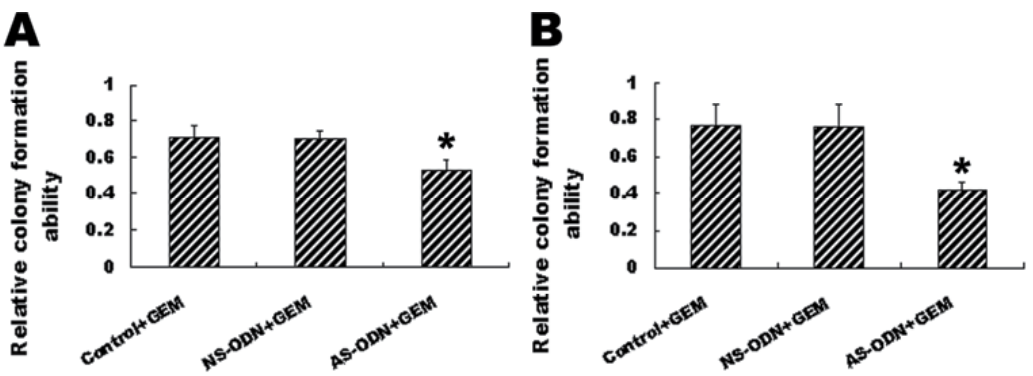

Fig. 4. Cells treatment with gemcitabine (GEM) in presence of AS-ODN exhibited significantly decreased colony formation ability in BxPC-3 (A) and Panc-1(B) cells. Briefly, cells were transfected with 0.2μM AS-ODN or NS-ODN for 24 h, and then the transfected cells and parental cells were treated with gemcitabine at 0.05μM in BxPC-3 cells and 0.8μM in panc-1cells for 48 h. Subsequently, gemcitabine-treated cells and parental cells (300 cells/ well) were plated in triplicate in 60-mm Petri dishes. On day 7, the plates were fixed in 70% methanol and were treated with Giemsa stain. Clonogenic survival was determined by counting the macroscopically visible colonies. The relative colony formation ability normalized to the untreated parental control is displayed. Data represent the mean values ± SD, from three independent experiments. *P<0.05 vs. the group of (control +GEM).

3.4 Cells treatment with gemcitabine in presence of AS-ODN exhibited significantly decreased colony formation ability

To further investigate the combined antitumor efficacy of hTERT AS-ODN and gemcitabine in human pancreatic cancer cells, colony formation ability was evaluated by colony formation assays. The ability of isolated cells to proliferate and generate colonies was clearly reduced in the cells treatment with gemcitabine in presence of AS-ODN, but not in presence of NS-ODN (Fig. 4). In particular, treatment with 0.8μM gemcitabine for 48 h, the AS-ODN-transfected Panc-1 cells showed a remarkable decrease of ≥50% in the relative colony number. Furthermore, after treatment with gemcitabine for 48 h, colonies arising from AS-ODN- transfected cells were smaller than colonies originating from NS-ODN-treated cells or parental control cells.

3.5 hTERT antisense oligodeoxynucleotide increases gemcitabine- induced apoptosis in both cell lines

We further examined whether down-regulation of hTERT mRNA and telomerase activity could increase cytotoxicity of gemcitabine by induction of apoptosis. Gemcitabine single agent treatment at 0.2μM for 48 h resulted in 30.5% of early apoptosis in BxPC-3 cells, and at 7μM for 48 h resulted in 15.8% of early apoptosis in Panc-1 cells, but when 0.2μM AS-ODN was previously added to both cell lines for 24 h, the effects were dramatically increased to 58.5% and 29.2%, respectively. At the same time, the percentages of late apoptosis were increased to 21.3% in BxPC-3 cells and 18.5% in Panc-1 cells. While when AS-ODN was added alone at 0.2μM for 24 h, the percentages of early apoptosis in BxPC-3 and Panc-1 cells were only 8.4% and 5.2%, respectively, and the same concentration of NS-ODN control sequence resulted in the similar percentage of early apoptosis as parental cells (data not shown). Thus, it appears that hTERT suppressing might increase gemcitabine -induced apoptosis in both cell lines and subsequently lead to an increased cytotoxicity of gemcitabine (see Fig. 5).

4. Discussion

Pancreatic cancer has a poor prognosis, even after curative resection. Gemcitabine is established as the reference treatment for pancreatic cancer patients [28]. However, clinical efficacy with gemcitabine as a single agent remains poor. Gemcitabine-based combinations are needed to improve outcomes. In the present study, we evaluated the effect of a combined gemcitabine and antisense hTERT gene therapy on tumor growth in human BxPC-3 and Panc-1 pancreatic cancer cell lines in vitro. We initially demonstrated that an AS-ODN complementary to the translation region of hTERT mRNA inhibited the expression of hTERT mRNA and telomerase activity in both cell lines, while gemcitabine alone resulted in only moderate down-regulation of hTERT expression in BxPC-3 cells and slight up-regulation of hTERT expression in Panc-1 cell lines. Then we demonstrated that down-regulation of the human telomerase reverse transcriptase mRNA and inhibition of telomerase activity by AS-ODN could sensitize both cell lines to gemcitabine, leading to enhanced cytotoxicity in vitro. These consequences suggest that the anti-proliferative effect of the combination gemcitabine and antisense hTERT therapy in human pancreatic cancer are mediated through the down-regulation of hTERT mRNA and inhibition of telomerase activity. These findings also make an antisense technology for hTERT inhibition therapy an attractive approach for the sensitization of pancreatic cancer cells to gemcitabine.

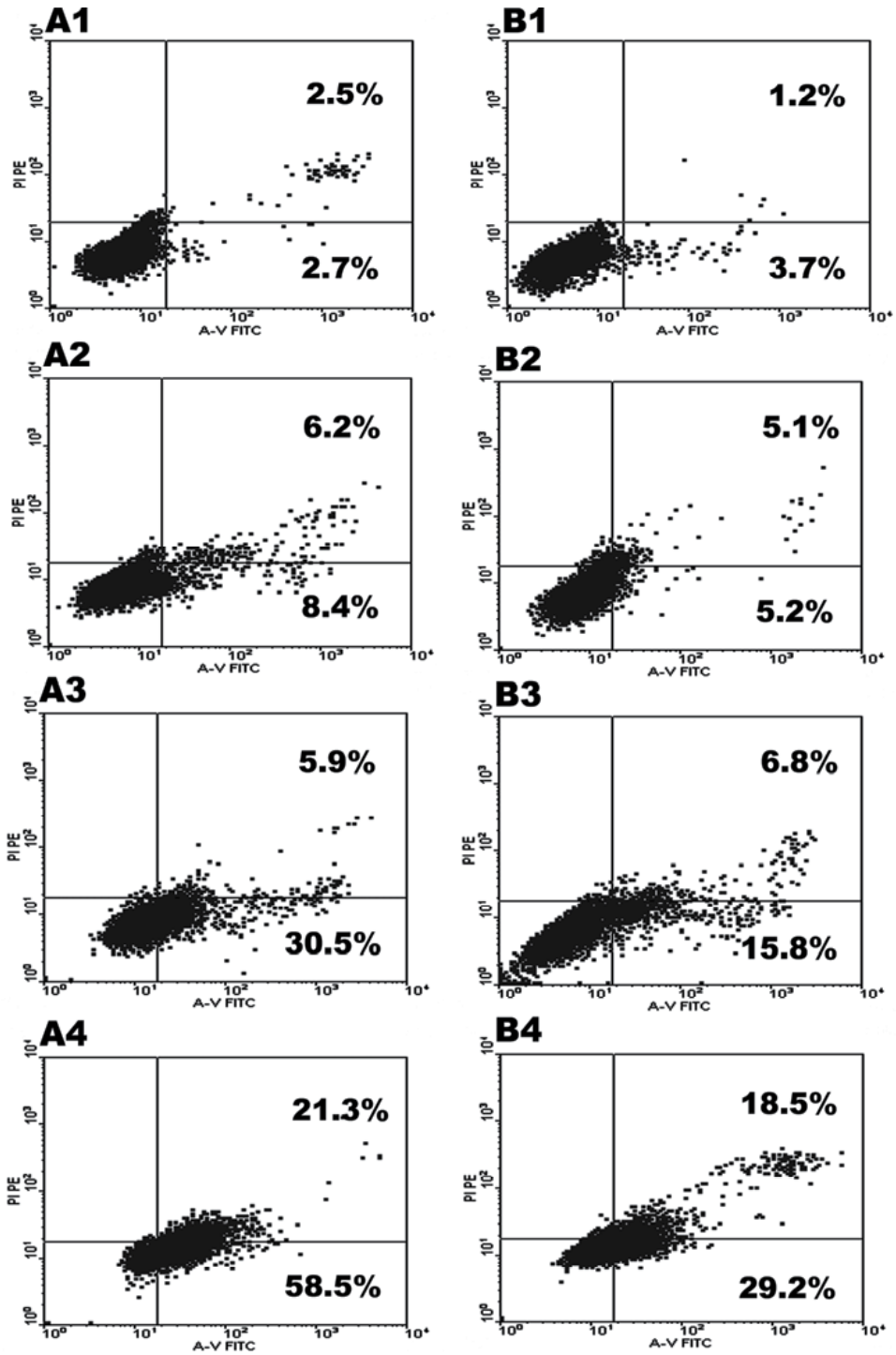

Fig. 5. hTERT Antisense oligodeoxynucleotide (AS-ODN) increases gemcitabine-induced apoptosis in BxPC-3 (A) and Panc-1(B) cells. Cells quantification of apoptosis cells was

performed using an Annexin-V- FITC Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, cells were plateded in a 60-mm Petri disk and treated with drugs for 48 h .Then cells were collected and resuspended in 500μl of binding buffer, and 5μl of Annexin-V-fluoresce in isothiocyanate (FITC) and 5μl of propidium iodide (PI) were added. Analyses were performed with a flow cytometer. A1, B1: both cell lines were treated without drug.A2, B2: both cell lines were treated with AS-ODN alone.A3, B3: both cell lines were treated with gemcitabine alone.A4, B4: both cell lines were treated with AS-ODN for 24 h followed by gemcitabine treatment for 48 h. Early apoptotic cells are defined as Annexin V-positive, PI- negative cells, late apoptotic cells are defined as Annexin V-positive, PI- positive cells.

Telomerase is a ribonucleoprotein enzyme responsible for lengthening and capping the ends of linear chromosomes, the telomeres [13-15]. Telomerase activation is required for the survival and proliferation of the large majority of tumor cells. Uncapped or critically shortened telomeres cause cellular responses such as inhibition of cell proliferation and apoptosis. It is currently unclear how telomerase is regulated in human cancer cells. Previous studies indicated that telomerase activity is strongly correlated with the abundance of hTERT mRNA but not the hTER[29-31], and ectopic expression of hTERT in somatic cells is sufficient to restore telomerase activity[32-35].Thus, strategies targeting hTERT may be a new approach for inhibition of telomerase activity and gene therapy of cancer. Recent studies indicate that down-regulation of hTERT expression or expression of dominant -negative hTERT could inhibit telomerase activity and prevent the malignant proliferation of tumor cells after considerable passages in culture [21,36-38]. In our experiments, we have demonstrated that treatment of pancreatic cancer cells with hTERT AS-ODN could down-regulate the levels of hTERT mRNA expression, inhibit the telomerase activity, but result in a slightly attenuated ability of proliferation in both cell lines. In fact, as human cells reduce their telomere length by 50-100 base pairs per cell division, a long lag phase is required before growth arrest can be obtained, even in cancer cells with relatively short telomeres [10,39]. Thus , in present study, the moderate anticancer efficacy of hTERT AS-ODN in both cell lines may be independent of telomere shortening ,but partially dependent of the loss of the hTERT-mediated capping function of telomerase [40].

It was obvious that anti-telomerase therapy alone was not the best selection of cancer treatment for its requiring long time to reduce the telomere length [10,39].However, transiently transfection of hTERT AS-ODN may enhance the anticancer efficacy of other well known chemotherapy agents targeting DNA [25,26]. Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a synthetic pyrimidine nucleoside analogue, the diphosphate (dFdCDP) and triphosphate (dFdCTP) forms of the drug play an important role in the cytotoxic effect: dFdCDP is an inhibitor of ribonucleotide reductase, while dFdCTP is incorporated into DNA, both leading to the inhibition of DNA synthesis and making genomic instability[41]. Thus, we speculate that hTERT mRNA silencing may lead to sensitization of pancreatic cancer cells to gemcitabine. Our study showed that hTERT AS-ODN significantly increased the gemcitabine- induced cytotoxicity in both cell lines, especially sensitize the gemcitabine -resistant cells. Apoptosis test further demonstrated that hTERT suppressing could increase gemcitabine-induced apoptosis in both cell lines, but the same concentration NS-ODN control sequence could not increase gemcitabine-induced cytotoxicity in any of pancreatic cancer cell lines. The similar results were also acquired by

other report in the bladder cancer cell lines [26]. Assessment of AS-ODN uptake showed that Panc-1 cells are more easily to be transfected with Oligofectamine than BxPC-3 cells, which might partly explain why the sensitizing effects of hTERT AS-ODN on Panc-1 cells are more obvious than on BxPC-3 cells.

It is not clear of the relationship between the expression levels of telomerase activity or hTERT mRNA and chemotherapy resistanse. In our study, we initially found that the expression levels of hTERT mRNA and telomerase activity in Panc-1 cells were higher than those in BxPC-3 cells. Then we found Panc-1 cells were 31-fold more resistant to gemcitabine than BxPC-3 cells. It seems that the more higher expression of hTERT mRNA or telomerase activity, the more resistant of cancer cells to gemcitabine. Our following study showed that down- regulation of hTERT mRNA and telomerase activity could increase the sensitivity of cancer cells to gemcitabine, especially could restore the sensitivity of gemcitabine-resistant cells to gemcitabine, which indirectly denmonstrated hTERT mRNA or telomerase may be implicated in gemcitabine resistance. Xi and his associates introduced vectors encoding dominate negative (DN)-hTERT, wild-type (WT)-hTERT, or a control vector expressing only a drug-resistance marker into HeLa cells. Results showed that DN-hTERT transfected HeLa cells with shortened telomeres were more susceptible to multiple chemotherapeutic agents and radiation. WT-hTERT transfected HeLa cells with longer telomeres exhibited resistance to radiation and chemotherapeutic agents [42]. Our results showed that at least in part, gemcitabine resistance was associated with the high expression of hTERT mRNA or high telomerase activity.

In conclusion, our results demonstrate that down-regulation of hTERT mRNA and inhibition of telomerase activity by hTERT AS-ODN could increase the sensitivity of pancreatic cancer cells to gemcitabine and especially sensitize the gemcitabine -resistant cells. These findings should further be explored in vivo.

5. Acknowledgements

This study was supported by Science and Technology Planning Project of Changzhou, Jiangsu Province, China (CS2004214 and CS20092025) and the key medical innovation talents training project of Changzhou, Jiangsu Province, China

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Edited by Sanjay K. Srivastava

This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

ISBN 978-953-51-6935-2

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