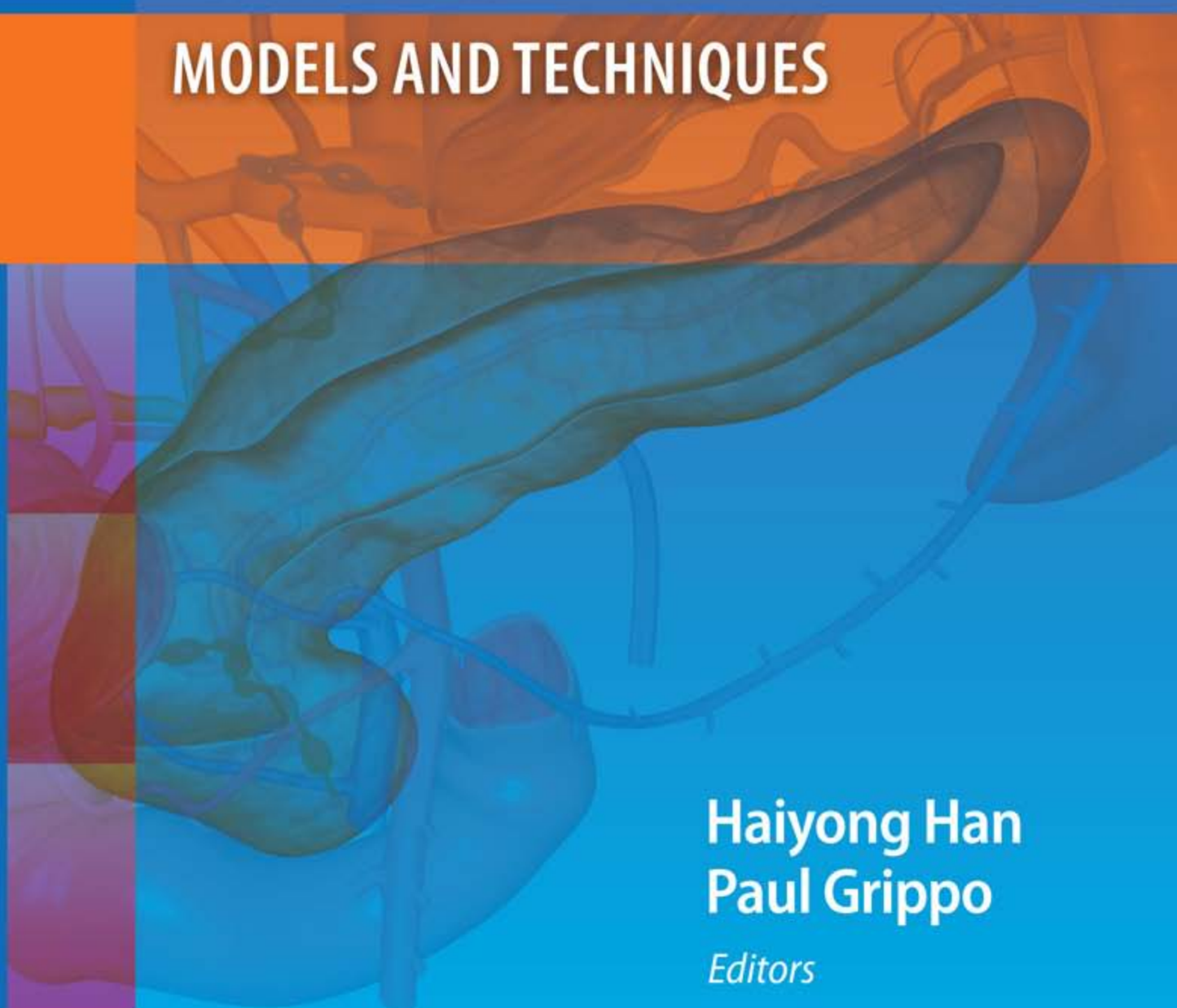
Drug Discovery in Pancreatic Cancer

MODELS AND TECHNIQUES



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Foreword

Everyone picking up this book will be aware of what a devastating disease pancreatic cancer is. And probably everyone picking up this book wants to make a difference in the fight against that disease. This book was designed by Drs. Han and Grippo for those who want to make a difference. And the book clearly hits the mark.

The contributing authors are extremely accomplished investigators who are sharing their state-of-the-art investigative knowledge on (1) molecular, proteomic, and bioinformatic techniques; (2) in vitro and in vivo systems (already established and new highly innovative models); and (3) new methods for clinical investigation. There is useful information for the beginning as well as for the accomplished investigator. The tables summarizing available cell lines, and available inhibitors and on special techniques are incredibly valuable. In addition to discussions of the more routine approaches to studying pancreatic cancer, the investigator is treated to insights on "cutting edge" approaches to studying the disease.

At the time of the writing of this book, pancreatic cancer is the fourth leading cause of death from cancer in the US. Worldwide more than 200,000 people will die each year, from the disease. It has the worst one-year and five-year survival of any cancer. Anyone using the information in this text will very likely have a better chance of making a dent in the morbidity and mortality caused by pancreatic cancer.

Daniel D. Von Hoff, M.D., F.A.C.P.

Preface

With a median survival of 6 months and a five-year survival rate of less than 5%, pancreatic cancer is the most lethal of all cancer types. This dismal picture of pancreatic cancer is mainly due to the lack of early diagnosis and effective treatment for patients with advanced disease. To increase the survival rate of pancreatic cancer patients, improved tumor markers for diagnosis and new molecular targets for drug development are desperately needed. A lot of effort has been made in searching for genes causative to or associated with the progression of malignant behavior in pancreatic cancer. As a result, alterations in a number of cancer-related genes have been identified in pancreatic tumors. The identification and characterization of these cancer-related genes have significantly increased our understanding of pancreatic cancer tumorigenesis, progression, and metastasis. Unfortunately, the treatment of pancreatic cancer has yet to benefit from these findings as therapy has not advanced much in the past 20 years.

Over the past decade, tremendous advances have been made in the field of cancer drug discovery, particularly in the area of molecular and genetic models and technologies. Many of those advanced models and technologies have been applied to the drug discovery processes for pancreatic cancer. In this book, a team of experts describe the latest development in the application of these models and technologies in pancreatic cancer. The authors include basic scientists as well as clinicians, all who work on the front-line of the war against this disease and have first-hand experience on these cutting-edge tools and techniques. It is our hope that the knowledge presented in this book will provide a strong background for those who want to make an impact in the treatment of patients with this dreadful disease.

We wish to thank Rachel Warren for inviting us to participate in this project and for entrusting it to us. We are especially grateful to all of the contributing authors for their hard work and enthusiasm in producing state-of-the-art chapters for this book.

Haiyong Han
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Chapter 1
Drug Evaluations in Pancreatic Cancer Culture Systems

Bhargava Mullapudi, Yongzeng Ding, Xianzhong Ding and Paul Grippo

Abstract Pancreatic cancer has proven to be one of the most difficult diseases to detect, diagnose, and treat due to both the location of the pancreas in the abdomen and the lack of overt symptoms before cancer dissemination. Thus, it is critical to have a variety of modeling systems that can be employed to evaluate drug responses/mechanisms while providing a relatively simple format for drug screening. Cell culture serves this purpose. In this context, it is necessary to review the available pancreatic cancer cell lines, culture techniques, predominant signaling pathways, and types of analyses that can be utilized to assess the effectiveness of drugs on pancreatic cancer cells, including aspects of pharmocotherapeutic strategies for the development of novel methodologies. Special consideration will be given to different signal transduction pathways like MAPK, JAK/STAT, PI_3K/AKT, RTKs, VEGF, and NF-κB, which are involved in various aspects of pancreatic cancer development and progression, complete with a repertoire of chemical inhibition at several levels within a cascade. Ultimately, abrogation of these cell signals can be detected through measurable variables at the cellular level, including apoptosis, proliferation, altered cell phenotype, the ability to invade and/or metastasize, and changes in cell cycle parameters. Evaluation of drugs at this level can set the stage for future strategies as well as exploration of novel compounds that inhibit other vital cancer signaling pathways.

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1.1 Introduction

With an average 5-year survival rate of 5% and no significant improvement in the past decade, pancreatic cancer still remains one of the most devastating cancers, posing a challenge to both early diagnosis and treatment strategies. Increased research in pancreatic cancer has brought some insight into the pathogenesis of this disease, but a more thorough understanding of disease etiology would lead to improved pharmacotherapy, which has yet to be seen. With surgery remaining the first line of curable treatment for most resectable tumors, chemotherapy or combined chemoradiotherapy is reserved to the locally advanced and unresectable tumors. In general, these approaches only modestly increase survival for some patients. This review focuses on the use of various pancreatic cancer cell lines employed in research, including their application in pharmacotherapy, the types of analyses used, the measurable variables, and the strengths and limitations of these applications.

1.2 Types of Cells and Cell Lines

A variety of human and rodent pancreatic cells lines are being used in research in order to characterize the processes that make a cancer cell and the types of compounds that inhibit these processes. Thus, it is important to review their principal features based on their origin, mutation status, histology and stage.

1.2.1 Normal (Non-Cancerous) Cells

The limitations involved in maintaining pancreatic ductal cells *in vitro* include the relatively small percentage of ductal cells (5–10%) in the human pancreas, their phenotypic instability in culture, their cellular senescence, and a decline in the normal enzymatic activity over time (Lawson et al. 2005). The ability to culture normal ductal epithelial cells allows for testing of various carcinogens and a more precise means of observing genetic changes that occur in the progression from a hyperplastic to malignant phenotype. It has been observed that adult human pancreatic duct cells (hDC) could be maintained for up to 3 weeks, with careful monitoring of fibroblast overgrowth (Gmyr et al. 2001), but, interestingly, adult acinar cells produce acinar and ductal antigens and, *in vitro,* have been reported to transdifferentiate to a ductal phenotype before eventually becoming senescent (De Lisle and Logsdon 1990). With each normal human cell division, telomeres are shortened and this eventually leads to telomere-controlled senescence in human cells (Ouellette and Lee 2001). The transfer of hTERT cDNA (which encodes the catalytic subunit of human telomerase) can immortalize human cells without changing their pheno-

type (Bodnar et al. 1998; Jiang et al. 1999; Morales et al. 1999). However, this has not been well established in hDC's.

1.2.1.1 HPDE (Human Pancreatic Ductal Epithelial Cells)

As of now, this premature senescence is overcome with use of viral oncogenes such as HPV 16 E6/E7 or the SV40 large T antigen (Furukawa et al. 1996; Jesnowski et al. 1998; Jesnowski et al. 1999). Several groups have established lines of these immortalized human pancreatic ductal epithelial (HPDE) cells using this method (Ouyang et al. 2000; Lawson et al. 2005). The major limitation for these techniques is that findings from molecular biological applications, with regard to genetic changes that occur with cell differentiation and transformation, must be carefully interpreted (Ulrich et al. 2002).

1.2.1.2 HPNE (Human Pancreatic Nestin Expressing Cells)

Cells expressing the neuronal stem cell marker nestin are present in the human pancreas but the biological role of these cells has yet to be resolved. Primary human cells derived from the ducts of the pancreas were transduced with an hTERT DNA. The infected cells became positive for telomerase and failed to senesce. The immortalized cells were positive for the expression of nestin (at both the mRNA and protein levels) and were found to be free of cancer-associated changes: diploid and expressing wild type $p16^{INK4a}$, p53, and K-Ras. An established line of normal human cells representing this cell type is of great value in defining the biological properties of this novel cell type and employing this cell type in research for the study of pancreatic cancer biology and pharmacotherapy.

In general, one major limitation of studying pancreatic cancer *in vitro* is that through isolation, purification, and maintenance of the cells, the interaction with the external milieu has been changed or lost at both the cellular and molecular levels. In an attempt to re-establish these and other interactions, cultured and primary pancreatic cancer cells can be introduced into immunocompromised mice.

1.2.2 Pancreatic Cancer Cell Lines in Culture

One of the major challenges in treating patients with pancreatic cancer is that over 80% of these patients present with advanced metastatic disease. It is important to study the morphologic and genetic changes that lead to the invasive and metastatic tumors. The study of these alterations is difficult at the tissue level because multiple cell types are involved. Therefore, pancreatic cancer cells offer an opportunity to study these phenotypic and molecular alterations in cells derived from various stages of invasion and metastasis, thus allowing for the study of different cell types within or associated to the pancreas.

Table 1.1 Cells of human origin

Cell line	Primary source	Genetic mutations	Histology/tumor grade
ASPC-1	Ascites	K-ras, p53, p16	PDAC, G2/G3
BxPc3	Primary tumor	p53, p16, DPC4	PDAC G2/G3
Capan-1	Liver metastasis	K-ras, p53, p16, DPC4	PDAC, G1
Capan-2	Primary tumor	K-ras, p16, DPC4	PDAC, G1
Colo357	Lymph node metastasis	K-ras, DPC4	PDAC, G2
HPAF-2	Ascites	K-ras, p53, p16	PDAC, G2
MiaPaCa-2	Primary tumor	K-ras, p53, p16	PDAC, G3
Panc1	Primary tumor	K-ras, p53, p16	PDAC, G3
Pan89	Lymph node metastasis	P53, p16	PDAC, G2
Pt 45P1	Primary tumor	K-ras, p53, p16	PDAC, G3

Source: Moore et al. (2001)

Currently, there are a large number of stable pancreatic cancer lines available (Table 1.1). Work has been done to characterize them by cataloging their primary source, genetic profile, histology, and tumor grade. The various sources of pancreatic cancer cell lines include: primary tumors, liver metastasis, ascites, and lymph node metastasis. Each of these sources yields a cancer that has both shared and unique molecular profiles evident in genetic mutations (Melstrom and Grippo 2008). Many of these cell types can be used in xenograft transplants into immunocompromised mice for *in vivo* evaluation of human pancreatic cancer in a mouse setting.

1.2.3 Rodent Pancreatic Cancer Cell Lines

The mechanism of malignant transformation of pancreatic cells from the pre-existing ductal, islet, or stem cells is obscure. Use of *in vitro* and *in vivo* studies could provide essential information on tumorigenesis and assist in the development of early diagnostic, therapeutic, and preventive measures. Only limited studies have been performed on cancers induced in the hamster, rat, and transgenic mouse, and only a few more have employed hamster and rat pancreatic cancer cells *in vitro*. In hamsters, only the gene sequences of c-ki-*ras* and p53 are known (Table 1.2).

Table 1.2 Cells of hamster origin

Cell line	Carcinogen	Origin	Mutation	Markers
H2T	BHP	Tumor	p53	–
HaP-T1	BOP	Tumor	–	–
HP-1	BOP	Tumor	–	No MUC1
HPC	BOP	Tumor	–	EGFR, No BGRA
PC1	BOP	Tumor	k-ras, p53	TGF-α, EGFR, BGA
PDPaCa	BOP	Tumor	k-ras	–
WDPaCA	BOP	Tumor	P53	–

Source: Townsend et al. (1982); Saito et al. (1988); Batra et al. (1992); Hirota et al. (1993); Morita et al. (1998)

1.2.4 Insulin-Secreting Cell Lines

The growth and differentiation patterns of human and hamster islets in culture have been found to be almost identical. The cells gradually lose their endocrine granules and appear to transform to ductal, acinar, and intermediary cells (Schmied et al. 2000). The formation of ductal elements within the cultured human islets has also been documented by different research groups (Lucas-Clerc et al. 1993; Yuan et al. 1996; Rozenblum et al. 1997; Bonner-Weir et al. 2000) and highlights the tendency of cultured islets (Table 1.3) to give rise to exocrine cells either by differentiation or activation and proliferation of pancreatic stem cells within the islets. The tendency of islets cells to give rise to cells of ductal morphology within islets is still not entirely understood.

1.2.5 Stem Cells

Emerging data suggest that malignant tumors are quite heterogeneous and that tumors are composed of a small set of distinct cells termed cancer stem cells, which are responsible for tumor initiation and propagation, and a much larger set of more differentiated cancer cells, which have very limited proliferative potential. These cells have been termed cancer stem cells (CSCs) because, like their normal stem cell counterparts, they possess the ability to self-renew and produce differentiated progeny.

Fluorescence-activated cell sorting and establishment of human tumor xenograft models in immunocompromised mice have been adapted by researchers studying stem cells in malignancies. Pancreatic cancer stem cells, defined by expression of the cell surface markers CD44+CD24+ESA+ (0.2–0.8% of all pancreatic cancer cells), were highly tumorigenic and possessed the ability to both self-renew and produce differentiated progeny that reflected the heterogeneity of the patient's primary tumor. The upregulation of developmental signaling molecules (Table 1.4), including sonic hedgehog and Bmi-1, in pancreatic cancer stem cells was also observed. Recently, Hermann and colleagues found that CD133+ cells in primary pancreatic

Table 1.3 Insulin-secreting cell lines from various species

Cell line	Cell origin	Species	Comments
MIN6	Insulinoma	Mouse	SV40 T-antigen transgenic mouse
Beta TC1	Insulinoma	Mouse	SV40 T-antigen transgenic mouse
HIT	Insulinoma	Hamster	SV40 T-antigen transfected
RINm	Insulinoma	Rat	Radiation-induced
RINm5F	Insulinoma	Rat	Radiation-induced
TRM-1	Fetal	Human	SV40 T-antigen, H-ras va112
Beta lox 5	Fetal	Human	SV40 T-antigen, H-ras va112, hTERT oncogene

Source: Gazdar et al. (1980); Praz et al. (1983); Efrat et al. (1988); Ishihara et al. (1993); Wang et al. (1997); de la Tour et al. (2001)

Table 1.4 Signaling pathways involved in stem cell function

Signaling pathway	Function
Hedgehog	Maintenance of stem cell pools by self-renewal, differentiation of progenitors/tissue patterning
Bmi-1 (Polycomb proteins)	Chromatin remodeling, cell cycle progression by regulation of cyclins/cyclin-dependent kinases
Wnt/Beta-catenin	Self-renewal of stem cell pools and tissue patterning/differentiation
Others: Pten, telomerase, Notch	Self-renewal and differentiation of stem cells and progenitors. Telomerase serves as a marker with high activity in stem cell pools

Source: Bednar and Simeone (2009)

cancers and pancreatic cancer cell lines also discriminate for cells with enhanced proliferative capacity (Hermann et al. 2007; Li et al. 2007).

From a clinical standpoint, the identification of cancer stem cells within human pancreatic cancers has important implications for treatment. Some recently published data suggest that pancreatic cancer stem cells may also be resistant to chemotherapy and radiation. Hermann and colleagues found that CD133+ populations in the L3.6p pancreatic cancer cell line were enriched after exposure to gemcitabine (Hermann et al. 2007).

Thus, if cancer stem cells seem to be the drivers in tumor initiation and maintenance, how should we proceed in determining the best way to target them? A next major step would be to perform more detailed studies to understand the biological properties of pancreatic cancer stem cells from primary human pancreatic cancers. Performing global gene profiling of pancreatic cancer stem cells compared with non-tumorigenic pancreatic cancer cells and normal pancreatic epithelial cells will identify the signaling pathways important in pancreatic cancer stem cell function and determine which pathway or combination of pathways should be targeted. This work is currently under way in a number of laboratories. An alternative approach is to perform a small interfering RNA library screen to identify genes that are important in pancreatic cancer stem cell self-renewal. In addition to the gold standard *in vitro* dilutional tumor propagation assays used to identify cancer stem cells, cancer stem cells have also been identified based on *in vitro* sphere-forming assays.

1.2.6 Advantages

Cell culture offers many research possibilities that are difficult or impossible to perform *in vivo*. The effects of certain drugs and toxins can be tested during various conditions in individual cells of a complex tissue, such as the pancreas. Many parameters, including the ingredients of the culture medium, culture conditions, population density, and growth rate can be controlled and/or measured. Furthermore, cells can be manipulated by transfection to investigate the role of various genes in the physiology or malignancy of the cells and their response to certain compounds. From a mixed cell population (most native cell lines are known to

have a heterogeneous cell population), clones can be established and the patterns and drug responses of individual clones can be studied. Functional and mechanistic studies following drug administration can also be more easily performed *in vitro*. Proteins or peptides produced or secreted by the cells can be measured in conditioned media during various culture conditions. Immunohistochemical, molecular, biological, and immunoelectron-microscopic examinations are other useful methods to gain additional pertinent information. Cell culture can likewise be used to test the sensitivity of patients' tumor cells to specific cytotoxic agents *in vitro* as well as *in vivo* after tumor cell transplantation into an immunocompromised animal (Ulrich et al. 2002).

1.2.7 Limitations

Cell–cell interactions are very important cellular functions in an organism, the disruption of which certainly has known and still-unknown consequences. Genetic manipulations of the cells can generate additional problems, ultimately altering some or many of the endogenous functions responsible for drug responses. A major problem with normal human pancreas cells is their tendency to become senescent, thus reducing their use for long-term studies. When targeted pharmacotherapy is studied *in vitro*, the predicted effects of the drugs may not be observed *in vivo* due to various cellular, endocrine, and metabolic differences. Also when a drug is tested *in vitro*, there are few pharmacokinetic variables that affect the drug uptake by the cells, whereas *in vivo* there are many factors such as absorption, renal and hepatic metabolisms, volume of distribution, and elimination constants that affect the delivery of the drug to the target organ.

1.3 Types of Culturing Systems

As well as a rather large selection of cultured cell lines, there are several types of culturing systems that can be employed.

1.3.1 Dish Cultures

Dish culture refers to the culture of cell lines of interest on culture plates, multi-well plates, or flasks. Providing an ideal growth medium is important along with an ideal environment for cell growth such as correct concentrations of O_2 and CO_2 for normal cell metabolism and the efficient maintenance of media pH. The growth medium requirements might be different from cell line to cell line. For example, HPDE cells require a medium with different antibiotics and growth factors than that used for maintenance of HPDE/K-ras cells. These systems are two-dimensional

thus providing ease of handling and usually very good growth rates, with some exceptions (Capan 1 and 2, for example). However, the major limitation is loss of extracellular matrix and cell–cell interactions, which define *in vivo* tumorigenesis.

1.3.2 3D Cultures

The 3D cell culture technique offers a better cell culture environment because it is one step closer to the type of growth environment observed *in vivo*. The latest designs include two types of inserts: one that can be inserted into a multiwell culture plate and the other that can be inserted into a culture flask. These inserts are a series of disposable three-dimensional materials made from both non-degradable and degradable polymers such as polystyrene and PCL. They are composed of struts/filaments which are joined together to form a well-defined porous structure, and these inserts come in a variety of porosity, pore size and surface area. The advantages include convenience, 100% porosity for cell seeding, improved cell culture efficiency, and easy separation of cytokines and growth factors secreted by cells without involving separation steps.

1.3.3 Co-Cultures

A technique of culturing mixed cell types *in vitro* allows for their synergistic or antagonistic interactions to be observed, such as changes in cell differentiation or apoptosis. Co-culture can be of different types of cells, tissues, or organs from normal or disease states. Interactions between tumor cells and surrounding stroma (notably fibroblasts) play a critical role in tumor growth, invasion, metastasis, angiogenesis, and chemoresistance (Gleave et al. 1991; Janvier et al. 1997; Nakamura et al. 1997; Anderson et al. 2000; Bhowmick et al. 2004; Muerkoster et al. 2004). Histologically, pancreatic ductal adenocarcinoma is almost uniformly characterized by a prominent host desmoplastic response at the site of primary invasion, suggestive of the presence of extensive tumor–stromal interactions. The importance of tumor–stromal interactions in the aggressive behavior of pancreatic cancer is also supported by experimental evidence that the invasive potential of pancreatic cancer cells can be greatly enhanced by co-culture with stromal fibroblasts (Maehara et al. 2001). Despite the importance of tumor–stromal interactions in cancer progression, underlying molecular mechanisms have not been well characterized, partly because of their diversity and complexity. Several molecules have been identified that participate in tumor–stromal interactions, including hepatocyte growth factor (Nakamura et al. 1997), interleukin (IL)-8 (Anderson et al. 2000), SPARC/osteonectin (Sato et al. 2003), transforming growth factor (Bhowmick et al. 2004), and several matrix metalloproteinases (MMPs) (Dong et al. 2001; Saad et al. 2002). The identification and characterization of genes/pathways involved in tumor–stromal interactions can identify targets for novel therapeutic strategies.

1.3.4 High-Throughput Screening

High-throughput screening (HTS) is an approach to drug discovery that has gained widespread popularity over the last three or four years. HTS is the process of assaying a large number of potential effectors of biological activity against targets (a biological event). The methods of HTS are applied to the screening of combinatorial chemistry, genomics, protein, and peptide libraries. The goal of HTS is to accelerate drug discovery by screening large libraries often composed of hundreds of thousands of compounds (drug candidates) at a rate that may exceed 20,000 compounds per week.

As a number of assays need to be processed per day, HTS employs multiple-well microplates along with the latest robotic processing. Previously HTS assays have been run in the standard 96-well microplate, whereas currently higher-density, lower-volume formats (e.g. 384- and 1536-well microplates) are now being employed. The advantage is increased throughput and lower volume, which reflects in lower cost per sample (Armstrong 1999).

1.4 Applications for Pharmacotherapeutic Evaluations

Pancreatic cancer poses a challenge to pharmacotherapy, which should be overcome with improved research strategies. This section summarizes the most popular signaling pathways involved in the pathogenesis of pancreatic cancer, including specific targets that have been used in pharmacotherapy. By presenting this data, knowledge gaps should become apparent, and future research in targeted therapy should improve treatment modalities and drug formulations, particularly at specific points in these signaling pathways. Employing pancreatic cell lines in these endeavors will serve as the platform for evaluating future applications with novel pharmacologic agents.

1.4.1 Signal Transduction Pathways

A variety of signal transduction pathways are activated in cells in response to growth factor, genetic, or mitogenic stimuli. These pathways represent a large cohort of potential targets for inhibition as a means of mitigating downstream events that alter cellular features leading to cancer. The response to these stimuli varies depending on the cell type and these pathways continue to converge and diverge, adding to their complexity. These types of pathways continue to be the focus of a great deal of research and, considering the importance of cell cycle regulation in biology, will continue to expand as investigations further delineate downstream events. This provides greater opportunity for novel inhibitors but also introduces the possibility of less specificity with known drugs.

1.4.1.1 MAPK

The MAP kinase (MAPK) type of pathways are triggered through a cascade of phosphorylation events that begins with a growth factor binding to a tyrosine kinase receptor at the cell surface. This causes dimerization of the receptor and an intermolecular cross-phosphorylation of the two receptor molecules. The phosphorylated receptors then interact with adaptor molecules that trigger downstream events in the cascade. The cascade works through the GTP exchange protein RAS, the protein kinase RAF (MAPKKK), the protein kinase MEK (MAPKK), and MAP kinase (Erk). MAPK then phosphorylates a variety of substrates that control transcription, the cell cycle, or rearrangements of the cytoskeleton.

Raf/MEK/ERK: The RAF/MEK/ERK cascade is the most widely known RAS-regulated pathway (Sridhar et al. 2005) and is thought to play an important role in the initiation and progression of pancreatic cancer. It is thus conceivable that this pathway could represent an important target for the treatment of this tumor type.

Sorafenib (Nexavar, BAY 43-9006) is a multikinase inhibitor that has shown promising *in vitro* and *in vivo* activity against a variety of tumor histotypes (Wilhelm et al. 2004; Wilhelm et al. 2008). It was initially developed as a specific inhibitor of the serine/threonine RAF kinase, in particular C-RAF (also known as RAF-1) and B-RAF, and it has been hypothesized that sorafenib exerts its anti-tumor effect by interrupting the MEK/ERK pathway (Wilhelm et al. 2004). However, the functional role of MEK/ERK inhibition in sorafenib-mediated lethality is not yet fully understood (Wilhelm et al. 2008).

1.4.1.2 JAK/STAT

The JAK/STAT pathway is activated by cytokine interaction with a family of receptors called the cytokine receptor superfamily. These receptors do not contain a protein kinase domain themselves, but they associate with and activate a family of protein kinases called the JAK (Just Another Kinase or JAnus Kinase) family. JAK family members are recruited to receptor complexes that are formed as a result of ligand binding. The high concentration of JAK in the complex leads to a cross-phosphorylation of JAK and thus activation. JAK then phosphorylates members of another protein family called STAT (signal transducers and activators of transcription). These proteins then translocate to the nucleus and directly modulate transcription.

IL-6 has been implicated as a mediator of growth promotion in pancreatic cancer (Barber et al. 1999). Suppressor of cytokine signaling-1 (SOCS-1) is a cytokine inducible protein that functions to negatively regulate cytokine signal transduction by directly interacting with JAK. It has been observed that aberrant methylation of SOCS-1 in pancreatic cancer caused the loss of inhibitory action on the JAK/STAT pathway (Fukushima et al. 2003). Markers can be developed to detect this methylation event, thereby detecting pancreatic cancer at an earlier stage. There is a growing need for the development of other inhibitors that can act at various levels of the JAK/STAT pathway.

1.4.1.3 Protein Kinase C

The protein kinase C (PKC) pathways consist of a family of phospholipid-dependent protein kinases. PKC is regulated by a large variety of metabolic pathways involving cellular levels of phospholipids and calcium. The main regulator of the pathway is diacylglycerol (DAG), which appears to recruit PKC to the plasma membrane leading to its activation. The activity of DAG is mimicked by the phorbol-ester tumor promoters. Once activated, PKC can phosphorylate a wide variety of cellular substrates that regulate cell proliferation and differentiation. Responses to PKC appear to vary with the types of PKCs expressed and the types of substrates available within a cell. Some evidence shows that the PKC pathway may interact with and exert effects through the MAPK pathway. In pancreatic cancer cell lines, inhibition of PKC could sensitize cells to the pro-apoptotic effects of chemotherapeutic agents like gemcitabine (El-Rayes et al. 2008).

PKC 412, a kinase inhibitor, significantly inhibits cell growth, orthotopic tumor growth *in vivo*, and cell cycle progression, while increasing programmed cell death (El Fitori et al. 2007). *Curcumin*, a dietary pigment responsible for the yellow color of curry, has been shown to be a potent inhibitor of PKC. Curcumin competes with calcium for the binding domain in PKC. Curcumin is currently being evaluated in multiple myeloma, pancreas and colorectal cancer (Dhillon et al. 2008). Further in-depth research on a broad range of kinase inhibitors may prove to be useful in therapies for pancreatic cancer patients.

1.4.1.4 PI_3K

The PI_3K family constitutes a large family of lipid and serine/threonine kinases, which includes a number of phosphatidylinositol kinases, as well as the related DNA-dependent protein, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) kinases (Hiles et al. 1992; Fruman et al. 1998; Jimenez et al. 1998; Pawson and Nash 2000). There are three classes of PI_3Ks.

Class IA PI_3Ks are composed of heterodimers of an inhibitory adaptor/regulatory (p85) and a catalytic (p110) subunit. p85 binds and integrates signals from various cellular proteins, including transmembrane tyrosine kinase-linked receptors and intracellular proteins such as protein kinase C (PKC), SHP1, Rac, Rho, hormonal receptors, mutated Ras and Src, providing an integration point for activation of p110 and downstream molecules. The SH2 domain of p85 has two major divergent functional activities: activation of small G-proteins and relief of *trans*-inhibition of p110.

Class IB PI_3Ks consist of p110γ and a regulatory subunit, p101, and are activated directly by G-protein-coupled receptors and indirectly by other receptors. All class I PI_3Ks possess intrinsic protein kinase activity; p110 autophosphorylation and phosphorylation of p85 downregulate activity of the complex. Two chemical inhibitors have been used to probe the function of PI_3K: *wortmannin*, a fungal metabolite that irreversibly inhibits p110 by reacting covalently with the catalytic site (Davies et al.

2000), and the flavenoid derivative *LY294002*, a reversible inhibitor which, like wortmannin, inhibits all class I PI_3Ks.

Class II PI_3Ks are monomeric, lack adapter subunits and preferentially use PI and PIP (Pawson and Nash 2000) as substrates (Oudit et al. 2004). Three mammalian class II isoforms have been identified: the ubiquitously expressed PI_3K-C2α and PI_3K-C2β, and liver-specific PI_3K-C2γ.

Class III PI_3Ks are heterodimeric enzymes consisting of adaptor (p150) and catalytic (Vps34, 100 kDa) subunits; the latter produce only PIP_3, are thought to be central to vesicle trafficking, and lack a Ras-binding domain. Intriguingly, class III PI_3K has been implicated in regulation of autophagy, a phylogenetically conserved process that enables survival under conditions of cell stress.

PI_3K inhibitors Wortmannin and LY294002 have antitumor activity *in vitro* and *in vivo*, and sensitize tumor cells to other targeted therapeutics, chemotherapy, and radiation (Schultz et al. 1995; Rosenzweig et al. 1997; Hu et al. 2000; Kim et al. 2000; Ng et al. 2001; Hu et al. 2002). Stable water-soluble conjugates of wortmannin are being developed to improve its pharmacological characteristics. LY294002 has a very short half-life and is insoluble in aqueous solutions. Novel LY294002 prodrugs are in development. A number of companies and academic sites have developed additional pan- and isoform-selective PI_3K inhibitors (Roberson et al. 2001; Ward and Finan 2003; Wymann et al. 2003; Knight et al. 2004). Although none of these inhibitors has been evaluated in human trials, preclinical data and animal models indicate that the new-generation inhibitors have much better pharmacological characteristics: greater water solubility, less protein binding, and better pharmacodynamics, as well as improved PI_3K selectivity. A number of isoform-specific inhibitors are under evaluation but as yet have not demonstrated superior activity to or less toxicity than LY294002 or wortmannin. With the advent of small interfering RNA (siRNA) technologies and recent demonstration of *in vivo* activity in several tumor models, it might be possible to selectively target the p110 or p85 isoforms.

1.4.1.5 AKT (Protein Kinase B)

AKT, the human homologue of the viral oncogene *v-akt*, is related to protein kinases A (PKA) and C (PKC) in humans (Bellacosa et al. 1991; Coffer and Woodgett 1991; Murthy et al. 2000). The three known AKT isoforms are derived from distinct genes (AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ). The PH domain in the N-terminal region of AKT interacts with 3′-phosphoinositides, contributing to recruitment of AKT to the plasma membrane. Recruitment to the membrane results in a conformational change that exposes two crucial amino acids that are phosphorylated and necessary for activation. One in the kinase domain (threonine 308 in AKT1) is phosphorylated by constitutively active phosphoinositide-dependent kinase 1 (PDK1), stabilizing the activation loop. Phosphorylation of the other amino acid in the hydrophobic C-terminal domain (serine 473 in AKT1) by PDK2 is necessary for full activation (Alessi et al. 1996; Blume-Jensen and Hunter 2001). Several

different potential PDK2s have been identified, including the mTOR rictor complex (but not the mTOR raptor complex inhibited by rapamycin and its analogues), integrin-linked kinase (ILK), PKCβII, and even AKT itself, thereby allowing the pathway potential for feedback control (Kawakami et al. 2004; Fruman et al. 1998; Lynch et al. 1999). How these potential PDK2s interact to regulate AKT is not yet understood. The relative roles of AKT signaling at the membrane, cytosol, and nucleus also remain to be determined.

AKT inhibitors: As with inhibitors of PI_3K that target the apex of the pathway, the theoretical advantage associated with AKT inhibition compared with downstream inhibition (for example, at mTOR), is that the PI_3K/AKT pathway bifurcates and integrates with signals from other pathways as the signal is propagated. The pathway can therefore act more globally and thereby effectively inhibit when targeting AKT (or indeed PI_3K) directly. Theoretically, such inhibition might also be less susceptible to the complicating and still largely unknown effects of feedback loops as opposed to the inhibition of single branches further downstream. This efficacy is balanced by potential for toxicity and a narrow therapeutic index (DeFeo-Jones et al. 2005). This might be particularly apparent with catalytic-domain inhibitors of AKT, which could demonstrate "gain of function" activity. For this reason, a number of isoform-selective AKT catalytic-domain inhibitors, as well as inhibitors of the PH domain, are currently under development (Barnett; DeFeo-Jones et al. 2005; Lindsley et al. 2005). Whether these more selective inhibitors will demonstrate wider therapeutic indices is unknown. Recently, interest has focused on the *alkylphosphocholines – miltefosine* and *perifosine* – as AKT inhibitors that prevent membrane localization, possibly by interacting with the PH domain (Kondapaka et al. 2003; Van Ummersen et al. 2004). The specificity of these two drugs to the multiple PH domain-containing proteins is unknown at present but there is some demonstrated selectivity to different PH domains. Whether this contributes to their apparently improved therapeutic indices and relative lack of toxicity compared to other AKT inhibitors is under evaluation in ongoing clinical trials.

1.4.2 Receptor Tyrosine Kinases

RTKs are anchored in the plasma membrane at the transmembrane domain, while the extracellular domains bind growth factors. Typically, extracellular domains comprise structural motifs including acidic regions, cadherin-like domains, cysteine-rich regions, discoidin-like domains, EGF-like domains, Factor VIII-like domains, fibronectin III-like domains, glycine-rich regions, immunoglobulin-like domains, kringle-like domains, and leucine-rich regions. There are a variety of RTKs that are organized into several classes of receptors:

- Class I: EGF receptor, NEU/HER2,HER3
- Class II: insulin receptor, IGF-1 receptor
- Class III: PDGF receptors, c-Kit
- Class IV: FGF receptors

- Class V: vascular endothelial cell growth factor (VEGF) receptor
- Class VI: hepatocyte growth factor (HGF) and scatter factor (SC) receptors
- Class VII: neurotrophin (NT) receptor family (trkA, trkB, trkC) and NGF receptor

The expression of the NTs and aberrant overexpression of the trk receptors has been demonstrated through IHC analysis and by *in situ* hybridization in human PDAC specimens relative to normal pancreas and in human PDAC-derived cell lines. In addition, NT and trk expression has also been demonstrated in PDAC-derived cell lines and normal pancreas (Oikawa et al. 1995; Shibayama and Koizumi 1996; Ohta et al. 1997). NGF expression correlates with perineural invasion and pain associated with PDAC (Zhu et al. 1999). As such, the NT-trk axis plays a role in the development and progression of pancreatic adenocarcinoma.

Tyrosine kinase inhibitors: Tyrosine kinase inhibitors (TKIs) were approved by the US FDA for treatment of patients with Non-Small Cell Lung Carcinoma (NSCLC), head and neck tumors, gastrointestinal stromal tumors, and, more recently, pancreatic cancer. TKIs are small molecules that inhibit the activity of the tyrosine kinase domain. *First-line TKIs* are reversible, competitive inhibitors of the EGFR tyrosine kinase domain; *second-line TKIs* are irreversible inhibitors. Second-line TKIs block the EGFR tyrosine kinase domain, but they also affect the tyrosine kinase domain in other receptors (e.g. HER-2, HER-4, and VEGFR) (Sequist 2007). Most of the EGFR TKIs used in clinical trials for pancreatic cancer are members of the first generation (gefitinib and erlotinib). Second-generation TKIs under evaluation are *EKB-569, HKI-272, CI-1033,* and *ZD6474*.

Gefitinib decreases VEGF and factor VIII levels in pancreatic cancer cell lines *in vitro* (Murthy et al. 2000). Compared with anti-EGFR monoclonal Abs, gefitinib has an inhibitory effect in cell growth that is dose-dependent. TKIs do not exhibit antibody-dependent cellular toxicity, but like other EGFR inhibitors, gefitinib is a good chemosensitizer and radiosensitizer because it suppresses DNA repair induced after these treatments (Maurel et al. 2006).

Erlotinib is the only TKI inhibitor approved by the US FDA for patients with advanced pancreatic cancer who have not received previous chemotherapy. When combined with gemcitabine, erlotinib decreases ERK1/2 phosphorylation and enhances apoptosis in pancreatic cancer cells (Ng et al. 2002). Erlotinib also inhibits cell growth in pancreatic cancer cells (Durkin et al. 2003). The clinical safety and effectiveness of erlotinib in cancer has been assessed in many trials (Dragovich et al. 2007). A phase III double-blind, placebo-controlled study comprising 569 patients with locally advanced and metastatic pancreatic cancer showed median survival of 6.24 months in the erlotinib/gemcitabine group compared with 5.91 months in the erlotinib/placebo group (P value = 0.038). The 1-year survival rate was 23% versus 17% (P value = 0.023). The overall disease-control rate was 55.7% on erlotinib/gemcitabine versus 49.2% on placebo and gemcitabine (P value = 0.07). EGFR levels in tumor samples did not correlate with the response to erlotinib. Erlotinib induces skin toxicity, but, similarly to other anti-EGFR drugs, there is a positive association between the presence of rash and response. In this study, patients who received erlotinib plus gemcitabine had a higher frequency of interstitial lung disease-like

syndrome compared with those receiving the drugs administered alone (Moore et al. 2007). This study led to approval of erlotinib by the US FDA.

1.4.3 VEGF

Angiogenesis provides the required substrates for tumor growth and dissemination. VEGF is a very important factor inducing angiogenesis, but EGF, TGF-β, fibroblast growth factor, and IL-8 also contribute to this process both *in vitro* and *in vivo* (Xie et al. 2006). Targeting angiogenesis is attractive because in theory it can reduce cancer progression, invasion, and metastasis. Different drugs inhibit angiogenesis including MMP inhibitors, COX-2 inhibitors, thalidomide, mammalian target of rapamycin, and EGFR inhibitors, but VEGF inhibitors are most effective in pancreatic cancer (Saif 2006).

The VEGF family has six different family members (VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor). VEGF, also known as VEGF-A, is the predominant member that binds to two different receptors: VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR). Both receptors have three different domains: (1) the extracellular domain, which has seven immunoglobulin-like regions, (2) the second and third domains, which constitute the transmembrane region, and (3) the intracellular tyrosine kinase domain (Tabernero 2007). VEGF receptor is similar to EGFR because after it binds to its ligand, the VEGF receptor forms a dimer, and the tyrosine kinase autophosphorylates and amplifies the initial signal by activating downstream intracellular pathways, such as MAPK and PI_3K (Xie et al. 2006). VEGF exerts most of its neoangiogenic effects through VEGF-R2 (Tabernero 2007). VEGF and VEGF-R are coexpressed in 88% to 93% of the patients with pancreatic adenocarcinoma (Seo et al. 2000; Niedergethmann et al. 2002). VEGF presence in tumor samples correlates with tumor size. VEGF and microvessel density (MVD) are also predictors of early recurrence after surgery, the development of liver metastasis, poor prognosis, and cancer-related death (Holloway et al. 2006).

There are a variety of therapies directed against VEGF:

1.4.3.1 Anti-VEGF Abs

Different Abs with anti-VEGF activity have been evaluated for use against pancreatic cancer. *Bevacizumab* is a human monoclonal anti-VEGF antibody that prevents VEGF binding to both VEGF receptors. 2C3 is a murine antibody with antihuman VEGF activity that prevents binding of VEGF to VEGF-R2 without preventing binding to VEGF-R1 (Holloway et al. 2006).

Bevacizumab decreases lymphangiogenesis and neovascularization *in vitro*; it increases the delivery of chemotherapy to the tumor in pancreatic cancer. The major concern with bevacizumab therapy in pancreatic cancer is bleeding and perforation;

therefore, it is not recommended in patients with tumors invading the duodenum or other organs (Kindler et al. 2005).

1.4.3.2 Other Anti-VEGF Therapies

VEGF can be inhibited with VEGF antisense oligonucleotides, VEGF-directed ribozymes, VEGF fused to diphtheria toxin, oncolytic adenoviruses, and VEGF-R TKI (Xie et al. 2006). Preclinical anti-VEGF therapy in pancreatic cancer models with antisense oligonucleotide AS-3 and oncolytic adenovirus replication seem to be promising therapies because they inhibit VEGF synthesis and angiogenesis in xenograft models, but they have not yet been tried in humans (Hotz et al. 2005; Saito et al. 2006). *Vatalanib* is an inhibitor of VEGF-R tyrosine kinase. In preclinical models, valatinib plus gemcitabine shows a decrease in tumor growth and metastasis in orthotopic pancreatic cancer models. This combination induces apoptosis in tumor-associated endothelial cells and decreases MVD. There are no clinical studies evaluating vatalanib and pancreatic cancer (Baker et al. 2002). *Sorafenib* is a pan-kinase inhibitor (Raf-kinase, VEGF-R2, and platelet-derived growth factor receptor). It is under evaluation as a therapy for NSCLC, liver, renal cell, colorectal, breast, melanoma, and ovarian cancers. One phase I study showed good tolerance after treatment with gemcitabine and sorafenib in pancreatic cancer (Siu et al. 2006).

1.4.4 NF-κB (Nuclear Factor-kappaB)

NF-κB is a transcription factor, which is activated by different pathways. Increased NF-κB activity can promote tumorigenesis, angiogenesis, promote invasion, metastasis, and chemoresistance in pancreatic cancer cells while inhibiting apoptosis. The cell surface receptors such as EGFR, IL-1 receptor, TRAIL receptor, TNF-α receptor, and CD-40/TRAF receptor, activate NF-κB. NF-κB is bound to two subunits IκB-α and IκB-β, which upon degradation provide stimulatory output via the classical pathway, requiring NEMO, or via the alternative pathway, using NIK. An atypical pathway results from casein kinase II phosphorylation of the PEST sequence of IκB. NF-κB moves into the nucleus, where it binds DNA to promote the production of the proteins (Holcomb et al. 2008).

Inhibiting NF-κB (Table 1.5) has been shown to decrease tumor growth and tumorigenesis in pancreatic cancer. One study showed that in the human pancreatic cancer cell line BxPC-3, proliferation is blocked with the administration of the proteosome inhibitor *PS-341*, which blocks activation of NF-κB (Shah et al. 2001). In a study in which mice were injected with Panc-1 human pancreatic cancer cells with or without IκB-α mutant protein, those injected with the IκB-α mutant that blocks NF-κB activity did not show any tumor formation. However, 100% of the mice injected with Panc-1 without the IκB-α mutant developed tumors (Fujioka et al. 2003). Another study using the NF-κB inhibitor *sulfasalazine* in combination with chemotherapy found decreased tumor growth as measured by Ki-67 staining

Table 1.5 List of NF-κB inhibitors and mechanism of action

Drug	Mechanism
Celecoxib (COX-2 inhibitor)	IKK inhibitor
IκB-α super suppressor	Resistant to enzymatic degradation and release of NF-κB
Bortezomib (PS-341)	26S proteosome inhibitor
Curcumin	Inhibits IKK and NF-κB activity
Parthenolide (or LC-1)	Inhibits IKK activation via classical and alternative pathways. Inhibits NF-κB translocation into the nucleus
Genistein	Blocks IKK activation and activity. Inhibits MAP kinase
Tyrosine kinase inhibitor	Inhibits activation of IKK via tyrosine kinase
NEMO-binding domain inhibitor	Blocks NEMO activation of IKK

IKK I Kappa B Kinase
Information of current ongoing trials can be found on the NCCN and NIH Web sites

(Muerkoster et al. 2003). Several mechanisms for the increased growth and tumorigenesis associated with increased NF-κB activity have been proposed. One possibility is supported by the finding that NF-κB increases cyclin D1 levels, which is important in promoting the G1 to S phase transition in fibroblasts (Guttridge et al. 1999). Biliran et al (Biliran et al. 2005) also demonstrated a link between increased cyclin D1 and cell growth in pancreatic cancer cells with increased NF-κB activity. Another possibility is that pancreatic cancer cells have been shown to secrete several NF-κB activators in an autocrine fashion (Niu et al. 2004). This was demonstrated by showing that a change to fresh media two hours before preparing extracts for an electrophoretic shift assay caused a significant drop in NF-κB activity. One of these factors has been shown to be secreted is interleukin (IL) 8, which is a growth-promoting cytokine (Le X et al. 2000).

In addition to promoting growth and tumorigenesis, there exists considerable evidence that NF-κB has anti-apoptotic effects that would favor tumor survival. NF-κB has been linked to the production of many antiapoptotic proteins such as cIAP1 (inhibitor of apoptosis protein), cIAP2, TRAF1 (TNF receptor Y associated factor), TRAF2, and the Bcl (B-cell leukemia) homolog (A1/BF1-1) (Wang et al. 1998; Wang et al. 1999; Yamamoto et al. 1999). Kreuz et al. (2001) demonstrated that cellular FLICE-inhibitory protein, a caspase 8 homolog, inhibits TNF-induced apoptosis. This effect can be blocked in SV80 fibroblast cells by inhibiting NF-κB (Kreuz et al. 2001). B-cell leukemia 2 and Bcl-xL are two other antiapoptotic proteins that have been found to be upregulated in pancreatic cancer cells (Fujioka et al. 2003). The expression of these proteins can be suppressed by inhibiting NF-κB with an IκB-α mutant or by blocking the epidermal growth factor receptor, which leads to a decrease in NF-κB activity (Fujioka et al. 2003; Sclabas et al. 2003). Muerkoster et al. (2003) observed that the combination of gemcitabine and/or etoposide with sulfasalazine, an NF-κB inhibitor, increased apoptosis more than with chemotherapy alone. Another study showed that Bcl-xL, which is upregulated in murine pancreatic tumor cells, could be blocked and apoptosis induced by inhibiting NF-κB along with Stat3, another transcription factor (Greten et al. 2002). McDade et al. (McDade

et al. 1999) also demonstrated that an NF-κB inhibitor could increase apoptosis in BxPC-3 cells. These data all indicate how NF-κB may enable pancreatic cancer cells to resist cell death, thereby challenging the efficacy of existing therapies.

1.4.5 Cell Cycle

The rate of cell division is a tightly regulated process that is intimately associated with growth, differentiation, and tissue turnover. Generally, cells do not undergo division unless they receive signals that instruct them to enter the active segments of the cell cycle. Resting cells are said to be in the G0 phase (quiescence) of the cell cycle. The signals that induce cells to divide are diverse and trigger a large number of signal transduction cascades.

1.5 *In Vitro* Analyses

The following analyses are used to measure the response to drug administration of cultured pancreatic cancer cells to determine the efficacy of the treatment as well as the mechanisms. Based on previous information or structure of a certain drug, the type of response expected can provide a relatively short list of parameters to investigate.

1.5.1 Apoptosis

Apoptosis is a physiologic and controlled process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis can be detected in populations of cells or in individual cells. Many different methods have been devised to detect apoptosis such as TUNEL (TdT-mediated dUTP Nick-End Labeling) analysis, ISEL (In Situ End Labeling), DNA laddering analysis (detect fragmentation of DNA in a population of cells), annexin-V staining (which measures alterations in plasma membranes via flow cytometry), caspase-3 staining, and detection of apoptosis-related proteins such p53 and Fas.

1.5.2 Cell Proliferation

Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations involving *in vitro* and

in vivo studies. Examples where determination of cell number is useful include the analysis of growth factor activity, serum batch testing, drug screening, and the determination of the cytostatic potential of anti-cancer compounds in toxicology testing. In such studies, *in vitro* testing techniques are very useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells.

Cell proliferation is the measurement of the number of cells that are dividing in a culture. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto the appropriate matrix and the number of colonies that are formed after a period of growth are enumerated.

Another way to analyze cell proliferation is the measurement of DNA synthesis as a marker for proliferation. In these assays, labeled DNA precursors (^{3}H-thymidine or bromodeoxyuridine) are added to cells and their incorporation into DNA is quantified after incubation (see DNA Synthesis section below). Proliferating cell nuclear antigen (PCNA) and Ki67 are often used as markers of proliferation, though they do not measure the number of cells going through DNA replication.

Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the cell cycle are measured either by their activity (e.g. CDK kinase assays) or by quantifying their amounts (e.g. Western blots, ELISA, or immunohistochemistry).

1.5.3 DNA Synthesis

During cell proliferation, the DNA has to be replicated before the cell can divide into two daughter cells. This close association between DNA synthesis and cell doubling makes the measurement of DNA synthesis very attractive for assessing cell proliferation. If labeled DNA precursors are added to the cell culture, cells that are about to divide incorporate the labeled nucleotide into their DNA. Traditionally, those assays involve the use of radiolabeled nucleosides, particularly tritiated thymidine ([^{3}H]-TdR). The amount of [^{3}H]-TdR incorporated into the cellular DNA is quantified by liquid scintillation counting (LSC).

Experiments have shown that the thymidine analogue 5-bromo-2′-deoxy-uridine (BrdU) is incorporated into cellular DNA like thymidine. The incorporated BrdU could be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies directed against BrdU. The use of BrdU for such proliferation assays circumvents the disadvantages associated with the radioactive compound [^{3}H]-TdR (for advantages and disadvantages of each approach, see Table 1.6). The amount of labeled precursor incorporated into DNA is quantified either by measuring the total amount of labeled DNA in a population, or by detecting the labeled nuclei microscopically. Incorporation of the labeled precursor into DNA is directly proportional to the amount of cell division occurring in the culture.

Table 1.6 Comparison of [^{3}H]-TdR and BrdU for DNA synthesis analyses

Assay	Advantages	Limitations
[^{3}H]-TdR proliferation assay	Sensitive (103–104 cells/test required) Linear measurement of cell proliferation over a broad, logarithmic range Low background	Radioactive isotope handling and storage problems Long half-life Radioactive waste disposal costs
BrdU-incorporation assay	No transfer of the cells; the entire assay is performed in a single MTP Non-radioactive	Assay is not linear over a broad logarithmic range of cell proliferation (limitation of the ELISA plate reader) Three washing and incubation steps Longer assay time

1.5.4 Cell Viability in Cell Populations

A number of methods have been developed to study cell viability and proliferation in cell populations. The most convenient modern assays have been developed in a microplate format (96-well plates). This miniaturization allows many samples to be analyzed rapidly and simultaneously. The microplate format also reduces the amount of culture medium and cells required as well as the cost of plasticware.

Table 1.7 Comparison of colorimetric assays for cell proliferation/metabolism analyses

Assay	Advantages	Limitations
MTT Assay	• No transfer of the cells; the entire assay is performed in a single MTP • MTT is metabolized by all cells; the assay can be used with all cell types • Inexpensive	• Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader • Insoluble reaction product; resolubilization of the reaction product required • Cannot take multiple time points in a single assay • Cells with low metabolic activity (e.g., lymphocytes) must be used in high numbers
XTT Assay	• No transfer of the cells; the entire assay is performed in a single MTP • Soluble reaction product • Can take multiple time points in a single assay	• Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader • XTT working solution has to be prepared shortly before use • XTT is not metabolized by all cell types
WST-1 Assay	• No transfer of the cells; the entire assay is performed in a single MTP • Soluble reaction product • Repeated measurement of the assay • Ready-to-use solution	• Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader • WST-1 is not metabolized by all cell types

Colorimetric assays allow samples to be measured directly in the microplate with an ELISA plate reader.

Microplate assays have been developed based on different parameters associated with cell viability and cell proliferation. The most important parameters used are metabolic activity and DNA synthesis for microplate format. Cellular damage will inevitably result in loss of the ability of the cell to maintain and provide energy for metabolic cell function and growth. Metabolic activity assays are based on this premise. Usually they measure mitochondrial activity. The cells are incubated with a colorometric substrate including one of the following: MTT, XTT, WST-1. A comparison of each is provided in Table 1.7.

1.5.5 Cell Cycle Parameters

Once the cell is instructed to divide, it enters the active phase of the cell cycle, which can be broken down into four segments:

- During G1 (G = gap), the cell prepares to synthesize DNA. In the latter stages of G1, the cell passes through a restriction point and is then committed to complete the cycle.
- During S phase the cell undergoes DNA synthesis and replicates its genome.
- During G2 the cell prepares to undergo division and checks its replication using DNA repair enzymes.
- During M phase, the cell undergoes division by mitosis or meiosis and then re-enters G1 or G0.

In most instances, the decision for a cell to undergo division is regulated by the passage of a cell from G1 to S phase. Progression through the cell cycle is controlled by a group of kinases called cyclin-dependent kinases (CDKs). CDKs are thought to phosphorylate cellular substrates, such as the retinoblastoma gene, that are responsible for progression into each of the phases of the cell cycle. CDKs are activated by associating with proteins whose levels of expression change during different phases of the cell cycle. These proteins are called cyclins. Once associated with cyclins, CDKs are activated by phosphorylation via CDK-activating kinase (CAKs) or by dephosphorylation via a phosphatase called CDC25.

D-type cyclins are the primary cyclins that respond to external cellular factors. Their levels start off low during G1 and increase towards the G1/S boundary. Cyclin D regulates CDK4 and CDK6. Cyclin E is expressed transiently during the G1/S transition and is rapidly degraded once the cell enters S. Cyclin E regulates CDK2 and perhaps CDK3. When S phase begins, levels of cyclin A increase and activate CDK2. The cyclin A/CDK2 complex is thought to have a direct role in DNA replication. The progression through mitosis is regulated by the presence of cyclin B. Cyclin B associates with CDC2 and forms the primary kinase present during mitosis (MPF = “M-phase/maturation promoting factor”). During anaphase, cyclin

B is degraded. This degradation of cyclin B appears to regulate the cell's progression out of mitosis and into G1. Flow cytometry and western analysis of these various cyclins, CDKs, and CDCs are employed to determine relative levels of these cells and proteins at time points during incubation of cells with certain compounds to verify that these drugs do affect cell cycling.

1.5.6 Invasion/Metastasis

The ability of a drug to affect the invasive and/or metastatic phenotype of a cancer cell can be determined by using an invasion assay or by introducing these cells into immunocompromised mice. In general, the *in vitro* invasion assay employs a multi-well plate coated with ECM proteins, usually collagen or laminin. This layer of ECM protein separates the top of the well from the bottom. Cells that invade through the matrix will either move far enough into the matrix or beyond so that when non-invasive cells are removed, any remaining cells can be stained and quantified. Comparisons with the number of cells originally plated and those moving into or past the protein layer provides a relative value of invasion. More cells that move through the matrix means greater levels of invasion. The *in vivo* invasion/metastasis assay requires an immunocompromised mouse for xenograft transplant, usually orthotopically in the pancreas. In this less quantifiable assay, invasion of cancer cells can be observed in microscopic sections to determine the relative extent of cell invasion among samples. Metastastic potential can be assessed by quantitating the number of metastases in an organ, usually the liver.

1.6 Concluding Remarks

Cultured pancreatic cells, particularly cancer lines, are highly valuable tools as a first line evaluation for the efficacy of a drug on a given cell type. In addition, cell culture systems allow for reliable, cost-effective analyses that define the relative potency, toxicity, and mechanism of a compound in a longitudinal yet relatively simple format. Drugs that fail to demonstrate any type of response in multiple cell lines and durations when evaluated for changes in apoptosis, proliferation, phenotype, viability, cell cycling, or invasion/metastasis would seem almost certain to fail in a preclinical setting, where dosing, clearance, target specificity, and overall toxicity would further challenge a response at the tumor level. Drugs that do provide a clear response in culture can be further evaluated in these systems to identify their specific mechanisms in cancer and "normal" cells *in vivo*. In addition, more advanced culture techniques can be employed to determine drug effects in a more realistic setting (3D cultures), in relationship to other cell types or secretions (co-cultures), or in regard to cell invasion and metastasis. This information will provide the rationale and area of focus for the next translational step in drug discovery,

where preclinical (including xenograft and genetically engineered) models of pancreatic cancer can be employed.

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Chapter 2
Mouse Xenograft Models for Drug Discovery in Pancreatic Cancer

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Abstract It is estimated that the development of a new anticancer agent costs US $800–1,000 million and takes over a decade between conception and approval. However, 90% of novel antineoplastic drugs fail in the clinic despite evidence of antitumor efficacy in classical preclinical models. This raises serious concerns whether such models are predictive of drug efficacy in humans, supporting the development of alternative approaches. Furthermore, a general transition has been observed from the empirical drug screening of cytotoxic agents against uncharacterized tumor models to target-orientated drug screening of agents with defined mechanisms of action. New approaches to anticancer drug development involve the molecular characterization of models along with an appreciation of the pharmacodynamic and pharmacokinetic properties of the tested drug. This chapter focuses on the classical and new preclinical screening models and techniques for anticancer drug development.

2.1 Introduction

Drug development is a multi-million dollar and time-consuming business. It is estimated that each new approved drug requires an expenditure of US $800 million in resources over 10–12 years in time between conception and approval (DiMasi et al. 2003). Although great progress has been made in understanding mechanisms of tumorigenesis resulting in development of many anticancer drugs, 90% of novel antineoplastic therapies fail in the clinic despite evidence of antitumor efficacy in classical preclinical models (Von Hoff 1998). Combined with this frustration is a

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special real sense of urgency in the field of pancreatic cancer, an almost uniformly fatal disease, accounting for >30,000 cancer deaths each year in the United States (Jemal et al. 2003). Lack of efficacy in late-phase clinical trials and rejection by the FDA are by far the most costly points of failure on drug development. This highlights the most important concept in preclinical modeling: predictive utility. Improving the ability of preclinical models to predict clinical efficacy would have a great impact.

Animal models have been used as the front line in predicting efficacy and finding toxicities for cancer chemotherapeutic agents before entering the clinic. Human tumor xenografts grown in nude or in mice with severe combined immunodeficiency (SCID) have covered all of the major tumor types and represented the mainstay of preclinical anticancer drug development testing in vivo. However, there has been considerable debate regarding the value of the classical xenograft model for predicting drug efficacy in humans (Takimoto 2001).

Furthermore, as opposed to the previous era of "cytotoxic" cancer drugs, there has been a major shift in the focus of modern drug discovery to therapies directed at cancer-associated molecular targets, where cytostatic rather than cytotoxic effects may often be predicted. This may require a re-evaluation of the in vivo models developed and validated using cytotoxic drugs when testing such agents. New approaches to anticancer drug development involve the molecular characterization of models along with an appreciation of the pharmacodynamic and pharmacokinetic properties and toxicity of the drug.

As modern drug development technologies increase the number of candidate therapeutics, and as the costs for preclinical and clinical testing escalate, the use of model systems to help prioritize compounds for clinical investigation becomes increasingly important.

Therefore, there is an obvious need for better tumor models, and perhaps equally importantly, better-defined questions and endpoints with which animal models are used to interrogate anticancer drug activity. This chapter summarizes the history of drug screening, with emphasis on pancreatic cancer, and aims to provide a critical survey of the role of the traditional and new in vivo xenograft models used for predicting chemotherapeutic drug response in patients in contemporary cancer drug development.

2.2 Classical Drug Development Program at the NCI

Systematic drug screening began at the National Cancer Institute (NCI) in 1955 with the establishment of the Cancer Chemotherapy National Service Center (NSC) screening program (Zubrod 1972), which tested agents in mice bearing rapidly growing murine leukemia cells injected intraperitoneally (i.p.) (Goldin et al. 1960, 1961; Stock et al. 1960). Later concerns were identified regarding reliance on a single leukemia tumor model because this could preferentially select for drugs targeting rapidly growing tumors (Venditti et al. 1984). These models were successful in

identifying active therapeutics against hematological malignancies, but not against solid tumors (Suggitt and Bibby 2005; Schein and Scheffler 2006). The discoveries leading to the establishment of human tumors in immunodeficient mice (Flanagan 1966; Rygaard and Povlsen 1969) led to the incorporation of solid human tumor xenografts representative of the major histologic types of cancer into this screening program in the 70s. The panel consisted of matched animal and human tumors of the breast, colon, and lung, in addition to the murine L1210 leukemia and B16 melanoma syngeneic models (Venditti et al. 1984; Plowman 1997). Subsequent analysis revealed that among the compounds that were disregarded by the syngeneic models, almost 30% of them were active in at least one human tumor xenograft (e.g., paclitaxel) (Venditti et al. 1984).

In 1982, the NCI implemented a cost-effective sequential screening strategy. Initially, drugs were examined against the P388 leukemia as a prescreen, followed by studies with a panel of murine tumor xenografts known to produce a relatively high yield of active compounds (Venditti et al. 1984). Drugs active in this panel were advanced to a secondary screen using compound-orientated tumors.

In 1989, the NCI changed its screening strategy from a "compound-orientated" to a "disease-orientated" strategy and launched the NCI-60 cell line panel incorporating human tumor cell lines of diverse histological backgrounds (brain, colon, leukemia, lung, melanoma, ovarian, breast, prostate, and renal cancers) (Boyd 1997). Because over 85% of the compounds submitted for screening are found to have no antiproliferative activity, the NCI adopted a three-cell-line prescreen in 1999, using three highly sensitive cell lines: MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), and SF-268 (glioma). Demonstration of growth inhibitory activity is required in this pre-screen panel before a compound can undergo advanced testing in the full 60-cell-line screen. The goal of the in vitro 60-cell-line screening assay was to select compounds for a secondary, more comprehensive, in vivo testing.

Given the large number of molecules emerging from the in vitro screen as candidates for xenograft testing and the time and resource requirements for the typical tumor implant studies, in an attempt to prioritize compounds for secondary xenograft screening, the shorter-term in vivo hollow fibre assay (HFA) was implemented in 1995, based on its good predictivity of xenograft activity (Johnson et al. 2001; Decker et al. 2004). In the HFA model, cells are introduced from tissue culture into semi-permeable fibres in mouse intraperitoneal (i.p.) or subcutaneous (s.c.) space. The assay has the potential to simultaneously evaluate compound efficacy against a maximum of six cell lines. Mice are treated with test compound at two doses for up to four days, and fibres excised and analyzed for cell viability using a tetrazolium-based colorimetric assay (Hollingshead et al. 1995; Decker et al. 2004). Total assay time is thus roughly one week. Supportive evidence for the use of the HFA as an in vivo prescreen is derived from the good predictivity of in vivo xenograft activity (Hollingshead et al. 1995; Johnson et al. 2001; Decker et al. 2004). Compounds considered active in the HFA are prioritized for in vivo xenograft testing.

To assess the predictive value of s.c. xenograft models, a retrospective review conducted by the NCI of 39 agents for which Phase II activity data were available

showed that, when compounds were active in at least one-third of xenograft models tested, there was a statistically significant correlation with activity in humans (Johnson et al. 2001). These data suggest that, at least for cytotoxic anticancer drugs, s.c. xenograft models may have some predictive value. However, activity within a particular histological type of xenograft generally did not predict for clinical activity against the same tumor, except for non-small-cell lung cancer.

It was suggested that the lack of histologically based correlations may be a consequence of experimental design which limited the number of tumors for each of the three major cancer types. Thus, it was concluded that a model system composed of several tumors with the same histiotype might better predict a clinical response against a specific tumor histiotype.

As a result, while the contribution of classical mouse models to understand cancer biology is inarguable, there has been considerable debate regarding their value for predicting drug efficacy. Furthermore, these studies were largely assessments of cytotoxic therapies, utilizing antitumor efficacy as the only endpoints. It is not clear whether these results extend to the testing of targeted therapies. New evolving strategies seemed to be in response to the advent of a new era of anticancer therapeutics.

2.3 New Animal Models

Clearly, the poor predictive power of our current models supports the use of alternative models and approaches. Table 2.1 summarizes the advantages and pitfalls of these models. Models which are more biologically representative of patient tumors could be valuable in predicting clinical outcome.

2.3.1 Orthotopic Tumor Models

Although s.c. xenografts may recapitulate certain aspects of the tumor–host microenvironment, it is clear that there are other aspects of the tumor microenvironment that are more closely mimicked by implanting tumor cells into the anatomic sites from which they originally arose. There are many reports of differences in biological behaviour when tumors are grown s.c. relative to orthotopically (Eccles 2002).

Clinical observations have suggested that the organ environment can influence the response of tumors to chemotherapy. For example, in women with breast cancer, lymph node and skin metastases are more sensitive to chemotherapeutic intervention than metastases in either the lung or bone (Donelli et al. 1967). The sensitivity of xenografted tumors to therapeutics may also be modulated by their location (Killion et al. 1998). For example, in one study, colon carcinoma cells implanted into different anatomical locations of nude mice showed different response to a drug in each area (Fidler et al. 1994).

Table 2.1 A comparison of the attributes of different animal models for pancreatic cancer

	Tumor origin				Site of implantation	
	Cell lines	vs	Fresh tumor tissue	vs Transgenic	Ectopic	vs Orthotopic
Human tumor similarity						
• Heterogeneity	More homogeneous		Different cell populations		Depend on the tumor origin	Depend on the tumor origin
• Microenvironment	Murine stroma		% Human stroma	Murine stroma	Artificial environment	The importance of organ environment
Metastatic potential	Depend on site of implantation		Depend on site of implantation	Depend on type of model	Gross metastasis occur infrequently	Metastasis occur frequently
Time-consuming	Short, often only several weeks		Several weeks or months	Long, with some models requiring one year or more	Short and easy	Long, due to complex surgeries
Cost	Cheap		Expensive, based in labor	More expensive, based in labor and housing	Inexpensive	Expensive, based on labor and skill techniques
Tumor burden asses	Depend on the site of implantation		Depend on the site of implantation	More difficult, because orthotopic growth	Easier, with calipers	Difficult

It has also been suggested that the disparity between preclinical and clinical activity for many drugs could be related to the treatment of advanced metastatic disease in the clinic, not represented by conventional s.c. xenograft models (Kerbel 2003). The propensity for metastasis is also influenced by tumor implant sites. In many cases, orthotopic implantation of tumor cells has been found to greatly enhance metastasis, by comparison with the same cells implanted subcutaneously.

Together, these results suggest that orthotopic transplant models may be superior to s.c transplant models, at least in recapitulating certain aspects of the tumor–host interactions. However, whether the orthotopic models are representative of clinical disease and should replace traditional s.c. nonmetastatic xenografts remains an unanswered, yet critical, question. Orthotopic models have also been observed to falsely predict clinical activity (e.g., batimastat; Wang et al. 1994). Furthermore, their application is hindered by several disadvantages. In contrast to conventional s.c. tumor xenografts, limitations include the need of higher technical skill because of the often complex surgeries; a more time-consuming process, and increased cost. Therapeutic efficacy is also more difficult to assess with orthotopic models in contrast to the relative ease of s.c. tumor measurements (Bibby 2004). Tumor burden can be assessed by serial sacrifice of cohorts of mice if tumor formation is highly penetrant and synchronous. However, this necessitates large numbers of animals. Noninvasive methods for assessing tumor burden, including small animal imaging (as magnetic resonance imaging and positron emission tomography), overcome these limitations (Hoffman 2002; Weissleder 2002; Bibby 2004). However, space and resource requirements may be prohibitive, and throughput may be inadequate for drug discovery purposes, as these modalities generally allow imaging of only one animal at a time. Reporter genes with specific fluorescence properties have been developed including the stable green fluorescent protein (GFP), red fluorescence protein (RFP), galactosidase lacZ gene, and the luciferase gene (Lin et al. 1990; El Hilali et al. 2002; Hoffman 2002).

2.3.2 Genetically Engineered Mouse Models

Genetically engineered mouse models (GEM) are a promising alternative to traditional preclinical assays. GEMs have been postulated as potentially more representative of specific human tumor histiotypes than classical xenografts because of their in situ and autochthonous origin (Sharpless and Depinho 2006). Over the past 20 years, GEMs have contributed to our understanding of the molecular pathways responsible for the initiation, progression, and metastasis of cancer cells, and have highlighted the importance of specific genes in particular types of cancer. The utilities of GEMs in drug discovery are further discussed in Chap. 4.

In comparison to traditional xenograft models, very few studies have tested known clinically effective agents using GEM models and these studies have not been encouraging (Bearss et al. 2000; Rego et al. 2000; Basu et al. 2004), making the general acceptance of the GEM model as preclinical tools questionable. In fact, they have not been used yet in development of any FDA approved anticancer agents.

Moreover, there are several limitations in using GEM in drug development, as their use is often restricted by intellectual property rights and patents (Weiss and Shannon 2003). Another major disadvantage of GEM models for drug discovery is the variable penetrance resulting in lack of synchrony in tumor development. In a setting where one wishes to test multiple doses of multiple compounds in appropriately powered studies, the synchronous development of tumors in sufficient numbers of mice to conduct such studies is difficult. The use of models in which tumor formation can be induced in a conditional manner, as several pancreatic models reported, improves the likelihood of having sufficient cohorts of mice. However, the CRE-lox system, which is the most commonly used approach for conditional induction of tumors, also carries additional intellectual property issues. Further, no one transgenic model is representative of all of the different forms of even one tumor histiotype; just as one human tumor cannot represent another human tumor of the same histiotype. Another subject to consider is that systemic disease, the principal cause of death in patients, is rarely observed in GEMs (Van Dyke and Jacks 2002), making the GEM that develop metastasis, very valuable. Some other limitations to the use of GEM in drug development are that breeding and maintaining a colony large enough to generate sufficient numbers of mice at the same age/gender can be very costly; that the stroma is rodent; that the tumors are difficult to follow, as we have described on orthotopic models; that the end point is frequently survival; and that statistics are difficult. We cannot forget that species-specific differences also exist, resulting in different mutant phenotypes in man and mouse (Jacks 1996).

2.3.3 Humanized Mice

The models discussed above lack human stroma and immune cells, which are important to the tumoral growth and metastatic process (De Wever and Mareel 2003; Kim et al. 2005).

The term humanized mice has been used to describe numerous animal models, including immunodeficient mice, reconstituted with human stem cells or lymphocytes (Legrand et al. 2006). This approach has also been combined with the transplantation of human thymic and/or bone marrow (BM) before stem cell injection to provide a human stromal environment. Since stromal cells contribute to tumorigenesis, fidelity of xenografts might be improved by establishing a fully human microenvironment. Recent studies have shown that when nonmalignant stromal cells are coinjected with transformed human cancer cells, the human stromal cells do contribute to eventual tumor formation (Chudnovsky et al. 2005). These studies have provided important insights into mechanisms responsible for tumor formation. Immunologically humanized mice have also been immunized to induce human hybridomas and to study T-cell responses against tumors and viruses. They can then be challenged with human tumor xenografts to study the effect of immunity on tumor/viral growth. However, further studies are needed to determine whether these models are an improvement over conventional xenograft or orthograft models for assessing therapeutic agents.

Another definition of humanized mice involves the insertion of a human gene into the mouse genome (Thomsen et al. 2005). Human tumor antigens have been expressed in GEMs, rendering them tolerant to human antigen and providing a model to study vaccine responses in the presence of immunologic tolerance.

2.3.4 Freshly Heterotransplanted Human Tumor Xenografts

One potential reason for the lack of predictability in existing xenograft models is that they are based on established human cancer cell lines. Xenografts derived from cell lines generally show a more homogeneous, undifferentiated histology, probably indicative of the higher selection pressure to in vitro conditions during extensive culturing (Engelholm et al. 1985; Hausser and Brenner 2005). It is likely that such xenografts no longer retain the original molecular characteristics of the original patient tumor. Moreover, these xenografts lack human stroma and immune cells, which are important for the tumoral growth and metastatic processes.

Xenografts derived directly from patient biopsies, with minimal in vitro manipulation, appear to retain better the morphologic and molecular markers of the source tumors, despite serial passing across several generations of mice (Perez-Soler et al. 2000; Bankert et al. 2001; Rubio-Viqueira et al. 2006). Because the tumor and stromal microenvironments are from the same individual, histocompatibility differences, which could affect the engraftment, growth, and response to drugs, are less of a concern. Many of the elements of a "natural" tumoral stroma will be present within the microenvironment, although the successful engraftment of all these components could depend on the strain of mice used (Williams et al. 1996; Simpson-Abelson et al. 2008). It should be noted, however, that several of the supporting elements in these tumors are indeed from mouse origin rather than human. Because it appears that model systems composed of a panel of several tumors with the same histiotype might better predict a clinical response against that specific tumor histiotype, several groups have established disease-specific panels of xenografts directly from patient tumors.(Perez-Soler et al. 2000; Fichtner et al. 2004; Rubio-Viqueira et al. 2006). They have shown that this approach is feasible, with a high rate of engraftment for some tumors (e.g., pancreatic, lung, and colon cancer). Some studies have shown response rates to several anticancer drugs in the xenograft model similar to the overall response rate recorded for monotherapy clinical trials with these agents (Perez-Soler et al. 2000; Fichtner et al. 2004; Fiebig et al. 2004; Rubio-Viqueira et al. 2006).

Our group at Johns Hopkins established a freshly heterotransplanted human pancreas tumor xenograft panel as an in vivo platform for late preclinical drug development in pancreatic cancer (Rubio-Viqueira et al. 2006). The goal was to develop well-defined cohorts of carcinomas, each one from a unique patient, which can be treated with new drugs as in human phase I–II clinical trials and used to address translational research questions. To date, we have more than 50 different

xenografts growing, 30 of them have been treated with several drugs, simulating a classic human two-stage phase II clinical trial. Our results show that this approach can be used as an in vivo screening modality to test novel drugs with therapeutic potential, at least in the context of pancreatic cancers. The take rate of the carcinoma is high (more than 70%) and predictable and the growth kinetics in athymic mice are very homogeneous and similar to published studies (Bocsi and Zalatnai 1999; Sorio et al. 2001). In addition, we have demonstrated that drug testing is perfectly feasible in this model as is routinely done with conventional xenografted cell lines. Our results showed no major variations in the status of the principal genes mutated in pancreatic cancer, indicating that the fundamental pathogenetic elements remain stable. Indeed, the frequency of these genetic alterations is well within the expected frequency for pancreatic cancer, suggesting that these carcinomas are representative of pancreatic cancer as a whole. Interestingly, we have compared the responses of the same pancreatic xenograft to gemcitabine across three generations (F3 and F6) and have not observed any variations in therapeutic response, suggesting that, indeed, susceptibility or resistance is a stable trait.

An important consideration of this model, in contrast to existing preclinical models that use commercially available cell line resources, is that it will permit prospective clinical validation of preclinical efficacy data. Because almost all pancreatic cancer patients are treated in the clinic with gemcitabine either in the adjuvant or metastatic setting, a clinical protocol to correlate the response of a patient's xenografted tumor to gemcitabine with the clinical outcome of the patient when treated with this agent is ongoing. Data is still preliminary and the study is still enrolling patients. So far, we have seen a correlation between xenograft and patient response to gemcitabine (the standard treatment for pancreatic cancer) both in adjuvant and metastatic settings, although this correlation is not significant, probably due to the still low numbers (Jimeno et al. 2008).

In recent years, the heterotransplanted human xenograft model has increasingly been used to screen for new drugs against specific disease types. As a practical matter, however, primary tumor explants are time- and resource-intensive, and there are barriers to their incorporation into a high-throughput drug discovery effort. It is also important to realize that the successful rate of engraftment and the chemosensitivity of human tumors heterografted into different strains might differ, suggesting that the genetic background of the host might be a factor in determining tumor behavior and responsiveness (Yoshimura et al. 1997).

2.4 Interpreting Results: Variables and Endpoints

It is critical to fully appreciate and understand each of the many variables related to the use of animal models in drug testing in order to correctly interpret study results. Major variables include not only the origin of the tumor and its site of implantation, but also the agent dose, formulation, schedule and route of administration, and experimental endpoints.

2.4.1 Growth Characteristics of the Model

Not all xenograft models are usable for compound screening because of their growth properties. In some tumors, growth is too slow, or too inconsistent/erratic/non-linear with time, or they possess cystic or necrotic areas, even at relatively small tumor volumes.

2.4.2 Strain of Mice

Tumor growth depends on intrinsic tumor cell properties and host factors. Xenograft models require nude (athymic) or severe combined immunodeficient (SCID) mice that are T- and B-cell-deficient, allowing the engraftment of human tumor cells. However, innate immunity, particularly natural killer (NK) cells, can limit tumor growth and prevent metastasis in nude mice (Habu et al. 1981). Mice with the nude mutation, although T-cell-deficient, have a compensatory increase in innate immunity, most notably increased NK activity and tumoricidal macrophages. The beige mutation results in a delay in NK activation, but not the loss of NK cell function (Talmadge et al. 1980). The immunomodulatory effect of some anticancer drugs could be no estimated or underestimated with these xenograft models.

2.4.3 Stage at Which the Treatment Begins

The activity of a test molecule can also be critically dependent upon the stage (size) of the tumor at the onset of treatment. In some instances, agents are administered at the same time as tumor implantation ("chemoprevention" strategy). Herein, a choice of model where the take rate is reproducibly greater than 90% and with consistent growth properties is critical. Other times, treatments may not begin until tumors are just palpable ("early-stage" strategy). Herein, one needs to be aware of the possibility that the residual immune system of the host can be responsible for tumor regression or cures. Finally, treatment may not begin until tumors have reached 8–10 mm diameter ("advanced" stage). In many cases, this is more representative of how chemotherapy will be applied in the clinic. However, there is the possibility that either target expression may decrease in larger tumors or that drug uptake/penetration may be compromised because of poor vasculature/increased areas of necrosis. In addition, for rapidly-growing tumors, tumor sizes/volumes may exceed those deemed to be ethically acceptable and result in the early termination of experiments before any delayed drug-induced toxicity may be evident.

2.4.4 The Test Compounds

Another critical variable concerns the test agent itself, the way it is administered (route, scheduling, dose) and how it is formulated. Too many mice have been used in the past with compounds given as coarse suspensions in dimethyl sulphoxide (DMSO) or detergents (e.g., Tweens), vehicles which may not be usable in man. The use of athymic mice bearing xenografts may be of limited value with naked humanized (or fully human) antibodies.

2.4.5 Pharmacokinetics

Species-specific differences in pharmacokinetics may lead to a lack of predictability. Dose and schedule are critical determinants of drug activity (Kerbel 2003; Peterson and Houghton 2004; Tan et al. 2005; Graham et al. 2006). Retrospective analysis of pharmacokinetic and pharmacodynamic parameters in preclinical and clinical studies can often logically explain the failure of compounds in the clinic (Peterson and Houghton 2004). For many agents, maximally tolerated levels in mice may exceed achievable or tolerable levels in humans and may thus produce false positive results. In studies in which multiple drugs were administered at "clinically equivalent dose" (i.e., reaching clinically achievable concentrations) in s.c xenograft tumor models, antitumor efficacy was highly predictive of clinical efficacy (Inaba et al. 1988, 1989).

2.4.6 Endpoints

Finally, the choice of endpoint is a critical variable. The predictive value may depend on the criterion used to define efficacy. Endpoints need to be matched to tumor type (solid, leukemia, or metastatic), study context, implantation site accessibility, type of implantation, and therapeutic drug class.

Antitumor efficacy is simple to measure in s.c tumors, thus accounting for their popularity. Caliper measurements can be used to calculate tumor volume. When comparing the growth of tumors in treated to control animals, a ratio of 20–30% (% T/C) is a common threshold utilized for establishing efficacy. The therapeutic response in xenograft models is typically based on slowed tumor growth kinetics as opposed to tumor regression required in humans. Thus, common outcome criteria for animal models and humans are disparate. The predictive value may be improved in mouse models by adopting tumor regression as the efficacy endpoint.

It should be noted that a chronic slowness of tumor growth rate can be considered a relevant outcome as compared with tumor regression if our goal is to

delay tumor progression. It is noted that a recent review of the athymic nude mouse model using human xenografts (Kelland 2004) suggested that an outcome focused on tumor growth rate or cytostasis may be more predictive of clinical activity than tumor shrinkage (cytotoxicity).

Nevertheless, in the era of targeted therapies, inhibition of tumor growth per se may no longer be the most relevant endpoint. The animal models should define if the drug modulates its target in vivo and if the associated biological effect impacts on tumor growth. If a drug has no effect on tumor growth, interpretation of the results depends on whether it hits its target. Another question is how to interpret antitumor efficacy if there is no effect on the target. Evaluation of informative pharmacodynamic markers is thus critical.

Conventional molecular analysis can be accomplished by sacrificing cohorts of mice over time, and using molecular techniques. The major disadvantage of these approaches is that they provide static data points, and a dynamic picture must be constructed by looking at the averages of cohorts of mice over time. Thus, large numbers of mice and significant time and resources need to be expended to establish a dynamic assessment of drug effect.

Noninvasive imaging can also be used to assess molecular effects of drugs. By virtue of being noninvasive and nonlethal, molecular imaging can provide serial measurements over time in animals, establishing a true pharmacodynamic readout. Furthermore, since many drugs can be radiolabeled, PET offers the possibility of directly monitoring drug distribution and clearance (Weissleder 2002). It is also possible to design PET probes that bind to specific drug targets (Smith-Jones et al. 2004). Obviously, these approaches require significant radiological, biological, and chemical expertise. Bioluminescence and fluorescence imaging can also be used for molecular imaging. These modalities are strictly research tools, as clinical translation will not be possible using existing technologies.

Despite the technical difficulty, labor intensity, and expense commonly cited as limitations for detailed examinations, the utility of mouse models is improved by implementing multiple rigorous endpoints.

2.5 Future Approaches in Drug Discovery: Biomarkers and Personalized Therapies

Despite increasing progress in our understanding of the molecular basis for pancreatic cancer development and progression and the unprecedented discovery of therapeutic targets, this has not translated into better therapeutic interventions (Moore et al. 2003; Van Cutsem et al. 2004). A common denominator in studies conducted in pancreatic cancer is that they have been based on a total empirical approach with no efforts to understand the reasons for success or failure. While cure of pancreatic cancer will come from discovery and implementation of novel therapeutics, given the heterogeneity of pancreatic cancer it is also possible that the outcome of patients can be improved by targeting existing therapies to the patients more likely

to respond to these agents in an individualized approach. Models or markers that predict individual patient susceptibility to existing specific drugs are needed.

2.5.1 Predictive Biomarkers

It is likely that some agents failed in the clinic because they were tested in unselected groups of patients and their potential beneficial effects were "diluted" by patients with no benefit. Therapy-predictive markers would guide the choice of treatment. The availability of predictive markers clearly allows for more efficient and cost-effective patient treatment. This strategy has been successful in other settings such as breast cancer and more recently lung cancer (Baselga et al. 1996; Pegram et al. 1998; Lynch et al. 2004; Paez et al. 2004).

Animal models may also be important testing grounds for biomarkers to be used in the initial clinical trials. For example, the development of informative immunodetection reagents, functional imaging modalities, and identifying molecular signatures of response may all be amenable to testing in preclinical models.

Due to a shift in the focus of modern drug discovery to biologically-targeted anticancer therapeutics, xenograft models should be characterized to ensure that the molecular target of interest is present as well as to incorporate pharmacokinetic and pharmacodynamic endpoints (Sausville and Feigal 1999). The freshly heterotransplanted human xenograft model has the advantage that these xenograft tumors can be characterized in full detail at the molecular level. Because the tumors are easily propagated in additional xenografts, this model has become an important source of tumor material for biological studies, overcoming the limitations often encountered with small human biopsies. Analytic methods can be applied to multiple samples within the same tumor so that issues of cancer heterogeneity can be assessed. For example, the tissue array built with our pancreas tumor panel contains eight cores from each patient's tumor, a sampling much larger than most conventional tumor biopsies (Rubio-Viqueira et al. 2006). The biological information obtained for each tumor, before and also after treatment, could be incorporated into a database and linked to drug response (Fig. 2.1). This process will facilitate the elucidation and development of pharmacodiagnostic and pharmacodynamic markers of drug response.

We define a predictive pharmacodiagnostic marker as a marker which helps us to predict the response to a drug before the treatment with it. For example, somatic *BRCA2*/Fanconi anemia gene mutations are present in a subset of pancreatic cancer patients, particularly in familial cases (Goggins et al. 1996; Hahn et al. 2003), and it has been recently documented that the presence of these mutations is predictive of sensitivity to cross-linking (mitomycin C, cisplatin, chlorambucil, and melphalan) chemotherapeutic agents in pancreatic cancer preclinical models (van der Heijden et al. 2005). Another pharmacodiagnostic biomarker in pancreatic cancer is deoxycytidine kinase, essential for the phosphorylation of gemcitabine (2′,2′-difluorodeoxycytidine), which has been related to gemcitabine sensitivity in

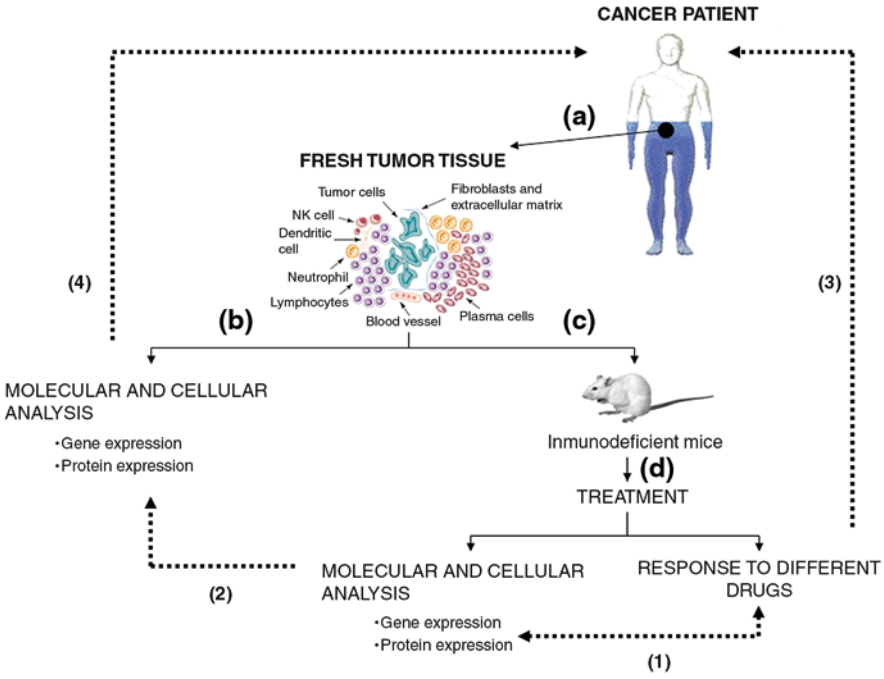

Fig. 2.1 A flowchart for the "Tailor-Made" treatment process using mouse xenograft models. The *solid line arrows* point to the different steps of the process: after surgical resection (**a**), a piece of the excess tumor tissues not needed for clinical diagnosis is saved for (**b**) molecular and cellular analysis and the remaining (**c**) is implanted s.c into 5- to 6-week-old female inmunodeficient mice. Xenografted tumors are expanded in successive groups of mice to develop cohorts of tumor-bearing mice suitable for drug therapy (**d**). The *dotted line arrows* show the potential correlations: (1) correlations among tumor response and molecular or cellular data are assessed to look for biomarkers of susceptibility to each drug; (2) our hypothesis is that each patient's tumor will respond best to the drug that is most active against that patients' personal panel of mouse tumor; (3) human tumors xenografted directly from patient biopsy retain their main molecular features; and (4) molecular features of the human tumor can also be correlated with individual tumor history, looking for biomarkers of response and prognosis

preclinical cell-line-based xenograft models including pancreatic cancer (Sebastiani et al. 2006). Deoxycytidine kinase protein expression measured by immunohistochemistry correlated with gemcitabine activity in our novel direct xenograft pancreatic cancer model, exemplifying a method of assessment more easily translated to the clinic.

However there are several limitations on the use of pharmacodiagnostic biomarkers, including the failure to consider that a pre-treatment sample could be sufficient to predict the antitumor activity since the anticancer activity of a targeted agent may largely depend on alterations in signaling both upstream and downstream of the target. This implies that the pharmacodynamic effects of the agent could, in fact, be more important. These differences could explain the range of response to

targeted therapy among individual subjects with apparently identical target protein expression levels (Bishop et al. 2002; Campiglio et al. 2004), making the pharmacodynamics biomarkers more interesting.

The development of assays to predict tumor response to treatment was also hindered in the past by problems of tissue acquisition. Previously explored chemosensitivity assays required relatively large tumor specimens (i.e., surgical biopsies). Fine-needle aspiration biopsy (FNAB) is a minimally invasive, established diagnostic procedure that allows acquisition of enriched tumor cell populations to perform analytic molecular tests (Pelosi et al. 1994; Assersohn et al. 2002; Pusztai et al. 2003). Using the freshly heterotransplanted human tumor xenograft model, we have shown that cytologically confirmed tumor FNAB samples can yield viable tumor cells and sufficient protein quantities to analyze the efficacy of targeted drugs before (ex vivo) and during (in vivo) systemic treatment. Given its safety, minimal morbidity, and relative technical ease, FNAB is also suitable for serial sampling over the course of treatment to monitor therapy effect in vivo (Rubio-Viqueira et al. 2007).

2.5.2 Models and Techniques for Personalized Treatments

2.5.2.1 "Tailor-Made" Treatments

One prospect for further application of preclinical models could be the individualization of models of human cancers which would account for inter-patient variability, by developing direct-patient cellular or xenograft models and treating them with multiple anticancer agents, thereby facilitating the selection of the best therapy for each individual patient. This approach best represents "tailor-made" treatment, where the optimal agent(s) is identified for a particular patient. Our group at Johns Hopkins is conducting a clinical trial with this principal objective (Fig. 2.1). The hypothesis is that each patient's tumor will respond best to the drug that is most active against that patient's personal panel of mouse tumors. Tumor pancreas xenografts from patients with resectable pancreas adenocarcinoma are treated with a battery of different approved anticancer agents with the goal of prioritizing the agents that work best in the model to be used in the treatment of patients should their disease recur following treatment with gemcitabine. We expect that this study will validate the freshly heterotransplanted human tumor model as a predictor of clinical efficacy for anticancer agents. This approach is suitable in resected pancreatic cancer because patients are treated with adjuvant chemotherapy after resection and >80% develop disease progression with a median time to progression of 12–18 months, which is enough for engraftment of the carcinoma and testing of candidate drugs in the xenograft. Data are very preliminary and the study is still enrolling patients. Interestedly, a patient, whose xenograft was resistant to gemcitabine but very sensitive to mitomycine C, progressed during the standard treatment and showed an impressive clinical and marker response to mitomycin (Jimeno et al. 2008).

However, generalizability and applicability remain the pitfalls for these tailor-made approaches, as they are restricted to: (1) patients undergoing surgical resection of their cancer with availability of excess tumor tissue; and (2) successful propagation in vitro or in vivo conditions. It is unlikely that these tailor-made strategies will be routinely used for personalized medicine.

2.5.2.2 "Prêt-à-porter" Treatments

The "prêt-à-porter" concept is based on the following key observations: (1) gene expression can be measured accurately and has shown promise as the "universal language" in disease characterization and prognostication; (2) the "*Connectivity Map* concept" (Lamb et al. 2006) suggests that gene expression can be used to connect different biological systems; and (3) biological pathways drive disease phenotypes and, therefore, can be used as the connectable traits.

With these premises, we hypothesized that a given tumor can be connected with another tumor based on pathway expression similarities and that drug response should be similar in closely connected tumors. To test this hypothesis, we first developed the "prêt-a porter" reference data base using the NCI-60 drug screening panel, developing a Gene Set Connectivity Map (GS-CMAP). This panel contains 60 diverse human cancer cell lines screened with >100,000 chemical compounds for anticancer activity. We then connect each one of a group of our thirty freshly heterotransplanted human pancreas tumor xenograft with a cell line from the NCI-60 panel. Connections were done on base on gene set enrichment analysis (GSEA) and pathway correlation analysis, using KEGG as the base for systematic pathway classification. We have shown that a given human xenograft connected with a cell line based on pathway expression similarities has a similar pattern of susceptibility to anticancer agents of that cell line (Tan et al. 2008). In that way, the GS-CMAP coupled with this in vivo freshly heterotransplanted human tumor xenograft platform could be used as a powerful translation research tool in identifying pathway-specific drugs, proposing effective drug combinations, providing opportunities for new target discovery and even realizing personalized medicine, since a given patient tumor could be connected with a human tumor xenograft whose susceptibility to different anticancer agents would be known.

2.6 Concluding Remarks

As the number of therapeutic candidates increases, and the costs for preclinical and clinical testing escalate, the use of model systems to help prioritize which compounds to select for clinical investigation becomes increasingly important. Indeed, the cost of preclinical development is minimal as compared with the cost of a failed drug candidate in phase II or III clinical studies. The constant failure to impact pancreatic cancer outcome has generated a sense of defeat among the research

community and pharmaceutical industry which may decrease interest in continuing to investigate novel therapeutic strategies in this disease even further. In an attempt to improve the predictive power of preclinical studies, several models have been developed, as we described above. The value of any preclinical model will ultimately depend on its ability to accurately predict clinical response.

As we have discovered more about the underlying mechanisms responsible for the initiation and progression of human cancers, we have experienced a move away from the development of classic cytotoxic agents to the rational design of targeted molecule anticancer therapeutics. The use of uncharacterized tumor models has to be continuously replaced by more clinically relevant and molecularly characterized models along with the integration of pharmacodynamic and pharmacokinetic approaches. As we described, GEMs models are good models for a better understanding of pancreatic cancer development, for the finding of potential drug targets, and for the evaluation of these novel therapies targeting specific molecular defects in an initial step in their development. However, very different molecular pathways are involved in the whole process of carcinogenesis, and tumors are characterized by heterogeneous cancer cells populations, probably making this model appropriate for understanding the mechanism of action of the drug and its pharmacodynamic effects, but inappropriate for efficacious drug screening in cancer. We have shown that freshly heterotransplanted pancreas human tumor xenografts mimic the molecular profile of the original tumor and also its heterogeneity. We can make a careful molecular characterization of these xenografts, postulating this model as a good platform for anticancer drug screening and development.

We cannot forget that most drugs fail phase II clinical trials largely because of inappropriate guidance from preclinical studies. Among many reasons why preclinical studies fail to correlate with clinical efficacy are differences in drug metabolism, pharmacokinetics, and pharmacodynamics, many of which are not addressed in most drug studies in mice. It might be reasonable to return to preclinical animal studies after phase I clinical trials, using different panels of heterotransplanted human xenografts to test the new candidate drug at the dose equivalent to the dose achieved in the human. With this approach, we could select the kind of tumor in which to test the agent in a phase II study. Such strategies may be very useful when testing existing or approved therapeutics for a potential new indication.

Furthermore, mouse models are an ideal platform for testing combinations of therapeutics. Combination testing in humans is encumbered for both practical and proprietary reasons. Yet, there is every reason to believe that certain combinations of drugs will be effective even when each of the individual agents has no effect. Mouse models offer the opportunity to test the combination therapy hypothesis and perhaps provide the data necessary to change how clinical drug testing proceeds.

In summary, there is an obvious need for better tumor models for drug development, especially in pancreatic cancer—but perhaps equally important is the need to use the right models to ask the right questions about the right therapies. While it is unrealistic to expect that any animal model will perfectly predict human outcome, even small improvements will translate into increased productivity and significant cost savings.

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Chapter 3
Fluorescent Metastatic Mouse Models of Pancreatic Cancer for Drug Discovery

Michael Bouvet and Robert M. Hoffman

Abstract Here we describe our cumulative experience with the development and preclinical application of imageable, clinically-relevant, metastatic orthotopic mouse models of pancreatic cancer. These models utilize the human pancreatic cancer cell lines which have been genetically engineered to selectively express high levels of green fluorescent protein (GFP) or red fluorescent protein (RFP). Fluorescent tumors are established subcutaneously in nude mice, and tumor fragments are then surgically transplanted onto the pancreas. Locoregional tumor growth and distant metastasis of these orthotopic implants occurs spontaneously and rapidly throughout the abdomen in a manner consistent with clinical human disease. Highly specific, high-resolution, real-time quantitative imaging of tumor growth and metastasis may be achieved in vivo without the need for contrast agents, invasive techniques, or expensive imaging equipment. We have shown a high correlation between florescent optical imaging and magnetic resonance imaging in these models. Transplantation of RFP-expressing tumor fragments onto the pancreas of GFP-expressing transgenic mice may be used to facilitate visualization of tumor–host interaction between the pancreatic cancer cells and host-derived stroma and vasculature. Such in vivo models have enabled us to serially visualize and acquire images of the progression of pancreatic cancer in the live animal, and to demonstrate the real-time antitumor and antimetastatic effects of novel therapeutic strategies on pancreatic malignancy. These fluorescent models are therefore powerful and reliable tools with which to investigate metastatic human pancreatic cancer and novel therapeutic strategies directed against it.

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3.1 Introduction

Pancreatic cancer is often a fatal disease with 5-year survival rates of only 1–4% (Bouvet et al. 2000a; Katz et al. 2004a). It is the fourth leading cause of cancer-related mortality in the United States. Reasons for low survival in this disease include aggressive tumor biology, high metastatic potential, and late presentation at the time of diagnosis (Bouvet et al. 2001; Moossa et al. 2002). The symptoms of pancreatic cancer may include jaundice, pain, weight loss, digestive problems, and new onset diabetes (Katz et al. 2005). By the time an individual with pancreatic cancer develops these symptoms, the tumor has often reached a large size and metastasized to other organs including liver, lung, and peritoneum (Moossa et al. 2002). Although chemotherapy can offer some palliation, it is not curative and the median survival is less than 6 months. For the few patients who have localized disease, surgical resection offers the only chance for cure. However, even with potentially curative surgery the 5-year survival rates are only 15–20% (Bouvet et al. 2000a). Clearly, techniques for earlier diagnosis and new treatment modalities need to be explored if progress is to be made. In an effort to help develop more effective treatment modalities for pancreatic cancer and improve detection, we and others have developed orthotopic models of human pancreatic cancer in the nude mouse that simulate tumor growth, progression, and metastasis and allow for testing of novel treatment strategies (Marincola et al. 1989; Vezeridis et al. 1989; Fu et al. 1992; Furukawa et al. 1993; An et al. 1996; Tomikawa et al. 1997; Kiguchi et al. 1998; Bruns et al. 1999; Bouvet et al. 2000b, 2001).

Mouse models based on athymic mice have been used for human cancer for the past several decades. However, metastatic rates from subcutaneous or intramuscular xenografts have been low or non-existent, even from tumors that were highly metastatic in the patient from whom the tissues were derived (Kyriazis et al. 1978; Fidler 1990). Work from a number of laboratories indicates that implanting human tumor cells orthotopically in the corresponding organ of nude mice results in much higher metastatic rates. For instance, dissociated human colon cancer cells, when grown in culture and subsequently injected into the cecum of nude mice, produce tumors that eventually metastasize to the liver, showing that orthotopic implantation can enhance the metastatic capability of human tumor cells in nude mice (Morikawa et al. 1988). Similar results have been achieved for orthotopic implantation of cell lines of human lung cancer, bladder cancer, melanoma, breast cancer, stomach, colon, and head and neck cancer (Hoffman 1999).

In the 1980s, investigators first reported metastatic models of human pancreatic cancer using orthotopic implantation of tumor-cell suspensions, which resulted in invasive tumor growth and subsequent metastases (Tan and Chu 1985; Marincola et al. 1989). Our laboratory first developed the concept of orthotopic implantation of tumor fragments which was found to greatly increase the metastatic potential of the implanted tumor (US Patents 5). Vezeridis et al. used pancreatic tumor tissue for orthotopic transplantation, resulting in extensive local growth, and metastasis to liver, lung, and lymph nodes (Vezeridis et al. 1989). In 1992, we used

histologically intact patient specimens of pancreatic cancer for orthotopic transplantation to nude mice to construct a metastatic model of human pancreatic cancer (Fu et al. 1992). This model resembled the clinical picture of pancreatic cancer including: (1) extensive local tumor growth, (2) extension of the locally growing human pancreatic cancer to the nude mouse stomach and duodenum, (3) metastases of the human pancreatic tumor to the nude-mouse liver and regional lymph nodes, and (4) distant metastases of the human pancreatic tumor to the nude-mouse adrenal gland, diaphragm, and mediastinal lymph nodes (Fu et al. 1992).

The following year, we described use of this patient-like model of human pancreatic cancer for the in-vitro and in-vivo evaluation of the antitumor activity of 5-fluorouracil (5-FU) and mitomycin-C (MMC) (Furukawa et al. 1993). The antitumor activity of these agents was initially determined in an in-vitro histoculture drug-response assay and inhibition rates were 5.6% for 5-FU and 39.4% for MMC. When the antitumor activity of 5-FU against PANC-4 was determined in vivo using the nude mouse orthotopic transplant treatment model, slight local tumor growth inhibition with equivalent incidence of metastases to the liver and the peritoneum as the control were observed in the mice treated with 5-FU. In contrast, mice treated with MMC had considerably reduced local tumor growth without liver and peritoneal metastases. Thus, the histoculture drug response assay in combination with the orthotopic transplant metastatic model provided for the first time a paradigm for evaluation of agents that may be effective against not only locally growing human pancreatic cancer but resulting metastases as well.

A number of approaches have been taken to label tumor cells to visualize and track them in vivo. Previous attempts to genetically label tumor cells for tracking purposes used the *Escherichia coli* β-galactosidase (*lacZ*) gene to detect micrometastases (Lin et al. 1990). However, detection of *lacZ* requires extensive histological preparation, with sacrifice of the tissue and/or animal; therefore, it was not possible to image, visualize, and study tumor cells in real- time in viable fresh tissue or in the live animal.

The ability to confer real-time visualization and imaging of tumor growth and progression in viable fresh tissue and in the live animal would be an important factor in the development of a real-time reporter gene for metastasis and recurrence. Several approaches have been developed with this goal in mind: Fukumura et al. and Chambers et al. labeled tumor tissue with fluorescent dyes. However, these methods are not suitable for long-term metastasis studies (Chambers et al. 1995; Fukumura et al. 1997). Weissleder et al. have infused tumor-bearing animals with protease-activated near-infrared fluorescent probes (Weissleder et al. 1999). Tumors with appropriate proteases could activate the probes and could be imaged externally. The limits to such a system include much higher liver to tumor background precluding liver metastasis imaging, which is among the most important metastatic sites; the stated time limit of 96 h, which precludes growth and efficacy studies; the requirement of appropriate tumor protease activity; and the requirement of selective tumor delivery of the probes.

Another attempt involved insertion of the luciferase gene into tumor cells such that they emit light (Sweeney et al. 1999). However, luciferase enzymes transferred

to mammalian cells require the exogenous injected delivery of their luciferin substrate, an invasive and impractical requirement in an intact animal. The resolution of this approach is low, due to low signal strength, requiring anesthesia since long periods are needed for photon counting. With this technology, there is insufficient photon flux to form a true image. Also, it is not known whether luciferase genes can function stably over significant time periods in tumors and in the metastases derived from them.

It became clear that higher signal strength, specificity, resolution, and physiologic conditions were necessary to report the natural course of tumor progression and metastasis on a real-time basis. The green fluorescent protein (GFP) gene, cloned from the bioluminescent jellyfish *Aequorea victoria,* was chosen to satisfy these conditions because it has demonstrated its great potential for use as a cellular marker (Astoul et al. 1994; Chalfie et al. 1994; Cheng et al. 1996). GFP cDNA encodes a 283-amino-acid monomeric polypeptide with a molecular mass of 27 kDa that requires no other *Aequorea* proteins, substrates, or cofactor to fluoresce (Prasher et al. 1992; Yang et al. 1996). GFP gene gain-of-function bright mutants have been generated by various techniques that have been humanized for high expression (Delagrave et al. 1995; Heim et al. 1995; Cormack et al. 1996; Zolotukhin et al. 1996).

A major advantage of GFP-expressing tumor cells is that imaging requires no preparative procedures, contrast agents, substrates, anesthesia, or light-tight boxes as do other imaging techniques (Hoffman 2005). GFP imaging is thus uniquely suited for whole-body imaging of tumor growth and metastases in live animals (Hoffman 2005). We have developed technology that has enabled the stable transduction of the GFP gene into a large series of human tumor cell lines (Chishima et al. 1997a–e; Yang et al. 1998, 1999a, b; 2000a, b, 2001). The tumor cell lines were able to stably express GFP at high levels both in vitro and in vivo. We have previously demonstrated the important parameter that GFP-expressing cancer cells could be directly visualized in fresh tissues of transplanted animals at a very high resolution: down to the single-cell level (Chishima et al. 1997a–e). With this technology, we were able to visualize tumor cells that had seeded with or without subsequent colonization in all the major organs, including liver, lung, brain, spinal cord, axial skeleton, and lymph nodes (Chishima et al. 1997a–e). We have developed orthotopic GFP metastatic models of lung cancer (Yang et al. 1998), prostate cancer (Yang et al. 1999b), melanoma (Yang et al. 1999a), colon cancer (Yang et al. 2000a), and other cancers as well (Hoffman 2005). These results demonstrated that GFP gene-transfected tumor cells represent a new tool to study tumor cell growth, dissemination, invasion, metastasis, and progression through all stages.

Recently, we described an in vivo model of GFP-expressing pancreatic cancer in the nude mouse (Bouvet et al. 2000b, 2002). To understand the metastatic pattern of pancreatic cancer, we developed stable high-expression GFP transductants of human pancreatic cancer cell lines. Fragments of subcutaneous-growing tumors were implanted by surgical orthotopic implantation (SOI) in the pancreas of nude

mice or by portal vein injection of a cell suspension. Subsequent micrometastases were visualized by GFP fluorescence in the peritoneum, periportal lymph nodes, liver, and lung as well as other sites in the abdominal cavity. The use of GFP-expressing MiaPaCa-2 and BxPC-3 cells transplanted by SOI or by portal vein injection revealed the extensive metastatic potential of pancreatic cancer. Furthermore, the primary tumor and subsequent metastasis were visualized by whole-body imaging through the skin of the nude mouse without the need for laparotomy (Bouvet et al. 2002). Such visualization can be a practical and convenient way to follow metastasis in a "real-time" fashion. We have also described a novel, highly metastatic model of pancreatic cancer that utilizes pancreatic cancer cells engineered to express very high levels of *Discosoma* sp. red fluorescent protein (RFP) (Katz et al. 2003a, c, 2004b; Bouvet et al. 2005; Tsuji et al. 2006). These RFP-expressing pancreatic tumors can be implanted into the pancreas of transgenic GFP nude mice to create dual-color models to study tumor host interactions (Amoh et al. 2006a, b). These new metastatic models can play a critical role in the study of the mechanism of metastasis in pancreatic cancer and in screening of therapeutics that prevent or reverse this process. These models are described in detail below.

3.2 Green Fluorescent Protein (GFP) Models of Pancreatic Cancer

3.2.1 GFP Models of Pancreatic Cancer

In the orthotopic models of pancreatic cancer described above, micrometastases in the peritoneum, periportal lymph nodes, liver, and lung as well as other sites in the abdominal cavity were visualized by GFP fluorescence (Fig. 3.1). Transplantation by SOI or by portal vein injection of GFP-expressing MiaPaCa-2 and BxPC-3 cells demonstrated the extensive metastatic potential of pancreatic cancer at the cellular level in vivo. In addition, these GFP models allowed the primary tumor and subsequent metastasis to be visualized by whole-body imaging through the skin of the nude mouse (Bouvet et al. 2002).

3.2.2 Tumor Selective Metastatic Organ Targeting (Bouvet et al. 2000b)

Figure 3.2 represents the incidence of metastasis in each model at week 20 post-SOI. The BxPC-3-GFP cell line produced locally-advanced, invasive tumors that metastasized in a regionally selective way to the spleen and the retroperitoneum,

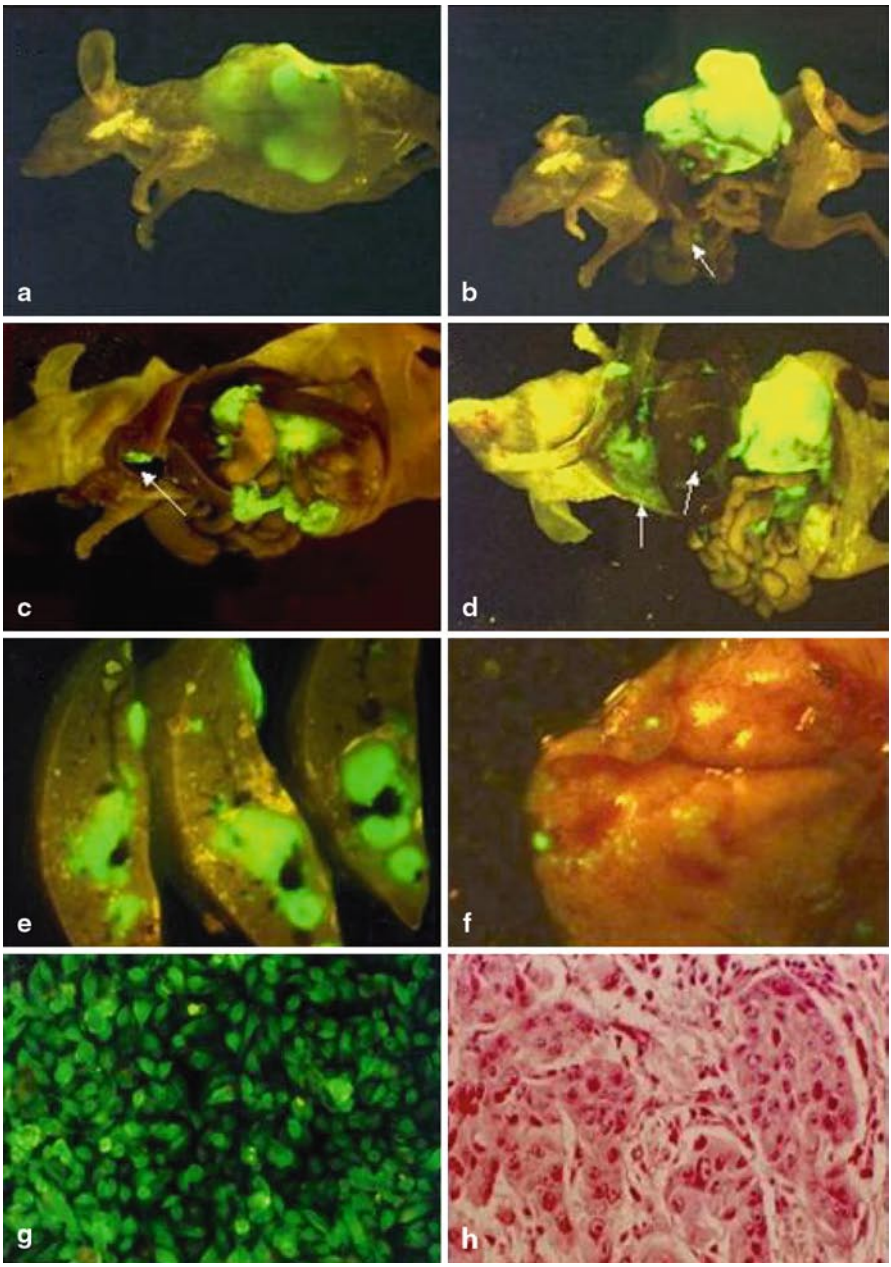

Fig. 3.1 **a** The BxPC-3-GFP pancreatic tumor, transplanted by surgical orthotopic implantation (SOI), is externally visualized with fluorescence through the skin of the nude mouse. **b** Laparotomy of the same mouse in (**a**) showing locally advanced BxPC-3-GFP tumor with portal lymph node metastases. **c** The primary tumor formed in the pancreas at 12 weeks after SOI is visualized under bright-field microscopy. Numerous metastases and micrometastases can be visualized by GFP under fluorescence microscopy to the stomach, spleen, periportal nodes (*arrow*), liver, and

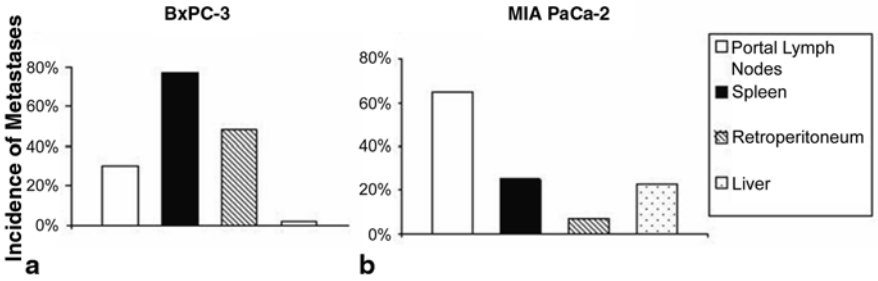

Fig. 3.2 Site-specific metastases in orthotopic models of human pancreatic cancer. **a** BxPC-3-GFP **b** MIA-PaCa-2-GFP. Forty-four and 26 mice were used for the BxPC-3-GFP and MIA-PaCa-2 models, respectively. The *y*-axis represents cumulative percentage of mice with metastasis (Bouvet et al. 2000b)

with distant liver metastases very rare (Fig. 3.2a). In contrast, metastases in the MIA-PaCa-2 model were selective to distant sites in the portal lymph nodes and liver with regional retroperitoneal lymph node metastasis very rare (Fig. 3.2b).

3.2.3 Real-Time Simultaneous Whole-Body Imaging of BxPC-3-GFP Tumor and Multiple Metastatic Growth (Bouvet et al. 2002)

Consecutive whole-body simultaneous images of the primary BxPC-3-GFP pancreatic tumor, spleen, bowel, and omentum metastases are shown in Fig. 3.3a. These images were simultaneously obtained in a single animal on day 46, day 50, day 57, and day 64 after SOI. In each of the sites, tumor growth and progression were quantified with image analysis. Growth curves (Fig. 3.3b) for the primary tumor and metastases at each of the above sites were constructed from the whole-body images. Thus, simultaneous metastases development can be quantitated with whole-body imaging.

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mediastinum (*arrow*). **d** MIA PaCa-2-GFP tumor at week 10 post-SOI. *Left arrow* shows diaphragm metastases and *right arrow* shows liver metastases. **e** Multiple high-expressing GFP liver metastases are noted under fluorescence microscopy. **f** GFP-expressing lung metastases are noted. **g** The human pancreatic cancer cell line MIA PaCa-2 was transduced with the RetroXpress vector pLEIN that expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message. The stable high expression clone was selected in 800 μg/ml of G418. **h** H&E section of MIA PaCa-2-GFP tumor (Bouvet et al. 2000b)

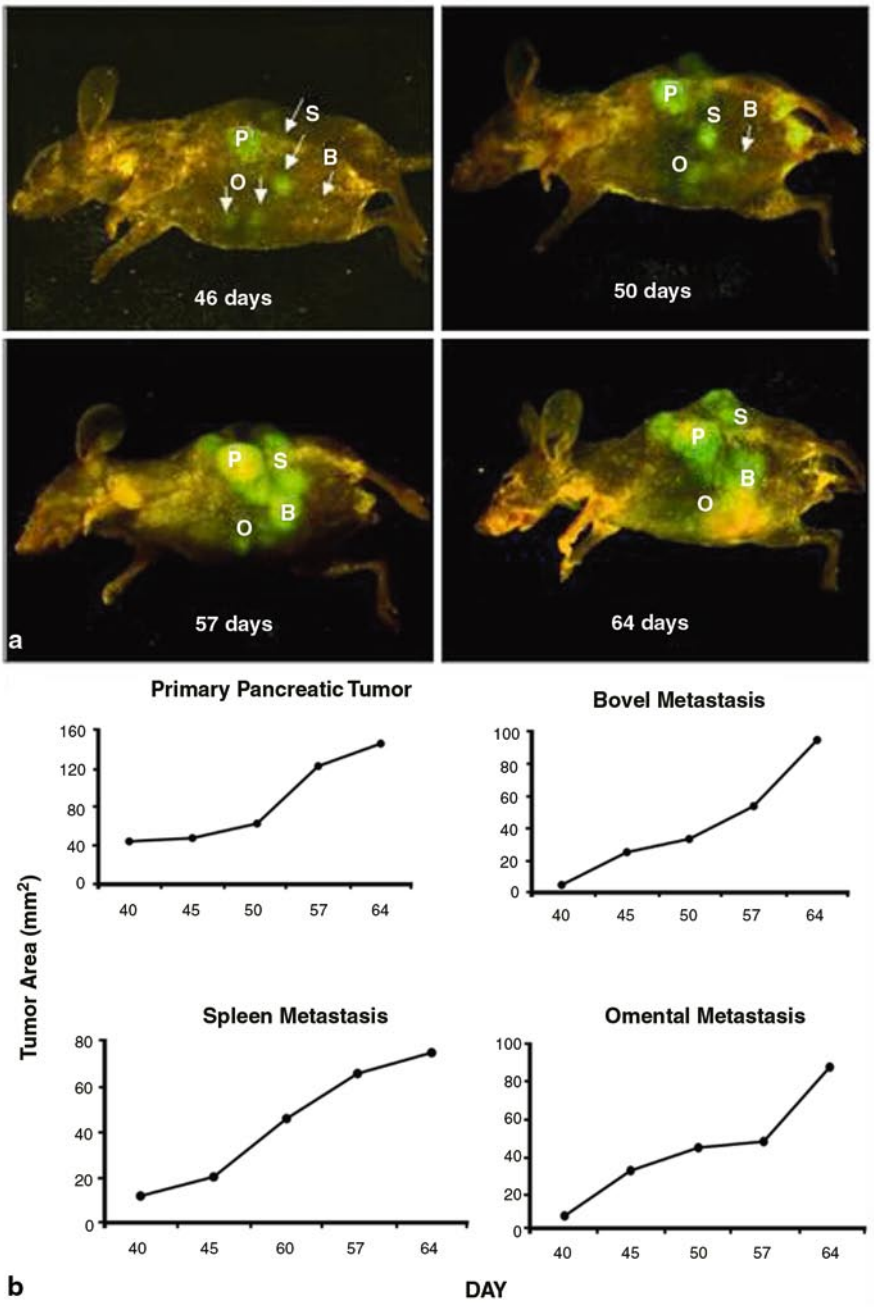

Fig. 3.3 **a** Consecutive external whole-body images of internally-growing BxPC-3-GFP tumors. A series of external fluorescence images of the BxPC-3-GFP pancreatic tumor in a single animal was obtained from day 46 to day 64 after SOI of BxPC-3-GFP in a nude mouse. **b** growth curves for primary pancreatic tumor (P), splenic metastasis (S), omental metastases (O), and bowel metastasis (B) as determined by whole-body imaging (Bouvet et al. 2002)

3.2.4 *Sequential Intravital Images of Omental and Liver Micrometastasis of BxPC-3-GFP (Bouvet et al. 2002)*

A series of internal intravital fluorescence images of an omental micrometastasis from a BxPC-3-GFP pancreatic tumor in a single animal was obtained from day 36 to day 70 after SOI of BxPC-3-GFP in a nude mouse. The images were acquired during a laparotomy procedure. As determined by intravital imaging, the size of the metastatic lesion grew progressively with time (Fig. 3.4). Figure 3.5 shows a series of intravital fluorescence images of liver micrometastases following SOI of BxPC-3-GFP in nude mice.

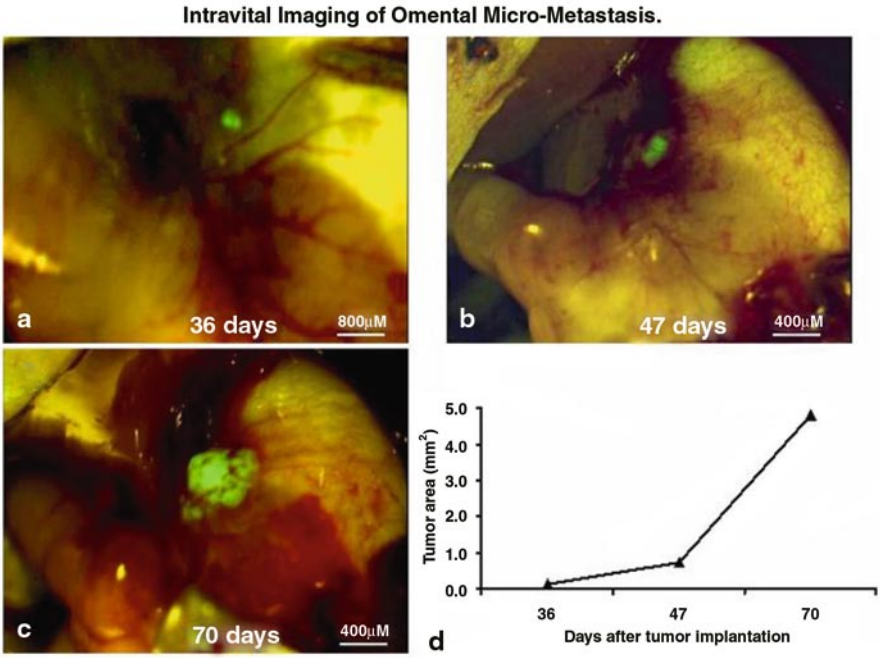

Fig. 3.4 Sequential intravital images of omental micrometastasis of BxPC-3-GFP. A series of internal fluorescence images of an omental micrometastasis from a BxPC-3-GFP pancreatic tumor in a single animal was obtained from day 36 to day 70 after SOI of BxPC-3-GFP in a nude mouse during a laparotomy procedure (Panels **a**–**c**). As determined by internal imaging, the size of the metastatic lesion grew progressively with time (Panel **d**) (Bouvet et al. 2002)

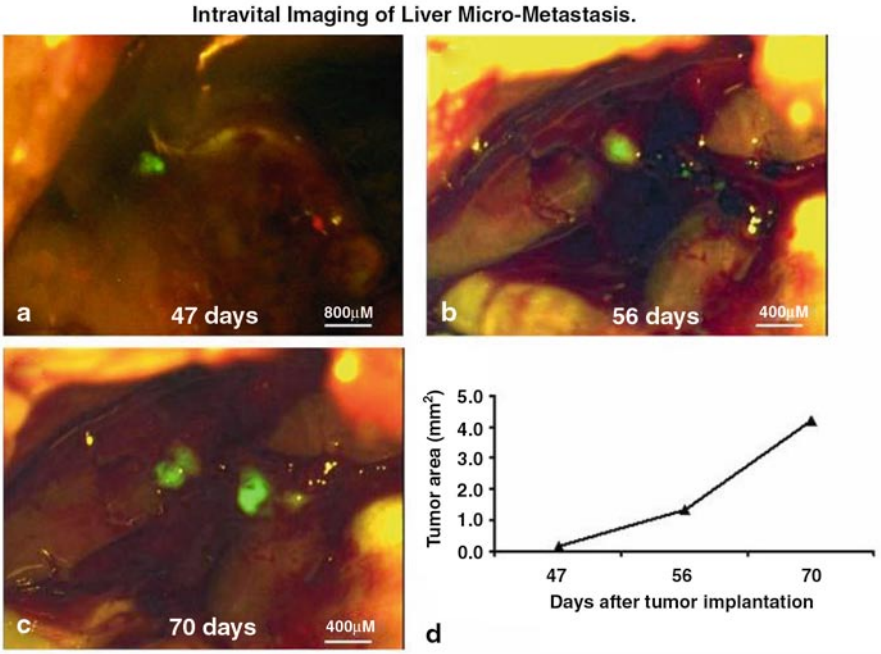

Fig. 3.5 Sequential intravital images of liver micrometastasis of BxPC-3-GFP. Internal images of liver metastases following SOI of BxPC-3-GFP tumor in nude mice were obtained during a laparotomy procedure (Panels **a**–**c**). As determined by internal imaging, the area of the metastatic lesions increased over time (Bouvet et al. 2002)

3.3 Red Fluorescent Protein (RFP) Models of Pancreatic Cancer

3.3.1 Red Fluorescent Protein Models of Pancreatic Cancer (Katz et al. 2003a, c, 2004b; Bouvet et al. 2005; Tsuji et al. 2006)

We described a novel, highly metastatic model of pancreatic cancer that utilizes pancreatic cancer cells engineered to express very high levels of *Discosoma* sp. red fluorescent protein (RFP). This model clinically resembles human pancreatic cancer in its pattern of growth and metastasis. It rapidly and reliably produces distant metastatic disease, and frequently gives rise to malignant abdominal ascites and peritoneal carcinomatosis. Moreover, the enhanced fluorescence of this model enables real-time visualization and imaging of pancreatic tumor growth and metastasis in the live animal, and permits identification of both macro- and micrometastases

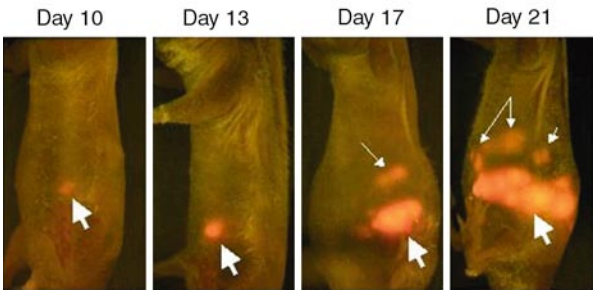

Fig. 3.6 RFP tumor fluorescence enabled real-time, whole-body imaging of tumor growth and metastasis of MIA-PaCa-2-RFP tumors after surgical orthotopic implantation. Panels represent sequential fluorescent imaging of a single mouse taken on days 10, 3, 17 and 21 after SOI. Progressive primary tumor growth and the development of metastases are clearly visualized through the skin in the live animal. *Heavy arrow* = primary tumor, *thin arrows* = metastatic tumor (Katz et al. 2004c)

(Fig. 3.6). These features make the model an ideal system with which to study the effects of novel antineoplastic agents on tumor growth and metastasis.

3.3.2 Sensitivity of Fluorescence Imaging

We have previously published data that describes in detail the reproducibility and sensitivity of our non-invasive fluorescence imaging systems, demonstrating that single cells can be imaged. Using fairly simple equipment including a CCD camera and light box, we have reproducibly and non-invasively quantitated tumor growth and response to therapy (Chishima et al. 1997d; Bouvet et al. 2000b, 2002; Lee et al. 2000; Yang et al. 2000a, 2002, 2003; Saito et al. 2002; Katz et al. 2003a, c, 2004b; Hoffman 2005; Hoffman and Yang 2006). With regard to the sensitivity of fluorescence imaging, we have shown that when human pancreatic cancer was surgically orthotopically implanted into nude mice, whole-body optical images showed, in real time, growth of the primary tumor and its metastatic lesions in the liver and other organs (Yang et al. 1999b; Bouvet et al. 2000b, 2002). The depth to which metastasis (Yang et al. 2000b) and micrometastasis could be imaged depends on their size. It should be noted that this sensitivity was achieved with a simple light box equipped with a 50-watt mercury lamp. Laser light sources now available to us enable greater sensitivity and resolution even deeper in the animal (Yang et al. 2007). Our recent studies (Bouvet et al. 2005) compared RFP-expressing pancreatic tumors to MRI (see Fig. 3.7). The fluorescent tumor size can be quantified using image quantification software as we have previously described (Katz et al. 2004b) and appears to be at least as sensitive as MRI.

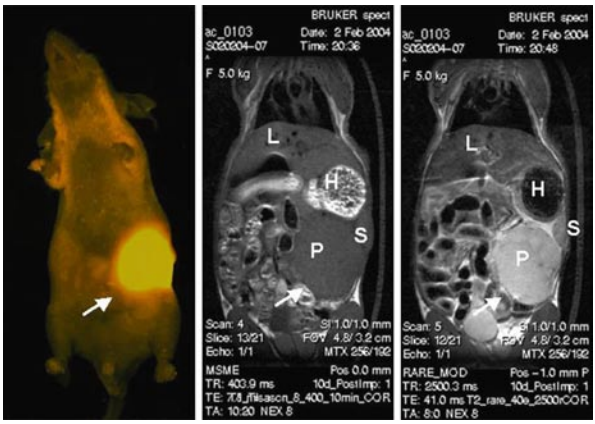

Fig. 3.7 Visualization of early and locally advanced pancreatic malignancy in an orthotopic nude mouse model by fluorescence optical imaging (FOI) and T1- and T2- weighted MRI. Panels represent a single mouse that underwent serial imaging on day 10 after surgical orthotopic implantation of fluorescent MIA-PaCa-2-RFP human pancreatic cancer fragments. Primary tumor was clearly visible using each imaging strategy. P = pancreas, L = liver, H = stomach, S = spleen (Bouvet et al. 2005)

In our recently described RFP model of pancreatic cancer, we have performed correlation studies to validate external imaging (Katz et al. 2003c). To confirm a correlation between tumor burden, as determined by externally visualized RFP fluorescence, and standard measurements of tumor volume, the primary tumor of each mouse was used. Primary tumor volume was first calculated using the formula (long diameter × short diameter^2)/2, where long diameter and short diameter measurements were precisely obtained in the open animal. The externally-visualized RFP fluorescent area was then determined as previously described by placing the mouse in a fluorescent light box equipped with a fiberoptic light source of wavelength 470 nm. At each imaging time point, real-time determination of tumor burden was performed by quantifying fluorescent surface area, as described previously (Bouvet et al. 2002; Katz et al. 2003c). The correlation coefficient ($r = 0.89$) between tumor volume and RFP fluorescence was highly significant ($p < 0.05$) (see Fig. 3.8).

We can increase the sensitivity of imaging down to the single cell level by the use of a simple skin flap in the mouse (Yang et al. 2002). Strong fluorescence labeling with green and red fluorescent protein along with inexpensive video detectors, positioned externally to the mouse, allows the monitoring through the skin flap of details of tumor growth, angiogenesis, and metastatic spread. Opening a reversible skin-flap in the light path markedly reduces signal attenuation, increasing detection sensitivity many-fold. The observable depth of tissue is thereby greatly increased and many cancer cells that were previously hidden are now clearly observable. We have shown that single tumor cells, expressing GFP, were seeded on the brain and imaged through a scalp skin-flap. Lung tumor micro-foci representing a few cells were viewed through a skin-flap over the chest wall, while contralateral micrometastases

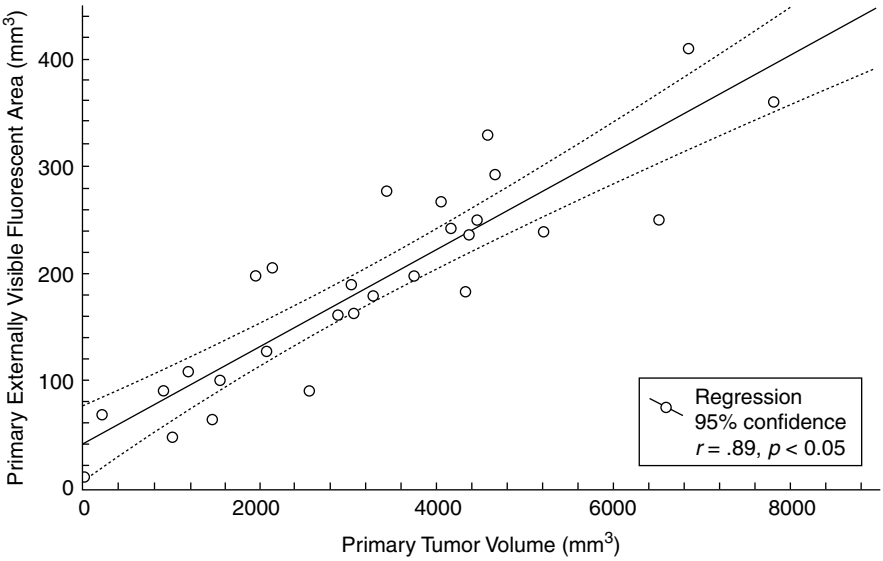

Fig. 3.8 Red fluorescent area quantified using external fluorescence imaging correlated strongly with tumor volume measured directly. At autopsy, measurement of externally visualized fluorescent area and direct measurements of the primary tumor of each mouse were obtained. A significant correlation ($r = 0.89$, $p < 0.05$) was observed between these values (Katz et al. 2003c)

were imaged through the corresponding skin-flap. Pancreatic tumors and their angiogenic microvessels were imaged by means of a peritoneal wall skin-flap (Yang et al. 2002).

3.3.3 Use of RFP Models for Drug Discovery and Evaluation

A novel, highly fluorescent, red fluorescent protein (RFP)-expressing pancreatic cancer model was orthotopically established in nude mice (Katz et al. 2003c). The MIA-PaCa-2 human pancreatic cancer cell line was transduced with RFP and grown subcutaneously. Fluorescent tumor fragments were then surgically transplanted onto the nude mouse pancreas. Groups treated with intraperitoneal gemcitabine or intravenous irinotecan were sequentially imaged to compare, in real time, the antimetastatic and antitumor effects of these agents compared with untreated controls (Fig. 3.9). Gemcitabine was shown to be far superior to irinotecan.

We subsequently demonstrated the ability of a novel, orally-administered cytosine analog, CS-682, to effectively prolong survival and inhibit metastatic growth in an imageable orthotopic mouse model of pancreatic cancer (Katz et al. 2003a). We have also used these RFP-expressing models to study adjuvant treatment with the cytosine analog CS-682 (Katz et al. 2004b). Seven days after implantation, mice were randomized into eight groups, depending on whether they were to be treated by tumor resection, 5 weeks of CS-682 chemotherapy at 40–60 mg/kg qd, or both.

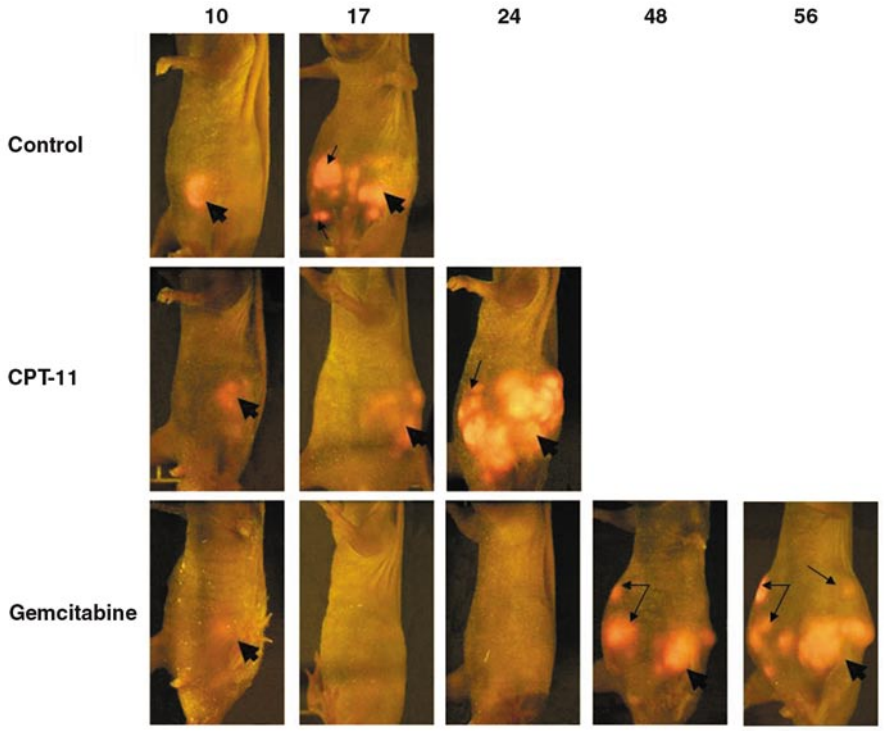

Fig. 3.9 Real-time, in vivo imaging of MIA-PaCa-2-RFP pancreatic cancer progression and evaluation of therapeutic efficacy over time. Representative mice from each treatment group on days 10, 17, 24, 48, and 56 after tumor implantation are shown. *Thick arrows* show primary tumor, and *thin arrows* indicate metastatic tumor. CPT-11 suppressed primary and metastatic tumor growth compared with controls. In contrast, gemcitabine successfully induced temporary regression of disease over the first month, after which growth and distant metastasis of tumor accelerated despite continued treatment (Katz et al. 2003c)

Throughout the course of treatment, non-invasive optical whole-body imaging, based on brilliant RFP expression of the tumor, permitted visualization and quantification of primary, metastatic, and recurrent disease (Fig. 3.10). Total tumor burden negatively correlated with survival. Untreated mice died of disseminated disease with a median survival of 26 days. Surgical resection alone conferred a small but significant survival advantage (median survival 28 days, $p = 0.03$). Primary CS-682 treatment at all doses also significantly prolonged survival compared to untreated animals ($p < 0.05$), and was more effective than surgery alone at doses of 50 mg/kg and 60 mg/kg (median survival 34 days, $p = 0.045$ and 38.5 days $p = 0.03$, respectively). Maximal survival (median 48 days, with 30% of animals surviving longer than 60 days) was achieved by adjuvant CS-682, 50 mg/kg, given after surgical resection of the primary pancreatic tumor ($p = 0.004$ compared to surgery alone). The results demonstrate that adjuvant oral administration of CS-682 for pancreatic cancer is highly effective with acceptable toxicity, suggesting its potential for cure of this disease in appropriate combinations.

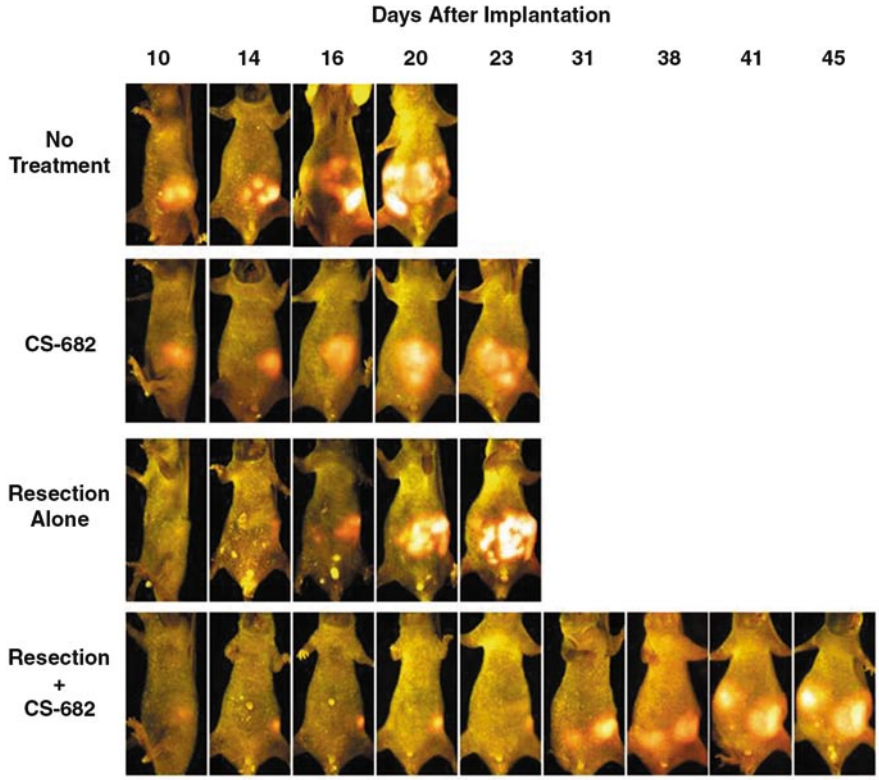

Fig. 3.10 Sequential, whole-body images of one representative mouse from each of four treatment groups. Real time evaluation of therapeutic interventions on pancreatic tumor growth and metastasis was facilitated in this non-invasive system using fluorescence visualization. Resection was performed on day 7 in both the resection and adjuvant groups; CS-682 was initiated in the primary and adjuvant groups at a dose of 50 mg/kg on day 9. The inhibitory effects of each treatment group on the growth and dissemination of orthotopically-implanted human pancreatic tumors were visible in the live mouse under UV light (Katz et al. 2004b)

3.4 Dual-Color Models of Pancreatic Cancer

3.4.1 Dual-Color Imaging of Nascent Blood Vessels Vascularizing Pancreatic Cancer in an Orthotopic Model Demonstrates Anti-Angiogenesis Efficacy of Gemcitabine (Amoh et al. 2006a)

The stem cell marker nestin recently has been shown to be expressed in nascent blood vessels in nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice. We visualized by dual-color fluorescence imaging tumor angiogenesis in the ND-GFP transgenic nude mice after orthotopic transplantation of the MIA PaCa-2

human pancreatic cancer line expressing RFP. Mice were treated with gemcitabine at 150 mg/kg/dose on days 3, 6, 10, and 13 after tumor implantation. At day 14, mice were sacrificed and mean nascent blood vessel density and tumor volume were calculated and compared to control mice. Nestin was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor (Figs. 3.11 and 3.12). Results of immunohistochemical staining showed that the endothelial marker CD31 co-localized in ND-GFP-expressing nascent blood vessels. The density of nascent blood vessels in the tumor was readily quantified by GFP imaging. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor as well as decreased tumor volume. The dual-color model of the ND-GFP nude mouse orthotopically implanted with RFP-expressing pancreatic tumor cells enabled the simultaneous visualization and quantification of tumor angiogenesis and tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer. The results have important implications for the clinical application of gemcitabine in this disease.

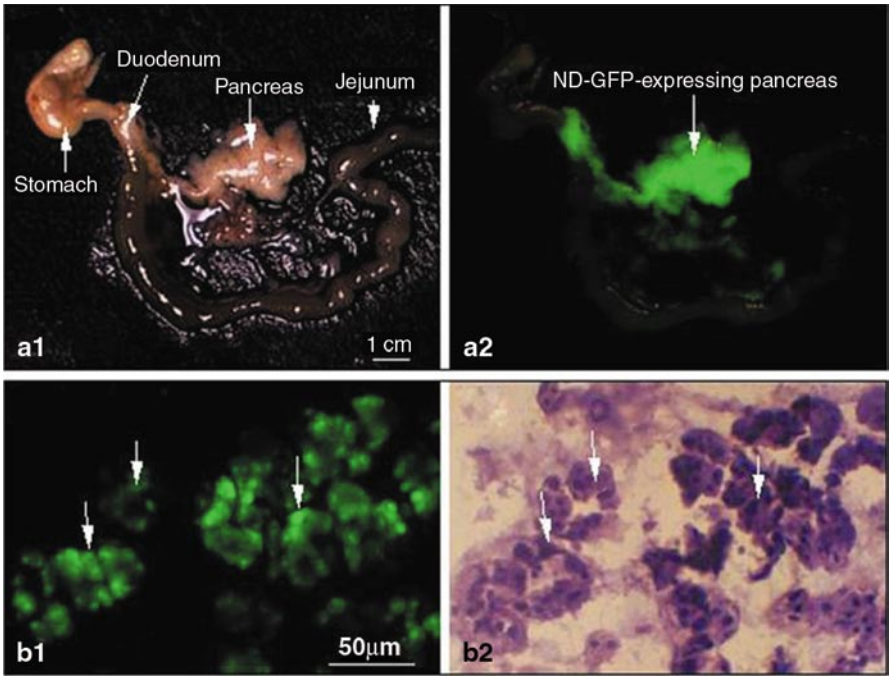

Fig. 3.11 ND-GFP expression in the pancreas. The pancreas in ND-GFP transgenic nude mice, **a1** bright field; **a2** fluorescence. Acinar cells of the pancreas in the ND-GFP transgenic nude mice (*arrows*), **b1** fluorescence; **b2** bright field (Amoh et al. 2006a)

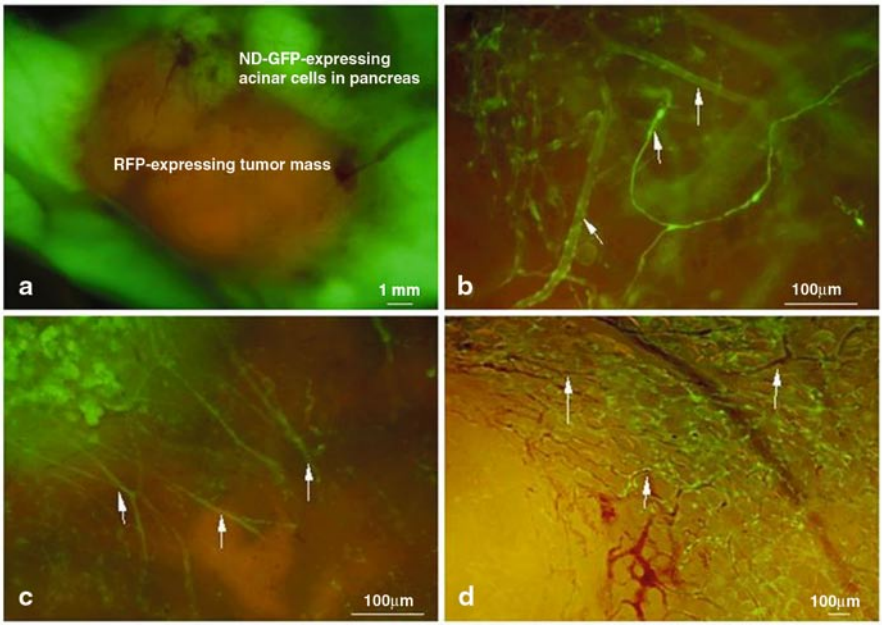

Fig. 3.12 Dual-color imaging of nascent blood vessels in the orthotopically growing MIAPaCa-2 pancreatic tumor. **a**–**d** Day 14 after orthotopic implantation of RFP-expressing MIAPaCa-2 human pancreatic cancer cells to ND-GFP transgenic nude mice. **a** The RFP-expressing MIAPaCa-2 human pancreas tumor growing in the ND-GFP expressing pancreas in an ND-GFP transgenic nude mouse. **b**–**d** The ND-GFP-expressing nascent blood vessels (*white arrows*) growing in the RFP-expressing tumor mass. **d** Newly formed ND-GFP-expressing blood vessels with blood flow (Amoh et al. 2006a)

3.4.2 Dual-Color Imaging of Nascent Blood Vessels Vascularizing Liver Metastasis of Pancreatic Cancer also Demonstrates Anti-Angiogenesis Efficacy of Gemcitabine (Amoh et al. 2006b)

We have also developed a model of liver metastases of pancreatic cancer in the nude mouse as outlined in Fig. 3.13. Nascent angiogenesis of pancreatic cancer liver metastasis in the ND-GFP transgenic nude mice, which formed after splenic injection of low-passage XPA-1 human pancreatic cancer cells expressing RFP, was visualized by dual-color fluorescence imaging. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. Immunohistochemical staining showed that CD31 co-localized with GFP in ND-GFP-expressing nascent blood vessels. The density of nascent blood vessels in the tumor was readily quantitated. Gemcitabine significantly decreased the mean nascent blood vessel density in the pancreatic liver metastases (Fig. 3.14). The dual-color model of the ND-GFP nude mouse, with RFP-expressing pancreatic cancer

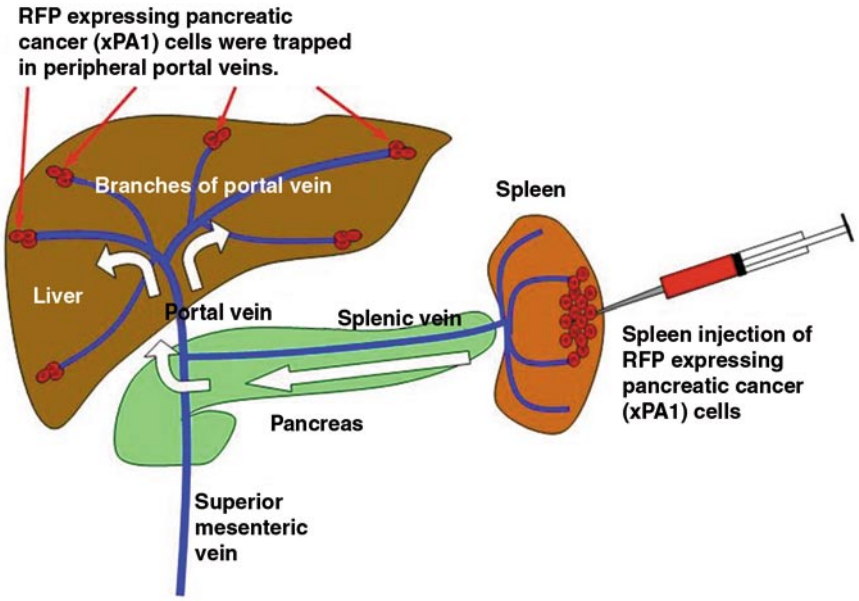

Fig. 3.13 Experimental model of liver metastases of pancreatic cancer. ND-GFP transgenic nude mice, 6–8 weeks old, were used. The mice were anesthetized with tribromoethanol. XPA1-RFP cells, grown in RPMI medium with 10% fetal bovine serum, were detached from the culture flask by a brief incubation with phosphate-buffered saline without calcium and magnesium containing 2 mM EDTA. The cancer cells were suspended in RPMI 1640/10% FBS, washed and resuspended in PBS. Fifty microliters containing 2×10^6 XPA1-RFP cells per mouse were injected in the spleen with a 27G syringe (Amoh et al. 2006b)

liver metastases, enabled the simultaneous visualization and quantitation of nascent angiogenesis and its response to angiogenesis inhibitors in the liver metastasis as well as the primary tumor.

3.5 Concluding Remarks

We have described in this report our cumulative experience with the development and preclinical application of several fluorescent, clinically-relevant, orthotopic metastatic mouse models of pancreatic cancer. These models utilize human pancreatic cancer cell lines which have been genetically engineered to selectively express high levels of GFP or RFP. Locoregional tumor growth and distant metastasis of these orthotopic implants occurs spontaneously and rapidly throughout the abdomen in a manner consistent with clinical human disease. Highly specific, high-resolution, real-time visualization of tumor growth and metastasis was achieved in vivo without the need for contrast agents, invasive techniques, or expensive imaging equipment. Transplantation of RFP-expressing tumor fragments onto the pancreas of GFP-expressing transgenic mice may be used to facilitate visualization of

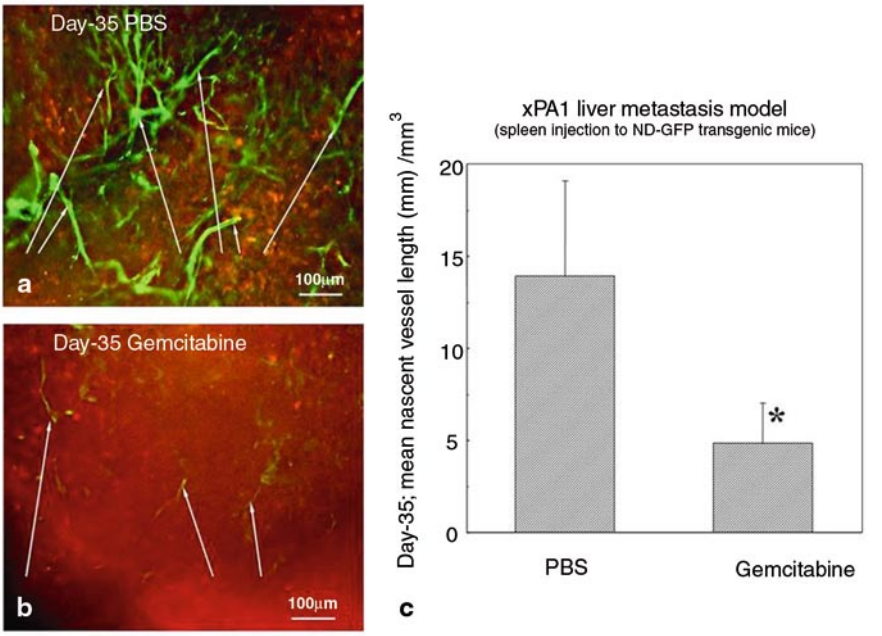

Fig. 3.14 Anti-angiogenic effect of gemcitabine on XPA-1-RFP pancreatic liver metastases. **a** Control mice were given ip injections of PBS. In the control mice, the ND-GFP-expressing nascent blood vessels formed a network in the growing tumor mass. **b** Mice were given ip injections of gemcitabine. In the treated mice, the ND-GFP-expressing nascent blood vessels were diminished. **c** By day-35 after splenic injection, the mean nascent blood vessel density in the gemcitabine-treated mice was significantly less than the PBS-injected mice ($p < 0.05$) (Amoh et al. 2006b)

tumor–host interaction between the pancreatic tumor fragments and host-derived stroma and vasculature. Such in vivo models have enabled us to serially visualize and acquire images of the progression of pancreatic cancer in the live animal, and to demonstrate the real-time antitumor, antimetastatic, and anti-angiogenesis efficacy of several novel therapeutic strategies in pancreatic malignancy. These fluorescent models are therefore powerful and reliable tools with which to investigate human pancreatic cancer and therapeutic strategies directed against it.

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Chapter 4
A New Preclinical Paradigm for Pancreas Cancer

Sunil R. Hingorani

Abstract Pancreas cancer confounds patient and physician alike. With an almost identical annual incidence and mortality, the disease has heretofore thwarted attempts to cure and even contain it. The approach to the patient with pancreas cancer is the same as for any cancer: detect it early, diagnose it accurately and eradicate it through a combination of surgery and chemical and radiotherapies. However, cancer of this organ eludes early detection, runs the risk of significant collateral injury when attempting to biopsy it for diagnosis, and resists all current forms of conventional chemotherapy and radiation. Moreover, the disease is as difficult to study in patients as it is to treat. Although classical experimental model systems have yielded significant information on genetic mutations of interest, they have not proved as useful in screening for drugs likely to be effective in patients. Mammalian model systems that faithfully mimic the full spectrum of the human disease from inception to invasion are needed. This chapter describes an integrated translational approach to developing and testing early detection, molecular diagnostic, chemopreventive and therapeutic strategies using state-of-the-art genetically engineered mouse models.

4.1 Introduction

Remarkable advances in biomedical research over the past few decades have uncovered the fundamental principles of carcinogenesis. These discoveries herald the promise of a modern era of personalized medicine. Translating these findings, however, to meaningfully impact the management of patients, particularly those with solid tumors, has proved unexpectedly daunting. Our increasingly sophisticated understanding of cancer pathogenesis has also highlighted the great heterogeneity

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and complexity of disease subtypes and presentations that exist even within a given histologically-defined cancer. The challenge now is to move from organ-specific to patient-specific management of disease. Major hurdles to truly individualized patient care include not only this molecular and phenotypic heterogeneity, but also a lack of early detection markers, a lack of molecular diagnostic markers, and the inability to identify specifically activated pathways in a given patient to direct treatment choices and to perform real-time assessments of target inhibition and therapeutic efficacy.

4.1.1 Delayed Diagnosis in a Rapidly Lethal Disease

Perhaps nowhere are these challenges more formidable than in pancreatic ductal adenocarcinoma (PDA). With the highest 1- and 5-year case mortality rate of any cancer, PDA presents a formidable challenge to patients, physicians, and scientists alike (Von Hoff et al. 2005). Vague and non-localizing symptoms preclude early diagnosis with the result that only 10–15% of patients have operable disease at the time of presentation. Median survival among the remaining 85–90% of patients is 4–6 months with only about 20% alive at 1 year (Von Hoff et al. 2005). As a result, pancreas cancer patients typically get *one* chance, if that, at an experimental therapy. They are sick and succumb quickly; second line therapy is rarely even an option or, sadly, even necessary. In fact, patients with PDA are frequently sufficiently ill at initial diagnosis as to preclude altogether their participation in studies of investigational agents, which generally have very strict performance status and physiologic criteria for enrollment.

4.1.2 Diagnostics and Measures of Treatment Efficacy

The same factors that conspire to obscure disease detection also hinder the study of its pathogenesis. The pancreas is difficult to access and hazardous to biopsy. The pancreas does not readily forgive manipulation, which can result in iatrogenic pancreatitis. Biopsy of the organ for diagnostic purposes in the setting of already suspected disease is challenging enough; biopsy for routine screening is logistically impractical, economically unfeasible, and medically unsound. This also raises an obvious conundrum for the development of better modalities for early detection: in the absence of knowing who has early stage disease, it is impossible to establish test and control populations. The only currently feasible approach is to prospectively bank serial blood samples from large cohorts of subjects and follow them over time for the potential *subsequent* development of PDA. Such cohorts do exist as rare exceptions to the rule (e.g., the Framingham Nurses' Study and the Women's Health Initiative), and are developed and maintained at enormous expense.

Given the presentation and natural history of this disease, it is not surprising that there is a relative dearth of tissue even from primary tumors and certainly for

metastatic disease. It is difficult if not impossible, therefore, to scrutinize simple measures of drug efficacy: Did sufficient drug reach the intended target? Was the target inhibited? Were there corresponding effects on proliferation, apoptosis and/or differentiation? If not, were alternate and compensatory pathways activated? In other words, what are the mechanisms of in vivo resistance? It stands to reason that before methods and agents can be developed to overcome resistance in pancreas cancer, we must first understand the mechanistic bases for resistance in the autochthonous setting.

4.1.3 Lost in Translation

The successful translation of promising therapies has historically been poorest in oncology (Kola and Landis 2004). More than 90% of cancer patients who enroll in early stage clinical trials experience no substantial objective benefit, while nevertheless sustaining exposure to potential drug-related toxicities (Roberts et al. 2004). One reason for the discordance between preclinical promise and clinical reality is the inability of traditional experimental systems to accurately represent the complex in vivo tumor microenvironment. For the purposes of identifying therapeutic vulnerabilities in a human cancer, tumor cells arising in situ in a native organ are categorically distinct from the same cells growing on plastic or under the skin of an immunocompromised mouse (Bissell and Radisky 2001). As a result, the ability to kill cancer cells in these contexts has had little bearing on treating the disease in patients.

Performing an adequately controlled and powered clinical trial entails considerable expense and daunting logistics. Recruiting enough patients at any one center to run a statistically significant Phase III trial is impractical for pancreas cancer; multicenter trials are therefore necessary. Such trials can cost hundreds of millions of dollars and take several years to complete and analyze (Dickson and Gagnon 2004). For all of these reasons, clinical trials are obviously not the best means to generate the *first* meaningful test of a new therapeutic agent and they should be restricted to those drugs with the highest likelihood of success. The question, of course, is how to establish that priority.

New paradigms for disease investigation, development of early detection and diagnostic strategies, identification of therapeutic vulnerabilities and the means to test them, and preclinical model systems that more accurately predict efficacy in patients are desperately needed. Our approach has been to undertake a systematic program to investigate the potential causal events associated with PDA by genetically engineering signature mutations, alone and in various combinations, into the laboratory mouse. By these means, it has been possible to develop highly faithful animal models of preinvasive (Hingorani et al. 2003), invasive, and metastatic PDA (Hingorani et al. 2003, 2005; Izeradjene et al. 2007). Even as these models continue to uncover important basic principles of pancreas cancer biology, they also serve as platforms for preclinical studies in what has recently revealed to be several distinct genetic and histologic pathways to PDA (reviewed in Rustgi 2006; Hingorani 2007).

4.2 Cancer as a Complex Organ

The stepwise development of a nascent neoplasm in its native organ involves a progression of genetic, cellular, morphologic and architectural changes in the tissue reflecting an evolving dialog among all of the cellular components normally resident in the organ, as well as those recruited to, or intruding upon, the neoplastic process. The resultant cancer can perhaps best be thought of as a complex "organ," comprised not only of the transformed epithelial cells, but also normal surrounding parenchyma including acinar, ductal and islet cells; an increasingly robust and abundant stromal reaction including pancreatic stellate cells and matrix deposition; multiple arms of immunity; neovasculature; and a brew of cytokines, growth factors, and other signaling factors (Radisky et al. 2001) (Fig. 4.1). In addition, the physicochemical environment of pancreas cancer is distinct from the normal tissue, including O_2 tension, pH, glucose concentration, and intratumoral or interstitial pressures. Each of these factors may contribute to therapeutic resistance while also presenting potential points of vulnerability to be exploited. In either case, these properties are unique to a given carcinoma and, in particular, to a carcinoma situated in its native site.

The in vitro tissue culture and in vivo xenograft model systems that have been instrumental in helping to elucidate the basic principles of cancer biology cannot recapitulate the complexity of the human malignancies with enough fidelity to identify detection and treatment strategies to impact the clinic. The very properties that enable the powerful reductionist approach to reveal fundamental mechanistic principles prevent these systems from serving as surrogates for the patient. Indeed, these earlier model systems were designed precisely to reduce the complexity inherent in the patient in order to generate manageable and testable hypotheses. What is needed now is to capitalize on this knowledge and understanding to build systems that allow us to cross the divide back to the patient; in other words, to create systems that reconstitute the genetic and biological diversity of the human disease.

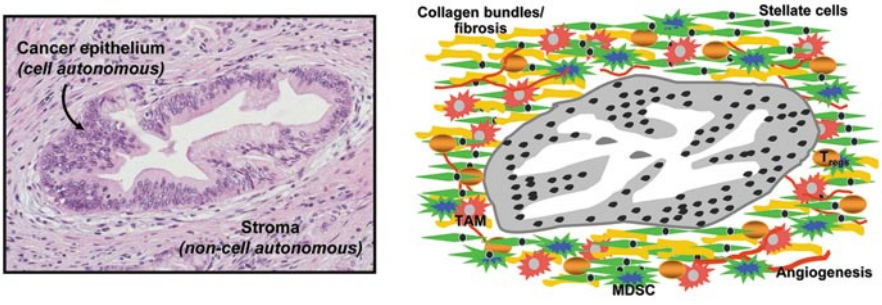

Fig. 4.1 Cancer as a complex organ. The multiple components that comprise an autochthonous cancer and the interdependencies among them result in a varied selection of cell and non-cell autonomous targets for therapeutic exploitation. Combining therapies targeted to distinct components within the cancer provides an alternative approach to the conventional concept of combination therapy and may increase efficacy and decrease the likelihood of emergent disease resistance. *Left*, ductal carcinoma-in-situ (PanIN-3) (photomicrograph courtesy of Dr. Ralph Hruban). *Right*, schematic representation of some of the constituents in the stromal reaction to pancreas cancer

4.3 Human Disease in a Mammalian Surrogate: Modeling Pancreas Cancer in Mice

The genome of the laboratory mouse, *Mus musculus*, is approximately 96% identical to our own (Waterston et al. 2002). At the histologic and physiologic levels, the murine pancreas is essentially indistinguishable from the human. Interestingly, however, mice do not get pancreas cancer spontaneously and they have been curiously resistant to efforts to induce pancreatic ductal adenocarcinomas either chemically or genetically. These challenges have recently been overcome. The history of genetically engineered mouse models of pancreas cancers has been extensively reviewed recently (see e.g., Grippo and Sandgren 2005; Tuveson and Hingorani 2005; Hingorani 2008) and only a brief overview will be provided here.

Static analyses of resected human pancreatic tumor specimens provided histologic and genetic frameworks for the initiation and progression of PDA (Hruban et al. 2001, 2004). These schema, in turn, generated hypotheses for mechanisms of pathogenesis and guided efforts to model the disease. Preinvasive disease begins most commonly in the terminal ductules, below the detection threshold of current imaging modalities. These preinvasive lesions, collectively termed pancreatic intraepithelial neoplasias (PanINs), manifest distinct nuclear and architectural changes as they progress from PanIN-1 to PanIN-2 to PanIN-3, or carcinoma-in-situ. A corresponding program of genetic progression involves prominent mutations in a circumscribed cast of players including the *KRAS2* proto-oncogene and the *CDKN2A/INK4A*, *TP53* and *SMAD4/DPC4* tumor suppressor genes. Not surprisingly, given their size and peripheral location, PanINs are asymptomatic (Fig. 4.2).

Though by far the most common path, the PanIN-to-PDA sequence is not the only route to invasive ductal adenocarcinoma of the pancreas. Distinct adenoma-to-carcinoma sequences exist in which invasive disease arises from cystic neoplasia (reviewed in Adsay 2005; Hruban 2006). Though much less common (<10% of all pancreatic neoplasms) and less likely to invade than PanINs, cystic neoplasms can be lethal once they do become invasive. As with PanINs, the two major classes of cystic neoplasms, mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs), have clearly definable histological, genetic, clinical and prognostic features (Fig. 4.2; reviewed in Maitra et al. 2005; Tanaka et al. 2006). However, some areas of overlap and difference are particularly noteworthy. IPMNs are centrally located and arise in the large main duct or immediate major branches. In addition, they have a very different genetic profile from PanINs and MCNs, being much less dependent on activating mutations in *KRAS* and frequently containing mutations not found at all in the other two precursor lesions, such as in *PI3KCA* and *LKB1/STK11*. However, the cystic neoplasms do share one important and distinguishing feature: they are often curable by resection. This finding is especially intriguing for invasive MCNs as these neoplasms share virtually identical spectra and incidences of key mutations as the PanIN-PDA route. PanINs and MCNs both arise in the periphery of the organ in the terminal branches, or ductules, of the ductal tree. Moreover, the invasive disease that arises out of MCNs strongly resembles the histopathology of conventional ductal adenocarcinoma and is distinguished

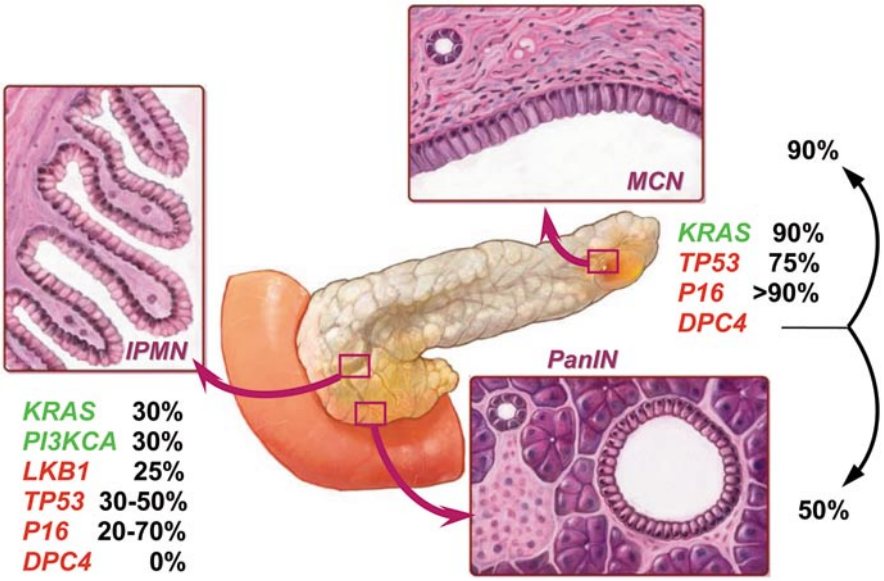

Fig. 4.2 Preinvasive neoplasms of the pancreatic ducts. PanINs, the most common precursors to PDA, arise in the terminal ductules and typically progress to invasive disease at the head of the gland. MCNs also develop in the periphery of the gland, though typically in the body and tail, and exhibit a characteristic "ovarian stroma." IPMNs are macroscopic cysts that form in the main or large branch ducts, most commonly in the pancreatic head, and have a distinct genetic profile from the other two classes of precursors (*boxes* indicate completely unique genetic events)

largely by the presence of associated cystic neoplasms. Thus, despite being relatively uncommon, understanding the critical differences in the MCN-PDA route as compared to the PanIN-PDA form of disease is likely to be informative. How does the same constellation of mutations sometimes result in conventional PDA that is essentially incurable, and other times invasive ductal carcinoma from cysts that can be cured? Static analyses of resected human tumor specimens have not resolved this paradox.

4.4 Lessons Learned from Modeling in Mice

A number of lessons have emerged over the past several years from systematic engineering of key mutations, alone or in combination, into endogenous loci of the mouse (reviewed in Leach 2004; Hingorani 2005; Rustgi 2006). Targeted expression of oncogenic $Kras^{G12D}$ to developing cells of the mouse embryo, for example, initiates the development of pancreatic intraepithelial neoplasias (PanINs) with complete penetrance (Hingorani et al. 2003). These neoplasms progress spontaneously in greater than 95% of these animals to invasive and metastatic PDA (Hingorani et al. 2003 and unpublished observations). These studies established PanINs as bona fide precursors to PDA, as mice have never before been described to develop PanINs

or PDA of their own accord. Disease progression can be hastened in the context of concomitant expression of point-mutant *Trp53*^{R172H} (Hingorani et al. 2005) or biallelic loss of the *p16*^{Ink4a}/*p19*^{Arf} loci (Aguirre et al. 2003). These latter studies have also revealed the existence of multiple distinct genetic trajectories to infiltrating PDA with sometimes subtle, but nevertheless important, differences in the clinical syndrome and histopathology of the resultant disease, differences that also reflect the heterogeneity of disease presentations encountered in patients (Table 4.1).

Although identifying the specific genetic and histologic subtype of pancreas cancer in a given patient will greatly refine the therapeutic plan, there will likely be further differences in specific signaling pathways activated even within a given subgroup. For example, even among patients with key mutations in *KRAS* and *TP53*, the considerable genomic instability that ensues means that each tumor will likely be characterized by different combinations of activated signaling pathways (Hingorani et al. 2005; Jones et al. 2008). Treating this subtly heterogeneous population as though it were homogeneous may mask the efficacy and potential utility of some targeted agents when otherwise used in a more narrowly defined and appropriate context.

These types of investigations also have the potential to uncover new fundamental principles about pancreas cancer pathogenesis that may also be applicable to carcinogenesis in general. For example, it appears that the specific sequence, in addition to the total complement, of mutations that arises during the course of tumor progression profoundly influences the phenotype of the invasive disease (Izeradjene et al. 2007). Early mutation of *Dpc4* after the development of *Kras*-initiated PanINs results in an altered differentiation state and the development of mucinous cystic neoplasms (MCN). These macroscopic cystic precursors can progress to infiltrating and metastatic PDA, albeit generally more slowly and with a lesser penchant for early dissemination. Disease progression invariably occurs in association with loss of heterozygosity (LOH) of *Dpc4*, followed by mutations of either, but not both, *Trp53* or *p16*. Interestingly, while appearing histologically identical in their invasive state to carcinoma cells from the classic PanIN-PDA route, MCN-PDA cells resist the ability of TGFβ to induce epithelial-mesenchymal transition (EMT) and increase migration.

A second study revealed surprising findings regarding the dynamics of the immune response to a developing pancreatic neoplasm (Clark et al. 2007). As noted above, the intense fibroinflammatory response that begins during the course of preinvasive disease and accompanies disease progression is essentially pathognomonic for human pancreas cancer and it is recapitulated histologically in the murine disease. Nevertheless, the specific cellular components and kinetics of this desmoplastic reaction have never been characterized in detail. The prevailing theory of immunosurveillance and the subsequent escape by an evolving neoplasm suggests that an early effective immune response is subsequently overwhelmed by counter-regulatory elements (reviewed in Dunn et al. 2006). Much to our surprise, we instead found immunosuppressive and tumor-promoting elements of the immune response specifically localized to preinvasive lesions from the very earliest stages of disease (Clark et al. 2007 and discussed further below). These findings again challenge an existent paradigm and re-frame the goals of a potentially vital component of the armamentarium against this disease.

Table 4.1 Preclinical applications of genetically engineered mouse models of pancreas cancer

Genotype	Preinvasive neoplasm	Median survival (months)	Key features	Potential applications					Refs.
				Ch	RF	Bio ED	Bio RR	Rx	
Kras$^{G12D/+}$	PanIN	15	Slow evolution of disease	×	×	×			Hingorani et al. (2003)
Kras$^{G12D/+}$;p53$^{R172H/+}$	PanIN	5	Faithful recapitulation of primary/metastatic human disease	×	×	×	×	×	Hingorani et al. (2005)
Kras$^{G12D/+}$;p16/p19$^{fl/fl}$	PanIN	2	Rapidly progressive primary tumor				×	×	Aguirre et al. (2003)
Kras$^{G12D/+}$;p53$^{flox/+}$;p16$^{(+/-)}$	PanIN	3.5	Rapidly progressive primary tumor				×	×	Bardeesy et al. (2006a)
Kras$^{G12D/+}$;Dpc4$^{fl/+}$	MCN	15	Slow evolution along distinct histopathologic route	×	×	×			Izeradjene et al. (2007)
Kras$^{G12D/+}$;Dpc4$^{fl/fl}$	MCN	8	Intermediate evolution along distinct histopathologic route	×	×	×	×	×	Izeradjene et al. (2007)
Kras$^{G12D/+}$;p16/p19$^{fl/fl}$;Dpc4$^{fl/fl}$	PanIN>IPMN	3.5	Rapid progression from mixed precursor populations				×	×	Bardeesy et al. (2006b)
Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$	PanIN	2	Rapid progression of primary and rare metastatic disease				×	×	Ijichi et al. (2006)
Kras$^{G12Vgeo/+}$;Elas-tTA/tetO-Cre	PanIN	variable	Inducible activation in acinar cell compartment	×	×	×			Guerra et al. (2007)

Distinct precursor lesions, tumor latencies, clinical syndromes and burdens of primary and metastatic disease distinguish the various pancreas cancer mouse models making them particularly suitable for specific types of investigations. The examples shown below and suggested potential roles in preclinical studies are meant to be illustrative only and by no means exhaustive or restrictive

Ch chemoprevention, *RF* risk factors, *Bio ED* biomarkers of early detection, *Bio RR* biomarkers of response and resistance, *Rx* treatment

4.5 Defining a Way Forward

The proposed program involves the coordinated and integrated execution of research protocols and clinical trials in genetically defined models of pancreas cancer subtypes and in patients. In the following, we describe a Murine Clinical Trials Program (MCTP), as part of a larger Center for Accelerated Translation in Pancreas Cancer (CATPAC), that is designed to prioritize treatment and detection strategies to be brought to the clinic in a comprehensive effort to accelerate translation for this devastating disease. This Center involves the bi-directional flow of information between mice and humans on a number of investigational levels: epidemiology; longitudinal studies; biomarkers for disease detection and response to therapy; chemoprevention; targeted therapies for early and advanced disease; and studies of familial disease (Fig. 4.3). The approach also entails a departure from more piecemeal approaches in which promising insights or leads are potentially obscured because of the uni-dimensional nature of data collection. Studies performed in defined genetic backgrounds also have the advantage of decreased "noise," allowing the most relevant factors to emerge so that the resulting data can also serve as a

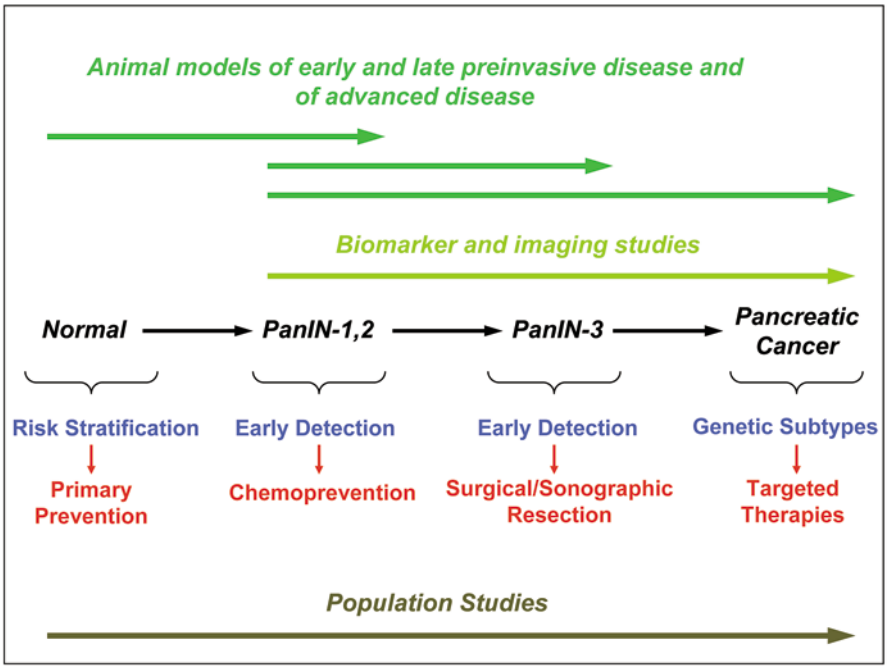

Fig. 4.3 Multi-dimensional analysis of pancreatic cancer initiation, progression, early detection, risk factors, and natural history. Studies of various stages of disease (*black*) are geared toward certain objectives (*blue*) allowing appropriate interventions (*red*). These types of studies are predicated upon the development and existence of highly faithful animal models that recapitulate all stages of the human disease (*dark green*). Information derived from these preclinical platforms can be integrated with findings from population studies and translated to the delivery of patient care

filter or lens through which to focus and scrutinize patient data. These multi-dimensional analyses for each stage of disease progression should reveal qualitatively different types of information and help pinpoint the most promising avenues for further pursuit.

4.6 Criteria for a Valid Mouse Model of Disease

We have stringently applied the following criteria for a valid mouse model of disease in preclinical studies. The model must:

1. Incorporate signature genetic events implicated in the human disease
2. Recapitulate the clinical syndrome of disease
3. Recapitulate the histopathology from preinvasive to invasive to metastatic disease
4. Recapitulate genetic progression implicated in human disease
5. Recapitulate treatment response to known agents and *increase predictive power* of new therapies brought to the clinic.

As described above, the first four criteria have been met (Hingorani et al. 2003, 2005) and the histopathology independently verified (Hruban et al. 2006). The last criterion was recently satisfied by repeating the preclinical studies performed with gemcitabine which contributed to its introduction to the clinic (Hertel et al. 1990). Gemcitabine is a deoxycytidine analog and inhibitor of nucleic acid synthesis which prolongs survival by a few weeks and provides symptomatic improvement in a minority of patients (Burris et al. 1997). As reported previously in the literature, we found that gemcitabine reliably inhibited the growth of xenografted human PDA cell lines (K. Olive, S. Hingorani, D. Tuveson, unpublished observations). Interestingly, the same was true for primary cell lines derived from genetically engineered *Kras*$^{LSL\text{-}G12D/+}$*;Trp53*$^{LSL\text{-}R172H/+}$*;Pdx-1-Cre* (Hingorani et al. 2005) mice. However, approximately 90% of primary in situ PDAs in these mice were unaffected by treatment with gemcitabine; 10% showed transient arrest and/or minimal regression before continuing to grow – the same relative frequencies observed in patients (Burris et al. 1997). Thus, just as for human cell lines and patients, cell lines from genetically engineered mice grown as xenografts were inhibited by the drug, while in situ tumors in littermates were largely resistant. These models now form the basis for our preclinical efforts in biomarker discovery and therapeutics.

4.7 Coordinated Targeting of Stroma and Epithelium: Getting Through the Shield to Hit the Heel

4.7.1 Cell Autonomous Targets

A number of cell autonomous signaling pathways have been identified over the past few decades of research in pancreas cancer and several of these have been

investigated in clinical trials (reviewed in Izeradjene and Hingorani 2007; Yeh and Der 2007). To date, since the introduction of gemcitabine as the modern standard of care for unresectable pancreas cancer (Burris et al. 1997), only a single additional agent has been found to improve survival when used in conjunction with gemcitabine. The addition of erlotinib (Tarceva), an ERBB1/EGFR antagonist, improves median survival by 12 days and 1-year survival from 17 to 23% over gemcitabine alone in unresectable disease (Moore et al. 2007). Needless to say, the search for additional targets, drugs and combinations actively continues.

The unique capabilities of cancer cells may come at the price of unique vulnerabilities as well, a premise that underlies the concept of "oncogene dependence" or "addiction" (Weinstein 2002). The idea suggests that the reorganization of intracellular signaling networks driven by a critical oncogenic mutation creates a state of dependency and thereby provides an "Achilles heel," an ideal target whose inhibition should be catastrophic for the cancer, but not normal, cell. Whether the experience with imatinib mesylate (Gleevec) in chronic myelogenous leukemia represents the exception or the rule remains to be seen (reviewed in Hingorani and Tuveson 2003), although it can reasonably be hypothesized that mutant *RAS* would represent such a dominant oncogene for pancreas cancer (Yeh and Der 2007). The recent deep sequencing study of the pancreas cancer genome by Kinzler, Vogelstein and colleagues (Jones et al. 2008) has nevertheless confirmed and elaborated upon another sobering and disheartening reality for pancreas cancer: the high degree of both numerical and structural genomic instability and large number of distinct signaling and regulatory pathways that are mutated in these cancers. Targeting the epithelial component of these cancers, therefore, is like trying to hit a moving target, as each round of cell division further extends the genomic diversity, expanding the possibilities for the development of resistant clones. Even should a therapy work initially, it is likely that resistance will rapidly emerge. This reality motivates, in part, the desire to target so-called cancer stem cells (Hermann et al. 2007; Li et al. 2007); should they exist (see e.g., Quintana et al. 2008), they are thought to represent not only the pool of cells required to sustain and re-populate the bulk tumor, but also a potentially more stable genome as a result of less frequent cell divisions. That such cells may be altered or experience artificial selection pressures during the course of culturing argues further for their study in an autochthonous setting. These possibilities notwithstanding, 1163 distinct mutations discovered across only 24 different human pancreas cancers gives pause (Jones et al. 2008). In this regard, targeting the stromal environment has added appeal and urgency.

4.7.2 Non-cell Autonomous Targets

The tumor microenvironment presents a number of mechanisms that contribute to drug resistance and must therefore be overcome in order to improve response. Before an anti-cancer agent can exert a therapeutic effect, it must surmount a number of barriers: the agent must enter and persist in the circulation at sufficient concentrations and for a sufficient period of time; it must enter the tumor

sub-circulation, traverse the capillary walls and diffuse across the tumor tissue; finally, it must encounter the cancer cell and kill it. It must also accomplish each of the latter tasks essentially uniformly across the tumor mass in order to have a clinically meaningful impact. Not only are microenvironmental factors, including pO_2, pH, glucose concentration, and interstitial fluid pressure extreme in some portions of the tumor, these parameters also vary heterogeneously across the tumor bed and over time. It is already well-appreciated that tumor cell killing is non-uniform across a carcinoma (Tannock 1978). Activated fibroblasts, aberrant and leaky tumor vasculature, decreased lymphatic drainage, and increased interstitial oncotic pressures all combine to increase interstitial fluid pressures which, in turn, limit the ability of drugs, large and small, to penetrate the tumor (reviewed in Heldin et al. 2004). Gradients in critical physicochemical parameters across a tumor not only contribute to generating corresponding gradients in drug concentration (Lankelma et al. 1999; Huxham et al. 2004), but also influence the local cellular response to those drugs.

The intense fibroinflammatory response, or desmoplastic reaction, that begins with the development of preinvasive lesions and increases during disease progression is virtually pathognomonic for human pancreas cancer (Mahadevan and Von Hoff 2007) and is recapitulated in the corresponding genetically engineered animal models. A rich panoply of cells and signaling molecules evolve coordinately during tumor progression, conspiring to promote and sustain the carcinoma, while contributing to its ability to resist chemical and radiotherapies. The cells of the stroma are likely to be far more genetically stable (Walter et al. 2008) than their intermingled epithelial counterparts, suggesting the very real possibility that a large number of tumors will respond to a limited number of "stromal" therapies. For example, it may be possible to cluster pancreas cancers into a limited number of "stromal subtypes" such as T-cell predominant or stellate cell predominant. In such circumstances, one would choose an agent targeting a specific pathway(s) found to be mutated in a patient's carcinoma cells (cell autonomous therapy) together with one or more stromal therapies (non-cell autonomous therapy). A few of the cellular and structural components of the stroma, together with strategies to disrupt them, are discussed further below.

4.7.2.1 Immune Cells

A number of theories abound regarding the evolution of the immune response during the inception and progression of neoplastic disease, but it had not previously been possible to study these processes as they develop in situ. The prevailing theory of immune surveillance of an evolving neoplasm posits that an early effective immune response is subsequently overwhelmed by counter-regulatory elements (Dunn et al. 2006). This conceptualization in part underlies the major focus in immunotherapy on augmenting the effector T-cell response. We found, however, that distinct classes of tumor-promoting and immunosuppressive cells invade nascent neoplastic ductal lesions and *precede* the development of an effective immune response (Clark et al. 2007). Specifically, we found early influx of tumor-associated macrophages (TAMs)

and regulatory T-cells, followed by myeloid derived suppressor cells (MDSC), and a complete absence of an effector response. In other words, the potential benefit of immunosurveillance is aborted before it has a chance to begin. These studies suggest that therapies geared toward *inhibiting* the tumor-promoting and immunosuppressive elements, and doing so from as early in the course of disease as possible, may be required before an effective immune response can be mounted.

4.7.2.2 Pancreatic Stellate Cells

Pancreatic stellate cells (PSC) are myofibroblast-like cells that are the presumptive counterpart of hepatic stellate cells (reviewed in Omary et al. 2007). PSC have been implicated as the source of matrix deposition in chronic pancreatitis and in pancreas cancer (Apte et al. 1998, 2004; Bachem et al. 1998); they have a mixture of properties of both fibroblasts and smooth muscle cells and are therefore distinct from both. Quiescent PSC express a number of intermediate filament proteins, including desmin, vimentin, glial fibrillary acidic protein (GFAP), and nestin, and also contain intracellular fat droplets. Activated PSC lose these fat droplets and express α-smooth muscle actin (SMA); they also bind to collagen fibers and organize them into a more rigid framework (Heldin et al. 2004). PSC secrete and respond to a number of growth factors, cytokines, and proinflammatory molecules (Omary et al. 2007). These molecules and the implicated signaling pathways represent potential targets for reverting activated PSC to a quiescent state and include, as examples, COX-2, PDGF, TGFβ, and vitamin A.

4.7.2.3 Angiogenesis

The insight that neoplasms must augment their local blood supply to grow beyond a critical limit represents one of the major conceptual advances in clinical oncology over the past few decades (Folkman 1972; Hanahan and Folkman 1996). Angiogenesis inhibitors are now a key part of the therapeutic armamentarium in colorectal, breast and lung cancers, and are being explored in virtually all other solid tumors. It is not clear that the same principles of angiogenesis will apply in PDA, however. PDAs have long been described as radiographically "hypodense," a result of decreased perfusion by contrast material of tumor tissue relative to that of the surrounding normal parenchyma and other organs (Ikeda et al. 1999). These tumors are also known to be hard, fibrous and hypovascular at resection. We have found similar characteristics in murine PDAs with large areas of tumor tissue containing virtually no discernable vasculature; inexplicably, these regions are often not necrotic but rather contain viable cells (unpublished observations). Collectively, these findings suggest that angiogenesis inhibitors may be of limited efficacy in PDA, a prediction borne out by very recent clinical data (Kindler 2007). The potential of these agents to "normalize" the vasculature and help decrease interstitial pressures may yet provide a role for them, but strict inhibition of angiogenesis per se may not be useful in this disease.

4.8 Putting Principles into Practice

4.8.1 Risk Factors and Chemoprevention

A number of highly faithful animal models of human pancreas cancer now exist encompassing the diversity of disease presentations encountered in patients. A representative sampling is shown in Table 4.1. Each of these models has a distinct tumor latency, clinical syndrome of disease, median survival, histopathology, burden of primary vs. metastatic disease, and subsets of activated and mutated pathways. As such, each model lends itself to particular types of investigations and interventions. For example, the model based on targeted expression of activated $Kras^{G12D}$ alone is particularly well-suited for studies of early detection and chemoprevention as disease evolves slowly from preinvasive neoplasms to invasive and metastatic PDA (Hingorani et al. 2003). The quantifiable progression of precursors from PanIN-1 to PanIN-2 to PanIN-3 also permits rigorous evaluation of potential risk factors for disease such as tobacco exposure, chronic inflammatory injury, diabetes and so on. Thus, exposing cohorts of animals at distinct ages to varying doses of tobacco smoke, or potentiators or inhibitors of inflammation (see e.g., Funahashi et al. 2007) and assessing the effects on disease progression can be readily performed. At the other extreme, the $Kras^{LSL\text{-}G12D/+};p16/p19^{-/-}$ model develops a rapidly progressive disease with large primary tumors and relatively scant metastatic burden, which lends itself to rapid assessment of therapeutic strategies to prolong survival in this particular subtype of disease (Aguirre et al. 2003). We use the $Kras^{LSL\text{-}G12D/+};Trp53^{LSL\text{-}R172H/+};Cre$ model as our primary system for investigation because of its extremely faithful recapitulation of the classic syndrome of PDA in patients and median survival of approximately 5 months (Hingorani et al. 2005).

4.8.2 Early Detection, Disease Recurrence, and Minimum Residual Disease

It is axiomatic in clinical oncology that early detection leads to increased cures. In a disease with such an early and extreme penchant for metastasis, detection at the earliest possible stage may offer the only possible chance of cure. As discussed earlier, attempting to identify markers for early detection is particularly difficult in pancreas cancer patients. The animal models lend themselves readily to such investigations. As proof of principle, it has already been shown that a definable proteomic signature exists in mice with even a very low burden of *preinvasive* disease, distinguishing their sera from that of control littermates (Hingorani et al. 2003). More recently, specific markers discovered in murine serum were also identified in patients, further attesting to the fidelity of these model systems (Faca et al. 2008).

The models lend themselves to the discovery of both stage-specific and genetic subtype-specific markers of disease. Indeed, the potential utility of a whole range of markers including circulating tumor cells and cell fragments, proteins, nucleic acids

and glycostructures can be explored. The models can be used simultaneously to refine the various technological platforms while also identifying potential candidate markers for direct testing in the clinic.

4.8.3 Target Inhibition

One of the greatest impediments to rapid progress in drug development is the difficulty of addressing mechanisms of resistance. First and foremost, one must establish whether or not the drug reached the tissue of interest and then whether or not the presumptive target was indeed inhibited. In addition, are there different effects in primary lesions and metastases? Within different regions of the primary tumor and among different metastases? Can these differences in responses be correlated with measurable parameters? For example, do areas of response correlate with greater vascularity or proximity to vessels? Each of these questions can be readily addressed in animal models. Indeed, the ability to readily map local drug concentrations and correlate them with measures of activity and response across primary and metastatic lesions, and as a result of specific interventions, represents one of the great strengths of genetically engineered model systems.

4.8.4 Acquired Resistance

A nearly universal feature of clinical management of cancer patients, even in those who experience dramatic initial responses to therapy, is the emergence of resistant disease. While many of the underlying mechanisms of acquired resistance will be cell autonomous and therefore potentially anticipated by and subject to study in in vitro systems, some will be non-cell autonomous. It is also possible that the specific types of cell autonomous resistance that emerge in culture will be distinct from those in vivo. These can only feasibly be studied in autochthonous models. As various mechanisms of disease resistance are revealed, approaches to preempt their emergence can also be studied in these model systems. Indeed, one can envision bringing regimens to the clinic optimized to decrease the likelihood of emerging disease resistance and having already elucidated tailored second- and third-line remedies for each mechanism. These types of approaches will help collapse the time to incremental advances in care, particularly for a disease such as PDA, in which large clinical studies are so inherently difficult.

4.8.5 Imaging

The current model for determining therapeutic efficacy typically involves imaging by CT or MRI for extent and stage of tumor, treatment with a drug regimen for weeks to months, and then repeat imaging; cross sectional diameters of lesions are typically used to assess for disease response. The approach is both slow and

inexact: many months of treatment are given before any benefit can be ascertained and volume measurements are crude estimates, at best, of efficacy. What is needed is real-time, or near real-time evaluation of drug penetration and activity and rapid tailoring of regimens as indicated. Thus, ideally a regimen tailored for a given patient with maximal likelihood of benefit should be identified within the initial 7–10 days of treatment. The animal model systems can also help develop and test strategies to achieve this goal. In addition, a number of imaging modalities including contrast-enhanced high-frequency ultrasound; next generation MRI, CT and PET technologies; optical coherence tomography (OCT); emergent technologies involving targeted nanoparticles; and others can be optimized through the ready availability of animals at distinct stages of disease. Each of the imaging modalities can also be studied in conjunction with circulating biomarker analyses to complement efforts to identify stage-specific disease markers.

4.8.6 Sequencing of Agents and Dosing Schedules

Most chemotherapy regimens used in practice today follow a schedule of administration based on historical principles of the kinetics of tumor cell proliferation and death and the concept of maximum tolerated doses (MTD) (DeVita and Chu 2008). Among the most commonly used formulas is weekly administration of cytotoxic drugs on a 3 weeks on, 1 week off schedule for a total of 6 months; therapy is interrupted or dose-reduced for treatment-related toxicities or other untoward events. Combination regimens are devised by considering agents with different presumed mechanisms of action and, when possible, distinct toxicity profiles so as to be able to deliver as close to the MTD for each agent as possible. The optimal concentrations or schedules with which to use such agents in combination, however, are rarely examined, nor are the optimal sequences in which to give them. The concept of chronotropic or metronomic therapy, for example, would be completely missed by such an approach (Kerbel and Kamen 2004). Pharmacokinetic and pharmacodynamic measurements are frequently performed in xenograft models which also do not necessarily reflect the behavior of in situ tumors in patients. Of course, attempting to perform multi-combination, multi-dose, multi-schedule, multi-sequence studies in patients is not practical.

The number of potential combinations of primary (cell autonomous) and stromal (non-cell autonomous) therapies increases exponentially. To rationally and systematically test all such possible combinations is essential to developing the most potent regimens; to do so in patients is impractical, if not impossible. After determining which combinations are ideal, one must still figure out how to sequence the agents, how far apart to dose the drugs, how frequently to give them and so on. For example, how many days prior to administering a cytotoxic agent should an anti-stromal agent be given to improve tumor penetration? How many doses of the anti-stromal agent are required to reduce interstitial fluid pressures sufficiently to maximize tumor penetration?

Finally, the role of radiation therapy in pancreas cancer management remains a subject of intense controversy, with opinion divided largely along geographic lines (Berlin 2007; Mulcahy 2007). Although the instrumentation for small animal delivery of radiation treatment is still in development, understanding how best to incorporate and sequence radiation into the therapeutic plan for locally invasive, locally advanced and even metastatic disease could, in principle, also be greatly facilitated in mouse models. These types of complex trials involving multi-modality therapy in different burden of disease contexts are inordinately challenging to perform in patients, while once again exposing them to significant potential toxicities.

Each of these considerations can be more readily addressed in faithful animal surrogates of the human disease. A pilot program consisting of small cohorts of animals (e.g., 3–5) of a given genetic subtype of PDA can be used to explore various possibilities and the most promising schemes selected for evaluation in larger studies (Fig. 4.4). The latter can include serial collections of serum and plasma for

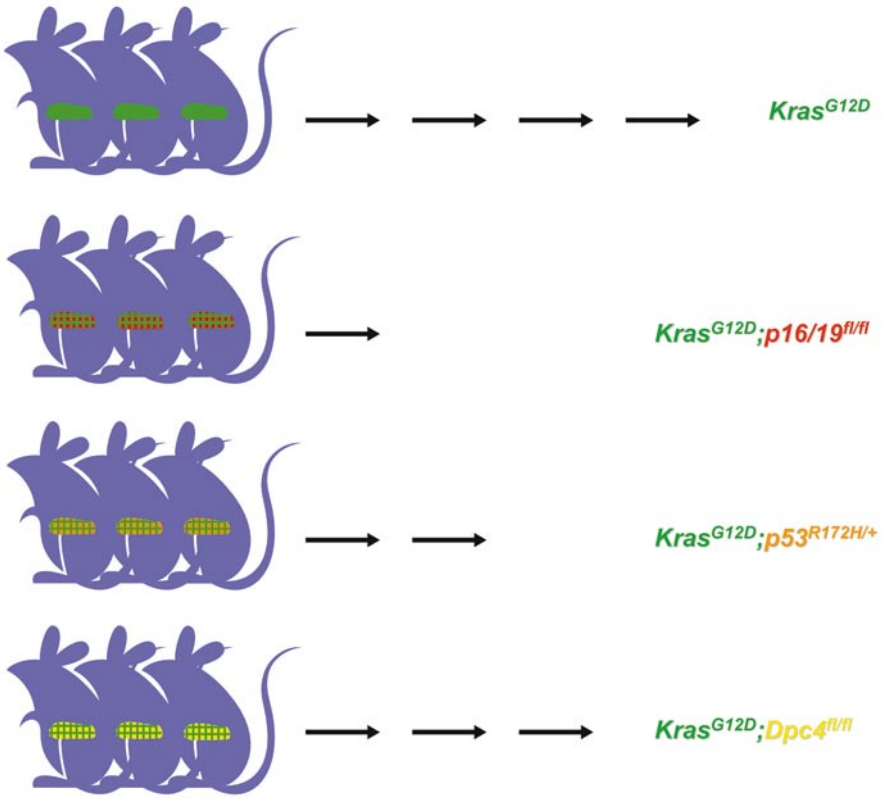

Fig. 4.4 Murine clinical trials program. Each of the described genetically engineered mouse models of pancreas cancer has a distinct rate of disease progression, spectrum of locally invasive and metastatic disease life expectancy, and life expectancy (indicated by length of *arrow*). As such, the models lend themselves to a wide range of potential clinical applications (see text for details)

studies to identify biomarkers of response and resistance to treatment; tissue samples collected prior to the initiation of therapy used to identify markers that *predict* sensitivity or not; and samples collected from all animals and all organs for a variety of detailed cellular and molecular analyses.

4.9 Concluding Remarks

We now possess an unprecedented level of information on the genomes, pathways, proteins, and fine structure of human cancers. The capability also exists to synthesize a seemingly endless array of small molecules that inhibit specifically (more or less) various essential processes in carcinogenesis. The challenge now is to integrate and harness this vast compendium of information and technical capabilities toward transforming the way we care for patients. For pancreas cancer, the recent development of genetically engineered mouse models that faithfully recapitulate the complexity and diversity of the cognate human disease provides an important piece in a comprehensive program that enables rigorous and systematic evaluation of the most promising detection, diagnostic, and therapeutic strategies for ultimate translation to the clinic.

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Chapter 5
Zebrafish as a Biological System for Identifying and Validating Therapeutic Targets and Compounds

Nelson S. Yee

Abstract Pancreatic cancer is a great oncologic challenge. Significant progress has been made in understanding the molecular genetics for transformation of pancreatic epithelia into pre-malignant neoplasia and eventually invasive carcinoma. In spite of these advances, effective treatment is still lacking, and the prognosis of most patients diagnosed with pancreatic cancer is dismal. Molecularly targeted agents show great potential for improving treatment in pancreatic cancer cells and animal models. However, translation of the preclinical studies into clinically useful drugs with meaningful benefits for the patients remains to be accomplished. Development of zebrafish models and application of innovative techniques will enable drug discovery for pancreatic cancer in a whole organism. The established models including wild-type zebrafish larvae, germ-line mutants, transgenics, and xenografts can be utilized to identify the genetic pathways and their interactions that control exocrine pancreatic development and cancer. Combined application of chemical genetic screens with radiographic imaging, nanoparticulate systems, and bioinformatics in the zebrafish models is expected to facilitate identification of drugs that specifically target the signaling networks in pancreatic cancer stem cells, and validation of candidate therapeutics by real-time monitoring of tumor growth. Ultimately, a systems-biology approach that applies the various techniques to the zebrafish models is predicted to lead to discovery of efficacious and safe drugs toward the goal of targeted and personalized therapy in pancreatic cancer.

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5.1 Introduction

5.1.1 Role of Zebrafish in Pancreatic Cancer

Pancreatic cancer is mostly fatal (Jemal et al. 2009; Parkin et al. 2002). It is typically diagnosed at the advanced stage, at which time it is generally resistant to the current treatments. Novel approaches to develop effective therapy are urgently needed, and the zebrafish provides new models and techniques that are complementary to the conventional mechanisms. The zebrafish (*Brachydanio rerio*) is a bony fish (teleost), and it was first described in the river Ganges in India (Hamilton 1822). The zebrafish was developed as homozygous diploid clones, and it has been utilized as a model organism to study vertebrate development (Streisinger et al. 1981). Because of the intricate relationship between development and cancer, the zebrafish has been demonstrated as a powerful model for understanding cancer biology and genetics. The combination of the unique features of zebrafish as a biological system and the evolving techniques to utilize the zebrafish models has demonstrated potential and value to facilitate drug discovery for prevention and treatment of human diseases. This is particularly relevant for solid tumors like pancreatic cancer, which is among the most aggressive and lethal of human malignancies.

5.1.2 Zebrafish Model as a Biological System to Identify Molecular Targets and Validate Drugs

The zebrafish is an established model for studying normal and cancer development of vertebrate organs. The unique features of zebrafish include small size, optical transparency, fecundity, relatively low cost for maintenance, and easy husbandry. Embryologic and genetic studies in the zebrafish have demonstrated conserved biological events and molecular mechanisms during pancreatic organogenesis (Yee et al. 2001, 2005, 2007; Field et al. 2003; Lin et al. 2004; Zecchin et al. 2004; Esni et al. 2004; Yee and Pack 2005, reviewed by Tiso et al. 2009). Accumulating evidence supports the zebrafish as a model of human pancreatic adenocarcinoma, as revealed by the similar histopathology and the conserved roles of oncogenic K-Ras and Hedgehog in pancreatic adenocarcinoma (Amatruda et al. 2002; Park et al. 2008). This evidence provides support for the utility of zebrafish as a biological platform to identify the developmental pathways in exocrine pancreas, understand the mechanisms underlying pancreatic carcinogenesis, discover molecular targets and drugs for treatment of pancreatic cancer, and evaluate therapeutic efficacy and toxicity.

5.2 Zebrafish Models and Techniques for Drug Discovery in Pancreatic Cancer

The combination of zebrafish models and the applied techniques will help facilitate novel approaches to identify new drugs for treatment and prevention of pancreatic cancer. The major zebrafish models include: (1) wild-type larvae based on the common genetic pathways in the developing exocrine pancreas and cancer; (2) germline mutations causing exocrine pancreatic phenotype in larvae; (3) transgenics carrying activated oncogenes and inactivated tumor suppressor genes; and (4) xenografts by transplantation of human pancreatic adenocarcinoma cells. These models enable studying mechanisms underlying pancreatic carcinogenesis, evaluating tumor growth in a developmental context, and determining drug response in preventing as well as suppressing tumor growth and metastasis.

Newly developed techniques are applicable to the zebrafish models for identifying therapeutic compounds and evaluating drug responses in a whole organism. Firstly, the zebrafish system is amendable to high throughput screens with small molecules that modify disease phenotypes (reviewed by Zon and Peterson 2005; Murphey and Zon 2006; Hong 2009). In essence, embryos are distributed to 96-well plates, with five embryos per well. Small molecules from a synthesized or an acquired library are added to the embryo medium. The phenotypic effects of the small molecules, such as suppression or reversal of an abnormality caused by a mutation or disease, are examined visually or detected with automatic devices. Advantages of small molecule screens in zebrafish include relatively small amounts of chemicals used, ease of administration, few animals, and low cost, as compared to conventionally used large animals. An example is illustrated by Stern et al. (2005) who identified small molecules that delay S phase to suppress the zebrafish mutation in *b-myb*. Therefore, the combination of chemical and genetic screens for modifiers of exocrine pancreas phenotype, genetic pathways, or tumor growth in zebrafish larvae is expected to help identify signaling pathways as biomarkers and molecular targets. With the advent of nanotechnology, specific targeting of biomarkers with nanoparticulate-delivered small molecules will provide powerful tools for developing preventive and therapeutic interventions in pancreatic cancer.

Secondly, microscopic radiographic imaging such as ultrasonography enables evaluation of therapeutic response by longitudinal characterization of tumor development in vivo, monitoring tumor size in real time within a living vertebrate organism, and repetitive sampling for microscopic and molecular analysis (Fig. 5.1) (Goessling et al. 2007; Spitsbergen 2007). Coupling Doppler with ultrasound can be applied to characterize the extent of tumor vascularization as non-invasive monitoring of vascular density and arterial blood flow as indicators of tumor progression and drug response. Generation of adult mutant zebrafish lacking skin pigment (White et al. 2008) will further facilitate visual detection and monitoring of tumor size and progression in chemical screens and in evaluation of drug responses (Fig. 5.2).

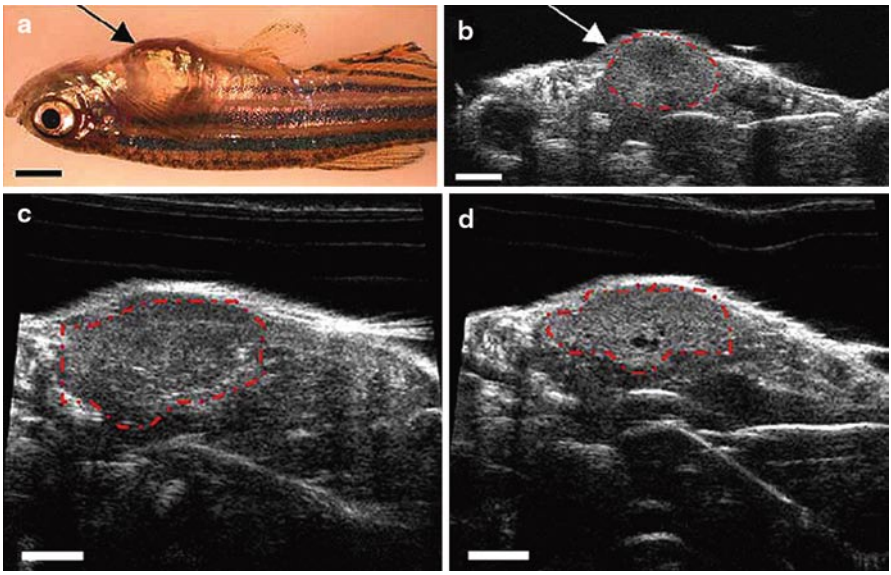

Fig. 5.1 Ultrasonographic imaging to evaluate and monitor therapeutic response of chemically (DMBA)-induced cancer in zebrafish. **a** adult zebrafish with abdominal tumor (*arrow*). **b** ultrasonographic examination of the tumor (*red dashed lines*) in (**a**). **c** and **d** ultrasonograms of hepatic tumor before (**c**) and after (**d**) treatment. Reprinted by permission from Goessling et al. (2007)

Fig. 5.2 Adult zebrafish *roy*$^{-/-}$; *nacre*$^{-/-}$ (*casper*) is devoid of skin pigment, thus facilitating visual detection of tumors in the internal organs and their response to treatment. Wild-type AB adult zebrafish is included for comparison. Reprinted with permission from White et al. (2008)

Thirdly, bioinformatics based on genomic, transcriptomic, and proteomic studies of zebrafish have provided valuable data that can be exploited for drug discovery. Chemogenomic analysis of adult zebrafish provides information on biomarkers and signaling pathways, and facilitates identification and validation of therapeutic targets and agents as well as evaluation of efficacy and toxicity (Lam et al. 2008). Transcriptomic analysis with oligo-microarray using total RNA extracted from wild-type and germ-line mutant zebrafish has generated novel information on gene functions during organogenesis (Stuckenholz et al. 2009). MicroRNA profiling of developmentally staged larvae and adult-derived cell lines showed that most of the miRNAs expressed in zebrafish are evolutionarily conserved as in mammals (Chen et al. 2005b). The zebrafish proteome obtained during early embryogenesis and organogenesis has revealed expression of proteins involved in diverse cellular functions, and these data will help refine analysis of the genetic network in vertebrate development (Tay et al. 2006; Lucitt et al. 2008). Recent studies have accumulated a large amount of bioinformatics data using human pancreatic adenocarcinoma by global genomic analysis (Jones et al. 2008), serial analysis of gene expression (Ryu et al. 2002), microRNA profiling (Bloomston et al. 2007; Lee et al. 2007; Szafranska et al. 2007), and proteomic analysis (Shen et al. 2004; Chen et al. 2005c). Taken together, the combination of the bioinformatics data in zebrafish and human pancreatic cancer will help elucidate the developmental and carcinogenic mechanisms, which can potentially be translated into therapeutic targets and biomarkers to improve diagnosis and optimize treatment response.

5.3 Wild-Type Zebrafish

5.3.1 Exocrine Pancreas of Wild-Type Zebrafish as Models

Exocrine pancreas development and cancer share common genetic pathways, such as epidermal growth factor, Ras, Sonic hedgehog (Shh), Notch, Wnt, fibroblast growth factor, and transforming growth factor-β (Yee et al. 2005; Yee and Pack 2005; Jones et al. 2008; reviewed by Koorstra et al. 2008). Some of these developmental pathways have been shown to play crucial roles in the production of pancreatic cancer stem cells and thus initiation of pancreatic tumorigenesis. Wild-type zebrafish embryos can be utilized as tools for reverse genetics (analysis of gene function as directed by the gene) and chemical genetics (analysis of gene function using chemical compounds) to determine the functional roles of the developmental pathways in pancreatic cancer. Using a reverse genetics approach, the functional roles of oncogenes and tumor suppressors in exocrine pancreas development can be determined by targeted disruption using morpholino oligomers, or ectopic forced expression under heat shock promoter (Yee et al. 2007). The exocrine pancreas of zebrafish larvae can be analyzed by in situ hybridization or immunohistochemistry using digestive enzymes (such as trypsin, carboxypeptidase A, elastase), ductal protein (cytokeratin), or pancreas-specific transcription factors (Ptf1a, Pdx1) as markers (Yee and Pack, 2005, Yee et al. 2005). Generation of

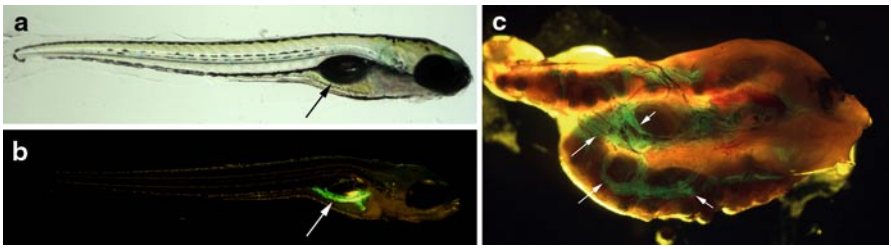

Fig. 5.3 Zebrafish exocrine pancreas during morphogenesis and in adult. **a** & **b** transgenic zebrafish larva expressing green fluorescent protein under control of elastase promoter (elastase: eGFP) on 5 days post-fertilization (dpf). **a** bright field. **b** green fluorescence; *arrow* pointing at exocrine pancreas expressing elastase:eGFP. **c** adult zebrafish at 1 year of age expressing elastase: eGFP. Note the exocrine pancreas in the adult zebrafish (**c**) is diffusely present within the mesentery, in contrast to the compact organ in the larva (**b**). The transgenic zebrafish elastase:eGFP was generously provided by Dr. Zhiyuan Gong

transgenic zebrafish lines expressing green fluorescent protein under control of exocrine pancreas-specific promoters such as elastase:eGFP (Wan et al. 2006) and ptfla: eGFP (Park et al. 2008) help facilitate analysis (Fig. 5.3).

Chemicals known to influence the growth of pancreatic cancer cells and alter signaling pathways involved in pancreatic carcinogenesis can be used to modulate developmental regulators of exocrine pancreas. In addition, toxicity of new chemicals and conventionally used chemotherapeutic agents can be visually monitored for cardiac edema or other developmental abnormalities. Targeting developmental regulators of exocrine pancreas has been translated into a potential therapeutic approach for pancreatic cancer. For instance, histone deacetylase 1 (Hdac1) regulates gene transcription by modulating the acetylation status of nucleosomal histones and other proteins, and it is implicated in normal physiology and disease states (reviewed by Haberland et al. 2008). Anti-sense oligonucleotide-mediated disruption of *hdac1* expression suppresses exocrine pancreatic growth (Yee et al. submitted). Treatment of larvae with chemical inhibitors of histone deacetylases (HDACs) such as trichostain A impairs epithelial proliferation in the exocrine pancreas (Yee et al. submitted). By combining the clinical HDAC inhibitor, suberoylanilide hydroxamic acid, with the small molecule antagonist of Smoothened that mediates Shh signaling (SANT-1), enhanced suppression of proliferation and induction of apoptotic death can be attained in human pancreatic adenocarcinoma cells, which are resistant to the standard chemotherapeutic drug gemcitabine (Chun et al. 2009). These data demonstrate that targeting developmental regulators, especially in combination, can be exploited as a potential therapeutic approach for pancreatic cancer.

5.3.2 Techniques in Wild-Type Zebrafish for Drug Discovery

Results of these studies demonstrate the utility for drug discovery of common signaling pathways between embryonic and cancer development in the exocrine pancreas

of wild-type zebrafish larvae. The combination of reverse genetics and chemical screening will enable identification of developmental regulators as potential drug targets, and facilitate discovery of small molecules that modify exocrine pancreas growth as candidate therapeutic agents. Integration of bioinformatics data with chemical and genetic studies of zebrafish is predicted to yield valuable information on the signaling mechanism of drug actions in cellular functions that are relevant to pancreatic cancer. Given the crucial roles of the developmental regulators in exocrine pancreas in the initiation of pancreatic tumorigenesis, application of nanoparticulate systems to selectively target the cancer stem cells is predicted to potentiate our ability to eradicate the cancer cells. Furthermore, wild-type zebrafish can be utilized as a predictive model for toxicity studies of drugs with potential clinical use.

5.4 Germ-Line Mutants

5.4.1 Zebrafish with Germ-Line Mutations Affecting Exocrine Pancreas as Models

The forward genetics approach (analysis of gene function directed by phenotype) has facilitated identification of germ-line mutations affecting the exocrine pancreas from genome-wide mutagenesis screens (Table 5.1). The genes involved and the molecular basis of some of these mutations have been identified. Some of these genes are known to have established roles in pancreatic carcinogenesis. For instance, the zebrafish mutations *sonic you* (*syu*) and *slow muscle omitted* (*smu*) affect Shh and its signal mediator Smoothened, respectively (Schauerte et al. 1998; Chen et al. 2001; Varga et al. 2001). The *syu* mutation produces a relatively small exocrine pancreas (Yee et al. submitted), and this is consistent with the proliferative effect of Shh in human pancreatic adenocarcinoma (Thayer et al. 2003; Berman et al. 2003; Morton et al. 2007). Another zebrafish mutation *mind bomb* (*mdb*) affects a RING domain-containing ubiquitin ligase that is required for activation of Notch (Itoh et al. 2003). Loss-of-function of Notch in the *mdb* mutation causes hypomorphic pancreatic ducts and enlarged acini (Yee et al. 2005), and this observation is in agreement with the role of activated Notch in pancreatic cancer (Miyamoto et al. 2003; Jones et al. 2008; De La O et al. 2008). The *digestive-organ expansion factor* (*def*) mutation affects a pan-endoderm-specific factor, which normally down-regulates the expression of an isoform of p53 (Chen et al. 2005a). The *def*-encoded protein promotes the growth of digestive organs including exocrine pancreas through the p53 pathway, which is implicated in the progression of pancreatic neoplasia (Hruban et al. 2000; Yee et al. 2003).

In addition, other genes affected by the zebrafish mutations causing the exocrine pancreas phenotype have potential roles in pancreatic carcinogenesis. For instance, the *slj* mutation affects the second largest subunit of RNA polymerase III (Polr3b), resulting in reduced transcription of tRNA, and disrupted epithelial proliferation of

Table 5.1 Zebrafish germ-line mutations affecting exocrine pancreas

Mutation	Exocrine pancreas phenotype	Genes	References
ductjam (djm)	Small acini, dysmorphic ducts	Unidentified	Yee et al. (2005)
ducttrip (dtp)	Diminished acini, dysmorphic ducts	*S-adenosylhomocysteine hydrolase (ahcy)*	Yee et al. (2005); Matthews et al. (2009)
def	Small, arrested growth	*Digestive-organ expansion factor (def)*	Chen et al. (2005a)
flotte lotte (flo)	Small, degenerated	*Embryonic lethal yolk sac (elys)*	Yee et al. (2005); Davuluri et al. (2008)
hdac1^{s436}	Small	*Histone deacetylase 1 (hdac1)*	Noel et al. (2008)
hdac1^{hi1618}	Small, arrest growth	*Histone deacetylase 1 (hdac1)*	Yee et al. (submitted)
mind bomb (mdb)	Hypomorphic ducts, enlarged acini	*Ubiquitin ligase*	Yee et al. (2005); Itoh et al. (2003)
mitomess (mms)	Decreased zymogen, hypomorphic ducts	Unidentified	Yee et al. (2005)
nil per os (npo)	Small	*RNA binding protein 19 (rbp 19)*	Mayer and Fishman (2003)
piebald (pie)	Small, degenerated	Unidentified	Yee et al. (2005)
ptf1a	Arrested growth	*ptf1a*	Dong et al. (2008)
slimjim (slj)	Small, degenerated	*RNA polymerase III subunit 2 (polr3b)*	Yee et al. (2005); Yee et al. (2007)
sonic you	Small	*Sonic hedgehog*	Schauerte et al. (1998); Yee et al. (submitted)
sweetbread (swd)	Small acini, hypomorphic ducts	Unidentified	Yee et al. (2005)
apc	Small	*Adenomatous polyposis coli (apc)*	Goessling et al. (2007)
K-ras	Arrested growth	*K-ras*	Park et al. (2008)

exocrine pancreas (Fig. 5.4; Yee et al. 2005). Accumulating evidence indicates that over-expression or activated Polr3 is involved in cancer, and ectopically increased tRNA levels can lead to malignant transformation (White 2008; Johnson et al. 2008; Marshall et al. 2008). Thus, the zebrafish *polr3b*^{slj} mutants can provide useful tools for determining genetic regulation of Polr3 activity and its transcripts in the formation of pancreatic neoplasia. In support of this idea, using a small molecule inhibitor of Polr3, we have demonstrated suppression of exocrine pancreatic epithelial proliferation in zebrafish larvae and in cultured human pancreatic adenocarcinoma cells (Yee et al. submitted).

For several mutations, such as *flotte lotte* (*flo*) and *ducttrip* (*dtp*), the gene-encoded products have been identified and are potentially important in the formation of pancreatic neoplasia. The *flo* mutation leads to exocrine pancreatic degeneration by causing loss-of-function in the zebrafish orthologue of *embryonic lethal yolk sac* (*elys*) (Fig. 5.4; Yee et al. 2005; Davuluri et al. 2008). The elys^{flo}-

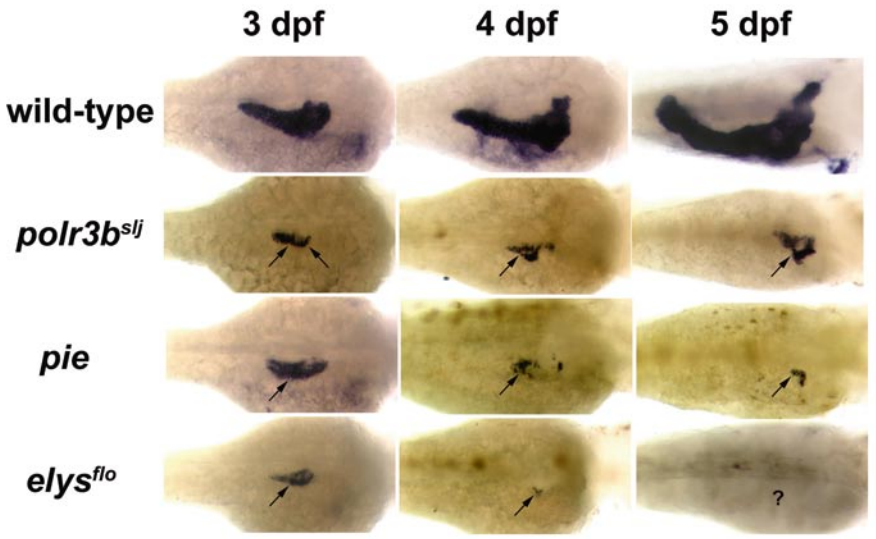

Fig. 5.4 Zebrafish mutations affecting early steps of morphogenesis in exocrine pancreas. Whole-mount in situ hybridization of *polr3b*^{slj}, *pie*, *elys*^{flo} mutant larvae and wild-type (wt) siblings on 3, 4, and 5 dpf using anti-trypsin riboprobes. Note in wt there is significant expansion of the exocrine pancreas between 3 dpf and 5 dpf, whereas the exocrine pancreas in each mutant on 3 dpf is relatively small, and it subsequently degenerates *dpf* day-post-fertilization. Modified and reprinted with permission from Yee et al. 2005

encoded nucleoporin is involved in the assembly of nuclear pore complex, and its role in the exocrine pancreatic epithelia remains to be defined. The *ducttrip* (*dtp*) mutation affects the gene that encodes *S*-adenosylhomocysteine hydrolase (Ahcy) (Yee et al. 2005; Matthews et al. 2009). The *ahcy*^{dtp} mutation disrupts cellular homeostasis of methionine and methylation reactions, and causes pancreatic acinar hypoplasia and ductal dysmorphogenesis (Fig. 5.5, Yee et al. 2005). Moreover, other zebrafish mutations such as *sweetbread* (*swd*), *mitomess* (*mms*), *ductjam* (*djm*), and *piebald* (*pie*) affect exocrine pancreas in unique ways (Fig. 5.4; Yee et al. 2005). The genes and the molecular basis of these mutations have not been identified. However, the zebrafish germ-line mutants will provide the tools for screens to identify genetic and chemical modifiers of the mutated genes and pathways in the exocrine pancreas.

5.4.2 Techniques in Zebrafish with Germ-Line Mutations for Drug Discovery

Zebrafish with germ-line mutations affecting exocrine pancreas will play a role as key intermediaries in the process of drug discovery in pancreatic cancer. Forward

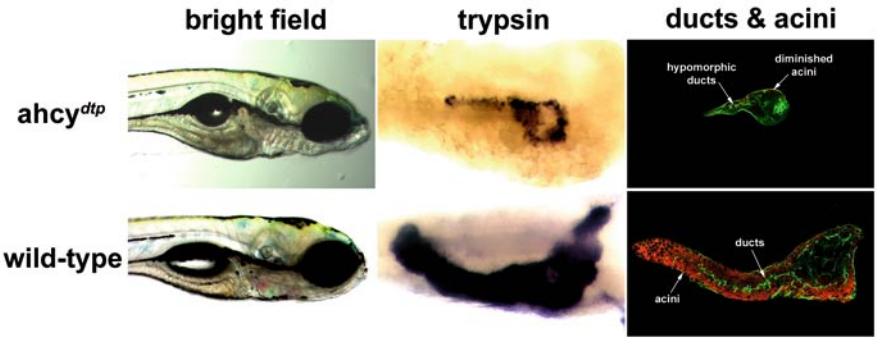

Fig. 5.5 Zebrafish *ducttrip* (*dtp*) locus encodes *S*-adenosylhomocysteine hydrolase (Ahcy) that controls cellular methionine homeostasis, and it is required for exocrine pancreatic growth, and acinar and ductal morphogenesis. Images of *ahcy*^{dtp} mutant larva and wild-type sibling on 5 dpf including bright field, whole-mount in situ hybridization using anti-trypsin riboprobes, and confocal analysis of pancreatic ducts (cytokeratin, *green*) and acini (carboxypeptidase A, *red*). Note in the *ahcy*^{dtp} mutant the pancreatic ducts are hypomorphic and the acini are diminished. Modified and reprinted with permission from Yee et al. 2005

genetic screens with these mutations are expected to identify modifiers that perturb the developmental pathways of exocrine pancreas and which can be translated into molecular targets for therapy. Chemical screens to identify small molecules and drugs that suppress or rescue the exocrine pancreatic phenotype of these mutations have the potential to discover novel therapeutic agents. Application of bioinformatics to genetic and chemical modifiers of the exocrine pancreas in those zebrafish mutations are predicted to generate useful data on the signaling mechanisms of growth control as well as the mechanism of drug actions in a developmental context.

5.5 Transgenics

5.5.1 Zebrafish Developing Pancreatic Cancer as Models

Zebrafish models for exocrine pancreatic tumors have been generated, and they exhibit features resembling those in human pancreatic cancer (Park et al. 2008; Haramis et al. 2006). A transgenic zebrafish model (*ptf1a:eGFP-Kras*^{G12V}) was established by expressing oncogenic *K-ras* under control of *ptf1a* promoter (Park et al. 2008). In the oncogenic K-Ras-expressing zebrafish, there is abnormal persistence of pancreatic progenitor cells which fail to differentiate in larvae, and they subsequently develop invasive carcinoma with acinar and/or ductal histological features (Fig. 5.6). The pancreatic tumors in the transgenic adult zebrafish exhibit abnormal activation of the hedgehog pathway, which is a prominent feature of human pancreatic adenocarcinoma (Thayer et al. 2003; Berman et al. 2003).

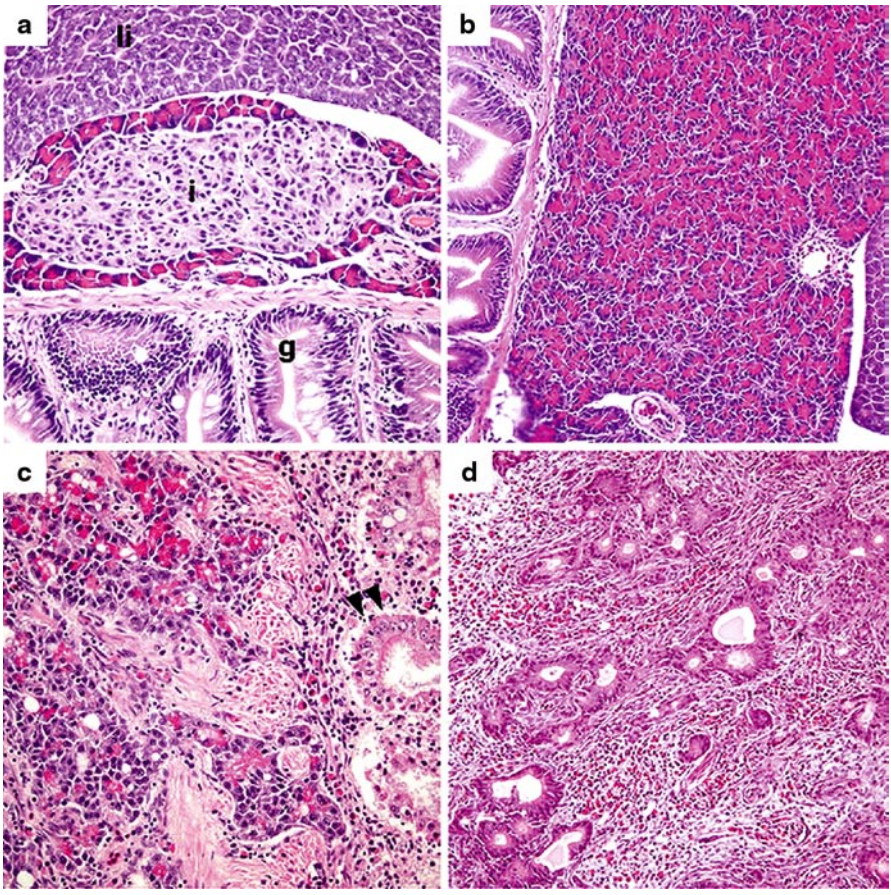

Fig. 5.6 Transgenic zebrafish expressing activated K-Ras develop pancreatic adenocarcinoma. pancreas. *li* liver, *i* islet, *g* gut. **b** acinar hyperplasia. **c** adenocarcinoma with mixed acinar and ductal differentiation. *Arrowheads* indicate base of intestinal crypt. **d** adenocarcinoma with ductal differentiation and desmoplastic reaction. Reprinted with permission from Park et al. 2008

Using target-selected screen (targeting-induced local lesions in genomes, TILLING) for an inactivating mutation in the tumor suppressor gene *adenomatous polyposis coli* (*apc*) from ethylnitrosourea (ENU)-mutagenized zebrafish, heterozygotes with the truncating mutation apc^{MCR} allele were recovered and analyzed for tumors. These mutant adult zebrafish were found to spontaneously develop intestinal and hepatic adenoma (Haramis et al. 2006). Treatment of the apc/+ zebrafish with the chemical carcinogen 7,12- dimethylbenz[a]anthracene (DMBA) induces pancreatic acinar adenoma and accelerates the formation of the intestinal and hepatic lesions.

Transgenic expression of oncogenic K-Ras and recovery of heterozygotes with the loss-of-function mutation in *apc* represents a complementary approach to generate zebrafish models of pancreatic cancer. These cancer models indicate conserved histopathology and genetic pathways in pancreatic cancer between zebrafish and human.

Continued efforts to generate pancreatic cancer models in zebrafish by transgenic expression of the other activated oncogenes and/or deletion of tumor suppressor genes, and by TILLING (Stemple, 2004), will expand the opportunities to further exploit these models to study the initiation and progression of pancreatic neoplasia.

5.5.2 Techniques in Genetically Engineered Zebrafish for Drug Discovery

Zebrafish expressing activated oncogenes or inactivated tumor suppressor genes are valuable tools for studying the cancer biology and genetics of pancreatic tumors. Application of chemical carcinogens to the genetically pre-disposed zebrafish that express transgenes under the control of pancreas-specific promoters (Park et al. 2008), or heat shock-inducible Cre/Lox-mediated recombination (Le et al. 2007), is predicted to expand the models for studying pancreatic cancer. Gene expression data obtained from the pancreatic cancer genome project (Jones et al. 2008) can be utilized to further exploit the transgenic zebrafish models by expressing the candidate genes under the control of exocrine pancreas-specific promoters. Application of chemical genetics to screen for modifiers of the pancreatic tumor phenotype is predicted to help identify potential carcinogens and drug candidates for prevention and treatment. Coupled with high-resolution imaging such as ultrasonography to monitor tumor growth, one can attempt to identify and validate compounds for preventing initiation of pancreatic neoplasia as well as progression of localized pancreatic tumor into invasive carcinoma. Integration of bioinformatics with the transgenic zebrafish models will help elucidate the molecular determinants for initiation and progression of pancreatic neoplasia. Moreover, selective targeting of biomarkers using nanoparticles to deliver toxic genes or chemicals in the transgenic zebrafish that develop pancreatic neoplasia at various stages will enable us to validate and maximize therapeutic efficacy in pancreatic cancer.

5.6 Transplants

5.6.1 Zebrafish Xenograft of Pancreatic Cancer as Models

Zebrafish xenograft models have been developed by microscopic transplantation of human cancer cells into the embryos, larvae, juveniles, and adults. Human metastatic melanoma cells have been injected into early zebrafish embryos, as reported by Lee et al. (2005), Topczewska et al. (2006), and Haldi et al. (2006). These studies support the xenograft model in zebrafish embryos to study tumor cell plasticity and the pathways involved as well as interaction between tumor cells and their microenvironment.

Larval and juvenile zebrafish have been employed for transplantation without rejection of the grafts. By injecting human tumor cells into the peri-vitelline space in zebrafish larvae expressing fluorescent reporter under vascular promoter at 48 hours post-fertilization, Nicole et al. (2007) showed induction of new blood vessels in the xenograft. Using dexamethasone-immunosuppressed 30-day-old zebrafish larvae genetically engineered to express fluorescence in blood vessels, Stoletov et al. (2007) microinjected fluorescently labeled tumor cells injected into the peritoneal cavity of the zebrafish, and dissected the interaction between the xenograft cells and the blood vessels.

Adult zebrafish xenografts have also been developed. Using heat shock procedure to generate homozygous diploid clonal zebrafish, Mizgireuv and Revskoy (2006) transplanted *N*-nitrosodiethyl-amine-induced tumor cells including pancreatic acinar cell carcinoma from one zebrafish into another. Alternatively, adult zebrafish have been irradiated to enable survival of transplanted melanoma cells from donor zebrafish (Patton et al. 2005).

We have microinjected fluorescently labeled human pancreatic adenocarcinoma cells (PANC-1) into the yolk sac of zebrafish larvae at 2 days post-fertilization (dpf). The larvae were incubated in embryo medium at 35°C, and the tumor growth was monitored under a stereo-dissecting microscope with fluorescent light (Fig. 5.7). We showed that the tumor cells remained as a mass at 5 dpf. Over the next 24 h, two smaller cell masses were observed, suggesting that the tumor cells have begun dissegregation. At 7 dpf, at least 3 discrete cell masses are evident, indicating further dispersion of the tumor cells. This demonstration supports the application of the zebrafish xenograft model to study the biology and genetic regulation of pancreatic

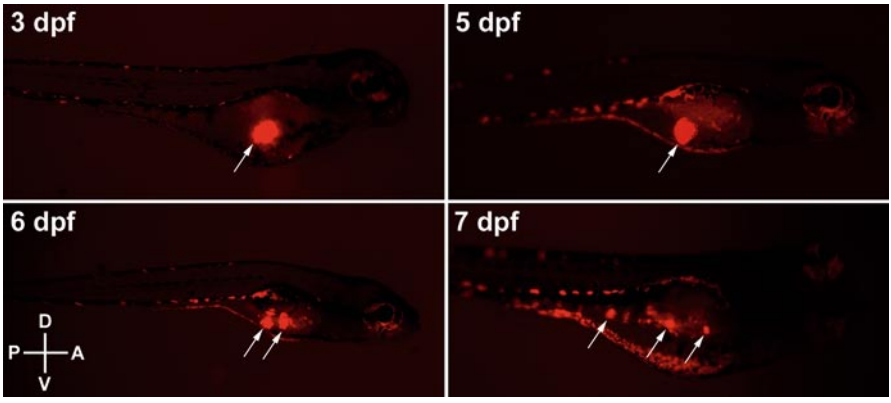

Fig. 5.7 Zebrafish xenograft of human pancreatic adenocarcinoma cells. Approximately 50 dil-CM labeled PANC-1 cells in 50 nl were micro-injected under a stereo-dissecting microscope (MZ16F, Leica) into the yolk sac of zebrafish larvae at 2 dpf, and the larvae were incubated in embryo medium (E3) at 35°C. Each larva was kept individually in a well of a 24-well tissue culture plate. The tumor xenograft in each zebrafish larva was examined daily under a stereo-dissecting microscope with fluorescent light emitting at 568 nm (MZ16F, Leica). Images of the xenograft in the individual larva were captured at the indicated time intervals. *D* dorsal, *V* ventral, *A* anterior, *P* posterior, *dpf* day-post-fertilization

tumors, using human pancreatic adenocarcinoma, either obtained from established cell lines, purified stem cells, or freshly resected surgical specimens.

Therefore, the zebrafish xenograft model allows visualization of tumor cells and monitoring of growth in a longitudinal manner in the same organism within a developmental context. These features provide unique advantages by reducing the number of cells injected and the number of organisms tested, and minimizing the variations among individual organisms. Moreover, direct examination of interaction between tumor cells and their stromal microenvironment, as well as the signaling pathways involved, will be facilitated.

5.6.2 Techniques in Zebrafish Xenograft Models for Drug Discovery

Application of chemical genetics to zebrafish with pancreatic cancer xenografts will provide complementary approaches to develop new drugs for its prevention and treatment. The tumor and the associated vasculatures may be modulated by adding chemicals in the zebrafish medium to inhibit the signaling pathways of carcinogenesis and angiogenesis, or injecting anti-sense morpholino oligomers to disrupt tumor and vascular growth in the zebrafish embryos. Zebrafish xenografts of pancreatic adenocarcinoma or the pre-malignant lesions can be utilized for screening and identifying chemicals as drug candidates either alone or in various combinations. Besides, using genetically modified cells by ectopic forced expression of genes or targeted disruption by RNA-mediated interference and adding signaling-specific chemical inhibitors, we can dissect the mechanism of drug actions in a living vertebrate organism. Therefore, studies using zebrafish xenograft models of human pancreatic cancer are expected to help understand the genetic mechanisms underlying tumorigenesis in a developmental context, identifying candidate therapeutic agents by high-throughput screens, and determining the mechanism of drug actions by chemical genetics.

5.7 Concluding Remarks

The combination of the zebrafish models and the various new techniques is expected to expand the opportunities for developing pathway-based drug targets and organism-based screening and validating of therapeutic compounds. The wild-type zebrafish larvae, germ-line mutants, transgenics, and xenografts will enable identification of the genes and pathways that regulate exocrine pancreatic development and cancer. The application of chemical genetic screens is expected to enable full exploitation of the zebrafish models for identification of drugs that target the signaling networks in pancreatic cancer. The application of microscopic ultrasonography and potentially microscopic positron emission tomography (PET)/computed

tomography (CT) to characterize pancreatic tumors in live zebrafish in real time will facilitate monitoring of tumor size and metastases, as well as pre-clinical validation of therapeutic efficacy of compounds. Generation of transparent zebrafish coupled with fluorescent or enzyme-based reporters is expected to further enhance visual detection of pancreatic tumors, and enhance the sensitivity of tumor detection. Ultimately, an integrative approach to utilizing zebrafish models in combination with chemical genetics and micro-radiography, and application of bioinformatics data and nanoparticulate systems, is expected to lead to systematic drug discovery toward the goal of personalized and targeted treatment of pancreatic cancer.

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Chapter 6
Gene Expression Arrays in Pancreatic Cancer Drug Discovery Research

Charles Gawad

Abstract The development of gene expression arrays to simultaneously quantify the expression of thousands of genes has been a leap forward in our attempt to understand the biology of pancreatic cancer in a variety of contexts. When combined with supervised and unsupervised interrogations using complex mathematical algorithms, researchers have been able to unveil specific molecular features of subsets of tumors. This has subsequently led to a greater understanding of the heterogeneity of pancreatic cancer genesis, metastasis, and resistance to drug therapy. Further, these studies have provided lists of proteins that could potentially be targeted to modify these phenomena, as well as serve as biomarkers during the drug discovery process and translation to the clinic. This chapter focuses on approaches to the application of gene expression arrays that have been able to address these questions, with special consideration of how they can and are being used to uncover and develop new therapies for pancreatic cancer.

6.1 Introduction

Cancer research has seen a significant transition in the past ten years, as many investigators have moved from studying single proteins or pathways towards global integrative analyses using high-throughput technologies. This change has allowed researchers unprecedented freedom to survey the global biology of a sample before reducing their focus to specific areas of interest. cDNA microarray was one of the first tools developed that allowed for the efficient generation of the global gene expression profiles required to perform these types of studies.

Since the initial experiments, gene expression array technologies have found widespread interest. There was only one publication using the terms “microarray”

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and "gene expression" found in PubMed in 1995, while that number soared to 3794 in 2007. As with any new, powerful tool identified in cancer research, the question that immediately arose was how can the data generated and conclusions drawn be applied to better diagnose or treat cancer patients? However, as with all the new high-throughput technologies, deciding how to mine the thousands to hundreds of thousands of data points generated by the experiments to identify meaningful inferences has posed significant challenges. Nevertheless, through the collaboration of physicians, scientists, and mathematicians, novel insights are being drawn about the diverse underlying biology of human cancers that are applicable to clinical oncology.

The utility of microarray technologies to identify important areas of gene expression alteration has been facilitated by the Human Genome Project, which identified and sequenced the 20–25 thousand genes in the human genome (Lander et al. 2001). This body of data has allowed researchers to print entire genomes on a single microarray slide, as well as to predict functional characteristics of genes that are differentially expressed. Nevertheless, the knowledge created from these studies has largely remained in research laboratories. This chapter will focus on what insights into clinically-relevant pancreatic cancer biology we have been able to gain from gene expression array technologies. More importantly, we also hope to articulate how those findings can be safely and efficiently translated into clinical practice to provide more objective data for the clinical decision-making of oncologists when caring for pancreatic cancer patients, with a concentration on the discovery of new therapeutic regimens that are so desperately needed.

6.2 Gene Expression Profiling Using DNA Microarray

6.2.1 Gene Expression Microarray Platforms

Gene expression arrays using cDNAs spotted on microscope slides were the first platforms developed for simultaneously analyzing the expression of a large number of genes in yeast (Schena et al. 1995). The technology was applied shortly thereafter to look for global changes in gene expression in human cancers (DeRisi et al. 1996). Since that time, it has been used to study many biological processes in various model organisms, and scaled up to cover entire genomes. Furthermore, oligonucleotide arrays, which use shorter DNA sequences, have been developed by several companies, including Affymetrix and Agilent. They have gained popularity with claims of greater quality control, genome coverage, versatility, and reproducibility, although those assertions have not been substantiated experimentally (Yuen et al. 2002).

Whether cDNA or oligonucleotide arrays are used, the general approach is similar, as outlined in Fig. 6.1. The slides for cDNA microarray are created by printing 100–300 μm spots for each gene, which are taken from cDNA libraries. Total RNA

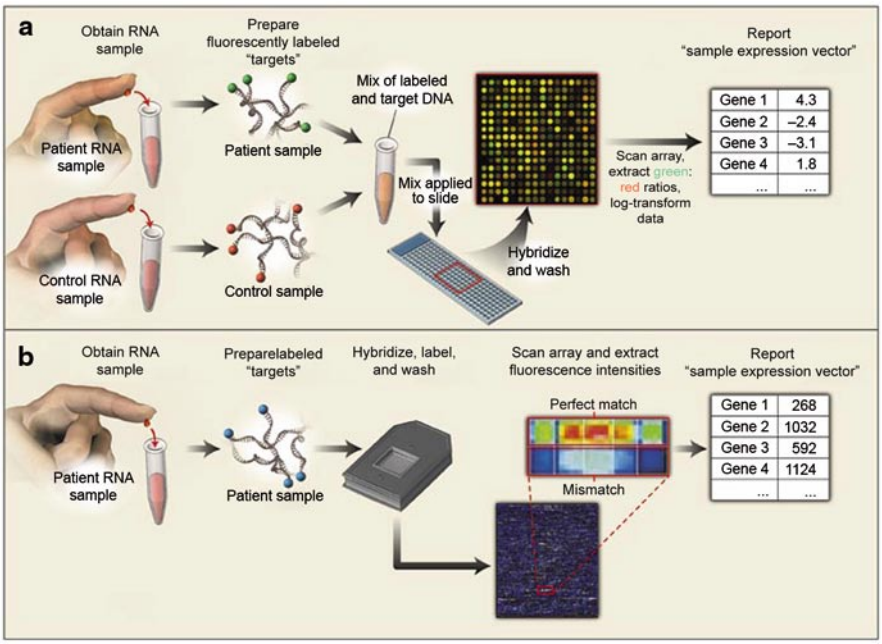

Fig. 6.1 An overview of DNA microarray analysis. **a** In a two-color analysis, RNA samples obtained from a test sample (patients) and a control subject are individually labeled with distinguishable fluorescent dyes and hybridized to a single DNA microarray consisting of individual gene-specific probes. Traditional spotted cDNA microarrays and Agilent's oligonucleotide DNA microarrays use this type of two-color platform. **b** In a single-color analysis, RNA from each biological sample is labeled and hybridized to a single array in which a series of gene-specific probes are arrayed. Affymetrix's GeneChip and Nimblegen's Gene Expression arrays use this platform. This figure was reproduced from Quackenbush (2006) with permission

is isolated from a tumor or cell line specimen using a number of approaches that involve homogenizing the sample, separation, isolation, and precipitation using an alcohol solution. The RNA is then simultaneously reverse transcribed and labeled. This is accomplished by incorporating Cy5 (red) and Cy3 (green) that are attached to nucleotides into their respective control and experimental samples. Those samples are then mixed and allowed to competitively hybridize to the cDNA already printed on the slides. The slide is then scanned for red and green wavelengths on each spot, which allows for the quantification of cDNA that hybridized to each spot from either the control or experimental samples. This allows for the quantification of the level of RNA or gene expression that was occurring in the sample at the time the RNA was extracted. There have been variations to these protocols, including the amp0lification of RNA from small samples, as well as labeling of cDNA with different molecules (Schulze and Downward 2001).

Oligonucleotide arrays are most commonly 25–60-mers synthesized in situ using one of two techniques. For Affymetrix arrays, the slides are constructed using photolithography on silicon wafers. Agilent arrays utilize ink-jet technology, and total

or mRNA is then isolated and reverse transcribed using the same techniques as cDNA microarray. For Affymetrix arrays, double-stranded cDNA is made, which is followed by in vitro transcription while simultaneously labeling by incorporating nucleotides attached to biotin. The sample is then hybridized to the slide, and detected using streptavidin (which binds biotin) attached to a fluorescent dye. The expression level of a specific gene can then be quantified using a scanner as outlined above. Affymetrix arrays only have single samples labeled, so controls must be hybridized to separate arrays. The Agilent samples are labeled, hybridized, scanned, and quantified in a similar manner to cDNA microarray. Further, this technology comes with the option of doing either one or two samples on a single slide by using only Cy5/Cy3, or both at the same time.

6.2.2 Technical Considerations

The first, and in many ways most important, step in producing global gene expression data is meticulously planning the methods to be used, and whether they are appropriate for the question being asked. The sensitivity of microarray technologies in detecting changes in gene expression requires compulsive attention to detail in order to generate reproducible data, as evidenced by wide interlaboratory variation in precision (Irizarry et al. 2005). Important considerations include choosing the best platform with an appropriate probe set for the question you are asking, identifying suitable controls with which to compare the samples, and insuring that the heterogeneity of the types of cells in a sample is consistent when using tissue (Churchill 2002). Tissue heterogeneity is an especially challenging issue when extracting RNA from pancreatic cancer specimens, as there is a variable amount of surrounding desmoplastic reaction. Once the data have been generated, tools need to be utilized to normalize the data, as well as assess its overall quality. These are complex undertakings that require thorough consideration in more detail elsewhere. In general, the background is subtracted, poor quality spots eliminated, and the global intensity between channels or arrays is made equal using one of a number of algorithms (Quackenbush 2002).

6.3 Application of Microarray Data in Drug Discovery for Pancreatic Cancer

Once overcoming the challenges of generating high quality, normalized data, an investigator can begin interrogating it with the help of powerful mathematical tools in an attempt to provide insight into previously inaccessible questions that are important to characterize pancreatic cancer biologists, as well as clinical oncologists and their patients. The general types of questions that can be formulated, which I will focus on in this chapter, include: (1) is there a way to understand and predict the underlying biology of known cancer classes, (2) based solely on biology,

can we "discover" new classes of cancer within histological tumor diagnoses, (3) is there a way to identify pathways or proteins that are involved in specific tumors, (4) can we identify an appropriate therapy or predict resistance to a prescribed regimen for a patient, (5) can gene expression profiles be used to screen drugs for specific cancers, and (6) can we monitor drug efficacy based on expression profiles? In the following pages I will summarize the approaches used in an attempt to accomplish these goals.

6.3.1 Reclassifying Cancer Types

One of the first papers to establish the value of cDNA microarray in cancer research showed that two general types of hypotheses can be evaluated with the data (Golub et al. 1999). The first strategy is called supervised interrogation, and works by breaking the samples into groups based upon some previously known characteristic, such as the presence of a mutation or patient outcome. The algorithms then describe the difference between the groups, and provide a list of genes that are most consistently different between them. The second method approaches the data in an unsupervised manner, whereby clustering algorithms are used to group samples based solely upon the similarity of gene expression patterns. These methods are used to take what are thought to be homogeneous groups, and then "discover" subsets within them that can be hypothesized to direct the biological difference between them. The investigator can then explore the differentially expressed genes within a specific subgroup.

6.3.2 Characterizing Known Cancer Classes

The mathematics required for interrogating microarray data with known characteristics have been adopted from the emerging field of artificial intelligence. Generally, these algorithms work by loading in a "training data set" with samples designated to a specific group, and then, through an iterative process, the genes that are most different between those groups are identified. The reproducibility of those differences is then confirmed using an independent experimental sample set. Two of the popular algorithms used for these experiments are support vector machines and artificial neural networks, but new approaches are continuously being developed (Brown et al. 2000; Ringner and Peterson 2003). Some groups that have been characterized using these techniques include those with different prognoses, genetic aberrations, stages, histological classifications, and metastatic potential, as summarized in Table 6.1.

Due to the homogeneity of known clinical and biological variables specific to pancreatic cancer, few studies have been done using supervised approaches for analyzing microarray data. Pancreatic cancer patients have an almost universally poor outcome, 85–90% are adenocarcinoma of the exocrine pancreas, greater than 90% of tumors harbor activating KRAS mutations, and other known molecular

Table 6.1 Summary of types of questions addressed using gene expression profiling combined with supervised interrogations

Type of question addressed	Example
Characterizing alterations between normal and malignant cells	Alizadeh et al. identified differences in gene expression between normal and malignant lymphocytes (Alizadeh et al. 1999)
Identifying the tissue of origin of a cancer	Khan et al. established the tissue of origin of small round blue cell tumors (Khan et al. 2001)
Discovering changes once a tumor has gained the ability to metastasize	Ramaswamy et al. identified distinct and universal changes in gene expression required for solid tumors to gain the ability to metastasize (Ramaswamy et al. 2003)
Differentiating tumors with different genetic aberrations	Various groups have characterized changes in gene expression that occur with activating mutations, tumor suppressor deletions, and gross cytogenetic abnormalities, e.g., gene expression differences between tumors with or without KIT mutations (Allander et al. 2001), between BRCA1 mutated tumors and BRCA2 mutated tumors in breast cancer (Hedenfalk et al. 2001), and between neuroblastoma cells with or without 1p36 deletion (Janoueix-Lerosey et al. 2004)
Identifying downstream transcriptional targets of characteristic genetic changes in a cancer	Khan et al. discovered targets of the PAX3-FKHR fusion protein, which is the result of a translocation commonly seen in alveolar rhabdomyosarcoma (Khan et al. 1999)
Prognostic signatures	Several groups have established potential prognostic signatures for central nervous system embryonal (Pomeroy et al. 2002), breast (van de Vijver et al. 2002), and prostate cancers (Singh et al. 2002)
Staging tumors	Xu et al. demonstrated changes that occur as normal epithelium progresses to Barrett's esophagus and esophageal cancer (Xu et al. 2002)
Characterizing the role of nonmalignant cells within a tumor	Dave et al. identified a signature associated with prognosis of follicular lymphoma, which was followed by sorting out the benign from malignant cells. They concluded that the presumably benign tumor-infiltrating immune cells were responsible for the favorable prognosis signature (Dave et al. 2004)

alterations only occur in a small subset of patients. Familial pancreatic cancer does account for about 10% of cases, but the gene with the largest known contribution, *BRCA2*, is mutated in 20% of families, which account for only about 2% of all pancreatic cancer cases (Hahn et al. 2003). Nevertheless, some studies have been able to differentiate benign inflammatory tumors of the pancreas from adenocarcinoma (Buchholz et al. 2005). Another group compared chronic pancreatitis that resembled adenocarcinoma histologically, and identified genes that discriminated between the two (Logsdon et al. 2003). One other set of researchers found genes associated with

liver/lymph node metastasis, as well as prognosis. This study also overcame the heterogeneity of pancreatic cancer tumors by microdissecting out the malignant cell and amplifying the isolated RNA (Nakamura et al. 2004). Hopefully, ongoing studies will continue to elucidate discriminating features of subpopulations of patients with pancreatic cancer so that more of these types of studies can be undertaken.

6.3.3 Identifying New Classes

The other strategy to explore microarray data is to apply one of a number of unsupervised clustering algorithms to discover groups with similar gene expression patterns (Shannon et al. 2003). This approach is useful when there are not known characteristics that can further subdivide a tumor type, as is the case with pancreatic cancer. This method then presumably subcategorizes different cancers based solely on the underlying biology of the tumors. The resulting subclasses can then be explored to create hypotheses about distinct phenotypic characteristics that represent those differences, such as clinical response to therapy, or the ability to invade and metastasize. To establish a causal relationship between the gene expression pattern and the observed phenotypic differences, verification must be done with independent approaches. This strategy has also been used to further subclassify tumors with differentiating characteristics, such as the presence of a genetic aberration, or distinct histological features. Regardless of the approach, these experiments provide a list of potentially important genes that have altered expression that is unique to each cancer "class" (Table 6.2).

Several cancer research groups have undertaken unsupervised approaches to evaluate microarray data produced from pancreas tumors (Crnogorac-Jurcevic et al. 2001; Han et al. 2002; Grutzmann et al. 2003; Iacobuzio-Donahue et al. 2003a, b). One study characterized intraductal papillary-mucinous tumors, and found with hierarchical clustering that the tumors with invasive features grouped together while their noninvasive counterparts formed their own cluster (Terris et al. 2002). Others produced oligonucleotide data, and compared their results to published serial analysis of gene expression (SAGE) libraries as a means to exclude genes already known to be expressed in normal pancreas (Iacobuzio-Donahue et al. 2003a, b). Still another approach subtracted the genes differentially expressed in tumor cell lines and normal pancreas from pancreatitis and adenocarcinoma specimens in an attempt to identify stromal genes with altered expression (Binkley et al. 2004). These studies have provided lists of differentially expressed genes that provide a snapshot of different facets of pancreatic cancer biology.

6.3.4 Expression Signatures of Cancer Subclasses

Both supervised and unsupervised approaches to analyzing microarray data have been important in defining alterations in gene expression associated with important characteristics of distinct populations of pancreatic malignancies. However,

Table 6.2 Summary of types of questions addressed using gene expression profiling combined with unsupervised interrogations

Type of study	Example
Subclassification of tumor type	This has been done for all major tumor types, including breast, lung, prostate, pancreas, and colon cancers (Alon et al. 1999; Xu et al. 2000; Han et al. 2002; Heighway et al. 2002; Sotiriou et al. 2003)
Identification of a subgroup of cancer with specific biological properties	Bittner et al. used hierarchical clustering to identify two distinct groups of melanoma samples. They then hypothesized and later confirmed that one group had a greater ability to invade and metastasize (Bittner et al. 2000). They also characterized *Wnt5a*, one of the genes overexpressed in the invasive group, as an important regulator of the phenotype (Weeraratna et al. 2002)
Confirmation and characterization of biological difference between unique histological tumor types	Higgins et al. found that renal cell carcinoma with clear cytoplasm had a distinct and homogenous gene expression patterns while those with granular cytoplasm were more heterogeneous (Higgins et al. 2003)
Identification of subgroups within tumors that harbor specific genetic alterations	Sorlie et al. have found that breast cancers with amplified ERBB2 or present estrogen receptor have unique expression profiles. They also suggested that there are at least two subclasses of ER+ tumors (Sorlie et al. 2001)
Subclassification of unique histological groups of cancer	Bertucci et al. have shown that there are distinct subgroups of inflammatory breast cancer (Bertucci et al. 2005). Others showed that non-BRCA familial breast cancer can be further subclassified (Hedenfalk et al. 2003)

how to apply those lists of genes to produce new diagnostic tools and treatments for cancer patients has remained a significant challenge. One approach has been to discover a subset of the most differentially expressed genes that can serve as a gene expression "signature" for that subclass. Identification of signatures associated with these groups could prove to be an important adjunct to established pathological interrogations that oncologists can use to provide their patients with more information about their specific tumor.

Many of the classes defined in the types of experiments outlined in Table 6.2 had expression signatures created to identify tumors that belong to that specific class. One potential application has been to identify the origin of a tumor that is difficult to diagnose by histology. Specifically, Bloom and colleagues used this approach with both oligonucleotide and cDNA microarray data from 463 metastatic adenocarcinomas that represented 21 cancer types, including pancreatic cancer, and were able to identify signatures using artificial neural networks that could recognize the tissue of origin of the cancer with about 85% accuracy. This could be an important tool for diagnosing the tissue of origin of the somewhat common presentation of a

metastatic adenocarcinoma of unknown origin (Bloom et al. 2004). The ability to correctly diagnose these patients would be an invaluable tool in guiding their treatment. Another group was able to identify a gene expression signature associated with prognosis in breast cancer (van de Vijver et al. 2002). Specific to pancreatic cancer, one group did a meta-analysis of the published gene expression studies, and found that they could create a classifier that could accurately recognize normal pancreas, chronic pancreatitis, and adenocarcinoma (Grutzmann et al. 2005).

The ability to distinguish cancer subclasses through the use of gene expression signatures that can classify unknown samples has been an important application of cDNA microarray data. However, most of the studies listed above were done on banked tumor specimens, and were thus retrospective. Recently, several groups have done prospective trials in an attempt to establish the clinical utility of these signatures. The only platform that the FDA has allowed to be commercially available in the US is Mammaprint, which provides prognostic information about a breast cancer patient's tumor. A recent trial with this technology showed prognostic discordance between the expected clinical outcome based histologic features with the signature. The authors suggested that the Mammaprint could identify patients that are mislabeled as having a poor prognosis, and thus protect them from aggressive chemotherapy regimens (Bueno-de-Mesquita et al. 2007). However, larger, more long-term studies are needed to verify these findings. A similar feasibility study also identified signatures that could prospectively identify lung cancer patients with poor prognoses. The signature showed even greater efficacy when combined with patient data, suggesting it could serve as an adjuvant prognostic indicator for patients with adenocarcinoma of the lung (Shedden et al. 2008). Lastly, there was also a prospective study done that was able to classify childhood leukemia samples with a 50-gene signature as either AML or ALL, and suggested diagnoses of AML for three patients who were unable to be classified by traditional techniques (van Delft et al. 2005). Although gene expression signatures have begun to show clinical utility in correctly diagnosing patients, as well as providing prognostic information, these approaches have not provided new treatments aimed at improving their survival. Nevertheless, they are likely to become more widely available in clinical oncology as the systems become validated.

6.3.5 From Lists of Differentially Expressed Genes to Drug Targets

The unprecedented success of imatinib (Gleevec) to treat CML catalyzed a profound transition in approaches to cancer drug discovery. Researchers have turned their focus from simply discovering drugs with anti-neoplastic activity to first identifying protein targets required for the growth of a tumor, such as BCR-Abl for CML. The hope has been that drugs could then be developed to inhibit the identified target that would exhibit the same level of anti-cancer activity as imatinib. The overexpressed genes identified in microarray experiments are all potential candidate targets for the treatment of that cancer. Further, the subclassification of tumors may facilitate the

discovery of proteins requisite for the survival of previously unrecognized cancer subclasses. Those who do studies under the assumption that all the tumors of the same histological class have uniform underlying biology may currently overlook these potential targets.

The first step in attempting to identify overexpressed genes that may be druggable cancer targets from a list generated by microarray analysis is accurate annotation. This process has been facilitated by the creation of common nomenclature by the HUGO Gene Nomenclature Committee (Wain et al. 2004). The standardization of gene names and symbols has made expression data easier to be analyzed between platforms, laboratories, and databases.

The Gene Ontology Consortium and other groups have also attached the known function of genes to their title in openly accessible databases (Ashburner et al. 2000). This process has helped identify the types of genes that are enriched in a gene expression dataset, and thus draw conclusions about which functional processes have altered activity in that group. One example of this is a study that characterized the changes in ontology classes that occurred in women after being treated with the EGFR inhibitor erlotinib based on gene expression data (Yang et al. 2005). The classes of dysregulated cellular processes that are identified in these studies could provide rationales for drug selection when choosing adjuvant or second-line therapies.

Another approach used to identify areas of signal transduction enriched with differentially expressed genes is the mapping of those genes onto pathway diagrams. The number of expected genes to change in each pathway by chance can then be compared to the observed number, and statistics can be computed to identify pathways that have the greatest proportion of genes with changes in expression. In our laboratory, for example, we have found a number of pathways enriched in our oligonucleotide array dataset of 22 pancreas tumors using the commercially available GeneGO software (unpublished data). One shortcoming of this approach is that it relies on the assumption that the chosen relationships of proteins in pathways being interrogated are true in the malignancy a researcher is studying. However, the technology for characterizing protein–protein interactions on the proteome scale, termed interactomes, has continued to improve (Gandhi et al. 2006). Thus, it is likely that pathway analyses will become more useful as the accuracy of the diagrams improves and the mathematics involved in identifying activated pathways mature (Ekins et al. 2007). The utility of this approach, if confirmed, would likely accelerate drug discovery, as small molecule inhibitors of many of the major pathways have already been identified.

Still, how to go from lists of differentially expressed genes, whether through function, pathway association, or consistency within the data, to the identification of a druggable target has currently proven to be an impassable bottleneck in the translation of microarray data to new treatments for cancer patients. Drugs have been developed for tumors with the mutation, as well as amplification of genes, as evidenced by the efficacy of imatinib in patients with gastrointestinal tumors harboring activating KIT mutation, as well as herceptin for breast cancers with amplified HER2. It is unclear if the overexpression of a gene has a strong enough biological influence over the survival of a tumor to be an efficacious target. Yet, recent studies have shown that the overexpression of as few as two genes can repro-

gram a differentiated cell back into a more pluripotent state, indicating gene expression levels can have significant influence on cellular phenotype (Kim et al. 2008). Still, whether the overexpression of genes has the same influence over genetically unstable cancer cells in human patients is unknown.

Nevertheless, there has been an abundance of studies showing that potential drug targets can be identified from many microarray datasets for most cancer types. The general approaches taken have been to first confirm the overexpression at the mRNA and proteins levels using qRT-PCR or northern blot analysis and western blotting, respectively. Some have also looked at patient samples using immunohistochemistry, which can also identify the location within the cell, as well as the part of the tumor the protein is being expressed in. These studies are followed by characterization of the function of the protein in that cancer through a combination of biological and/or pharmacological experiments. Inhibitors of the protein or its known downstream signals, if available, provide investigators with drugs that could potentially be readily translatable. The discovery of the ability of dsRNA to induce potent and specific suppression of gene expression led to the development of synthetically synthesized siRNA, which is commercially available to decrease the expression of potential targets identified in microarray experiments (Fire et al. 1998; Elbashir et al. 2001). siRNA technologies will be discussed in detail in Chap. 8. A complementary strategy is to overexpress the gene by transfecting a plasmid containing the gene, which should show the opposite effect of siRNA suppression. If potential targets are confirmed, monoclonal antibodies can be made or small molecules screened to identify drugs that could potentially be applied in the clinic. Furthermore, siRNA itself may prove to be a clinically-useful drug if vectors are developed to accurately and efficiently transfect it into tumors (Dykxhoorn and Lieberman 2006).

Using these approaches, pancreatic cancer researchers have detected several potential drug targets. One group found that P-cadherin may play an important role in metastasis, which is especially important in pancreatic cancer where more than half of patients have metastatic disease on presentation (Taniuchi et al. 2005). A similar study found that CD74 could be an important regulator of perineural invasion, which is associated with the local recurrence of pancreatic cancer (Koide et al. 2006). Several studies have also discovered proteins important for pancreatic cancer growth, which also make them logical drug targets. The proteins identified to date include GABRP, EphA4, FXYD3, and Plk1 (Gray et al. 2004; Iiizumi et al. 2006; Kayed et al. 2006; Takehara et al. 2007). Although early studies have shown some promise in targeting overexpressed genes identified by microarray analyses, the ability to bring these targets through preclinical models, and ultimately into clinical trials, has yet to be established.

6.3.6 Gene Expression Signatures as a Drug Discovery Tool

Although identifying single differentially expressed genes as drug targets may provide new cancer therapeutics for specific tumors, another approach that has shown greater early promise is the use of tumor signatures to do gene expression-based

high-throughput drug screening. The general strategy is to first identify a robust gene expression signature that represent a specific tumor class. Drugs are then screened using other technologies that can be more easily scaled up in an attempt to identify drugs that reverse the signature. The initial study used mass spectroscopy to confirm drugs found to reverse signatures when screened in AML cell lines. The compounds that were found as hits were then screened for their ability to differentiate AML cells (Stegmaier et al. 2004). One compound found in the group was DAPH1 (4,5-dianilinophthalimide), an EGFR inhibitor. Gefitinib, an FDA-approved EGFR inhibitor, showed similar results, and a clinical trial is currently under development (Stegmaier et al. 2005). The same group has performed similar studies in different tumor types that have incorporated in silico data to identify pathways involved in the reversal of gene expression, looked at combination treatments, and developed alternative confirmatory tests, as mass spectroscopy could be cost-prohibitive when trying to scale up to test larger drug libraries (Hieronymus et al. 2006; Peck et al. 2006; Stegmaier et al. 2007; Antipova et al. 2008; Hahn et al. 2008). Yet, whether the drugs identified will show clinical efficacy remains to be seen. There have been no published reports using this approach to identify small molecules that show efficacy in pancreatic cancer.

6.4 Other Potential Applications of DNA Microarray

6.4.1 Biomarkers

Although the focus of this book is the process of discovering novel pancreatic cancer treatments, characterization of genes or proteins confirmed to be consistently differentially expressed in tumor classes may allow for the identification of new biomarkers, which could then potentially expedite the drug development process. These markers could serve a variety of functions, from providing oncologists and their patients with prognostic information, to allowing for the use of surrogate endpoints in clinical trials. The latter would be especially important in accelerating the rate of drug development, as efficacy could be measured throughout the treatment rather than waiting to evaluate patient survival or recurrence. Surrogate endpoints could also mitigate some of the enormous costs associated with performing clinical trials, as those showing little efficacy could be stopped earlier, and patients could be diverted to newer, more promising drugs. It is also possible that gene expression signatures could serve as surrogate endpoints themselves.

Several potential biomarkers have been identified for pancreatic cancer through the use of microarray studies. The possible targets shown to be overexpressed in pancreatic cancer listed in Sect. 6.3.5 could serve as biomarkers in addition to points of therapeutic intervention. Further, there are other genes with biological function that are unknown or not targetable, but have altered expression, and have been identified as potential biomarkers. One study found that the sigma subunit of the adaptor protein 14-3-3 was overexpressed in intraductal papillary-mucinous

tumors, as well as in invasive ductal carcinomas (Okada et al. 2006). Another group identified increased nuclear staining of the protein S100A6 in a subset of pancreatic cancer patients, which was associated with poor survival (Vimalachandran et al. 2005). Still others discovered that neutrophil gelatinase-associated lipocalin is highly expressed in premalignant lesions of the pancreas, which is lost as the cells dedifferentiate into a more invasive phenotype. Further, they showed that serum levels could differentiate patients with a normal pancreas from those with an underlying pancreatic neoplasia. The authors suggest that it is a potential biomarker for early pancreatic cancer detection, which encompasses the period of time in which cases amenable to long-term survival are found (Moniaux et al. 2008). Lastly, a group showed that biomarkers could be characterized through microarray studies performed on samples obtained with endoscopic ultrasound-guided fine needle aspiration. This approach could minimize the invasiveness required to obtain a tissue sample (Laurell et al. 2006).

Although these limited studies have shown promise in identifying biomarkers that could potentially serve as surrogate endpoints for drug efficacy, it must be emphasized that they have not been tested prospectively in a clinical setting. The only serum biomarker approved for pancreatic cancer, CA 19-9, was recently shown to be a poor indicator of response to chemotherapy (Hess et al. 2008). Whether the biomarkers discovered through microarray studies will have greater clinical utility remains to be seen.

As previously noted, Mammaprint, a gene expression signature for breast cancer prognosis, is being used to provide information for clinicians and their patients (Lacal 2007). Unfortunately, there have not been gene expression profiles associated with pancreatic cancer that have been found to provide useful, reproducible information for oncologists and their patients. Nevertheless, biomarkers, whether they are single proteins or a larger signature, are likely to be more widely incorporated into clinical oncology, both to provide useful clinical information to individuals, as well as expedite the drug discovery process.

6.4.2 Drug Resistance

This book concentrates on describing technologies for identifying novel therapeutic regimens for patients with pancreatic cancer. However, the same technologies have also been employed in an attempt to better utilize the drugs we already have available. One of the earliest microarray studies in human cancer cells attempted to identify expression profiles associated with cells sensitive and resistant to the widely used chemotherapy drug doxorubicin (Kudoh et al. 2000). These strategies have also been employed to try and understand methods of resistance for some of the most common cancer treatments, such as androgens for prostate cancer, and cisplatin for ovarian cancer (Amler et al. 2000). Others have looked at genes that are induced by common chemotherapy agents, like 5FU, that could serve as targets to prevent drug resistance (Maxwell et al. 2003). Still another group evaluated

patients' samples for signatures associated with early relapse of standard chemotherapy regimens (Hartmann et al. 2005). Lastly, molecular mechanisms associated with resistance to newer targeted agents, like imatinib, have also been explored using gene expression array studies (Hofmann et al. 2002).

For pancreatic cancer patients, as well as gastrointestinal oncologists, understanding the robust mechanisms underlying the almost uniform resistance of pancreatic cancer cells to conventional therapies remains one of the most important questions to be answered. Investigators have looked at gene expression profiles associated with gemcitabine, which has become the cornerstone of most treatment strategies for pancreatic cancer (Nakai et al. 2005; Bai et al. 2007). Others have looked at the lists of differentially expressed genes in those studies, and found ribonucleotide reductase M1 subunit and selenoprotein P overexpression to be associated with gemcitabine resistance (Maehara et al. 2004; Nakahira et al. 2007). Another group found that decreased expression of BNIP3 was also associated with gemcitabine resistance (Akada et al. 2005). Still another study was able to extrapolate that inhibition of the anti-apoptotic pathway of p8 was involved in the cytotoxicity of gemcitabine in pancreatic cancer cells (Giroux et al. 2006). Lastly, others have found, by comparing global gene expression of radiosensitive to resistant pancreatic cancer cells, that decreased expression of caveolin-1 is associated with radiation sensitization (Cordes et al. 2007). If gene expression profiles are unable to identify new, more efficacious therapies for pancreatic cancer, it is still possible they will provide a means to better utilize those we already have available by elucidating the mechanisms of resistance.

6.4.3 Incorporating Gene Expression Arrays with Model Organisms

The completion and comparison of the yeast, mouse, and human genomes, among others, has allowed for a more seamless translation of discoveries from one organism to another through greater precision when performing comparative genomics studies (Johnston et al. 1994; Adams et al. 2000; Waterston et al. 2002). Although there is an entire other chapter of the book devoted to models of human pancreatic cancer, there are a few areas to mention that exemplify the power of incorporating gene expression profiling into these studies. One of the early experiments incorporated xenograft models of several cancers in an attempt to identify gene expression profiles that were associated with sensitivity to common chemotherapeutic drugs (Zembutsu et al. 2002). Others have looked at specific biological phenomena, such as metastasis in breast cancer, to better characterize the molecular events that occur during these processes, which could reveal new drug targets (Minn et al. 2005). By using animal models rather than cell lines, alterations that are produced by the incorporation of stromal and other signaling elements may provide a more accurate molecular portrait of what goes on in vivo, in actual cancer patients.

The ability to look at global gene expression profiles in mouse models has also enhanced our ability to understand basic questions about the biology of pancreatic cancer. One recent study was able to identify a gene expression signature associated with intraductal papillary mucinous neoplasms produced in mice that have mutant, constitutively active KRAS and overexpress TGFα (Siveke et al. 2007). Still another group found heterogeneity of gene expression depending on the zone within the tumor, as well as the microenvironment created by surrounding tissues (Nakamura et al. 2007a, b). The identification of the molecular cascade underlying the propensity of pancreatic cancer to metastasize early has also been an elusive question that has been interrogated with gene expression profiles in mouse models. *MSX2* and *CCN1* are two genes that have been shown to be upregulated in pancreatic cancer metastases in mice (Holloway et al. 2005; Satoh et al. 2008). Lastly, researchers looked at the response of pancreatic cancer mouse models to experimental therapies. One study found specific alterations in gene expression after treatment of mice with orthotopic pancreatic cancer xenografts with anti-VEGF antibodies (Bockhorn et al. 2003). The mechanism of the potential efficacy of treating pancreatic cancer with cyclopamine, an inhibitor of the sonic hedgehog pathway, was evaluated after treating Pdx1-Cre; LsL-Kras (G12D); Ink4a/Arf (lox/lox) transgenic mice. Several potential downstream transcriptional targets were identified using microarray studies (Feldmann et al. 2008). It is likely that the incorporation of microarray studies into mouse models will accelerate our ability to understand the underlying biology of pancreatic cancer, which will in turn facilitate the drug discovery process.

6.4.4 Integrative High-Throughput Analyses

Although gene expression profiles were the first efficient method for generating reliable, global datasets, other types of high-throughput technologies are continuously being developed. Unfortunately, proteins are more complex, which has made working with them in high-throughput platforms more technologically challenging, a topic addressed in another chapter. One approach to look at protein expression on a large scale was accomplished through the invention of the tissue microarray. This platform allows an investigator to simultaneously perform immunostaining of hundreds of samples on a single slide. It is a valuable tool for simultaneously confirming the altered expression of genes found in microarray experiments at the protein level in many samples at once (Moch et al. 1999). Another technology that has been developed to identify global changes in gene copy numbers using oligonucleotides printed in a manner similar to gene expression arrays is comparative genomic hybridization (CGH) arrays (Barrett et al. 2004). Still other technologies have been developed to look at global variations in DNA methylation patterns, DNA binding sites, and single nucleotide polymorphisms (Adorjan et al. 2002; Kondo et al. 2004; Gunderson et al. 2005). Lastly, high-throughput methods are being developed to identify mutations throughout the genome, such as the use of nonsense-mediated microarray to identify genes with mutations leading to premature stop codons, as

well as more streamlined sequencing approaches using beads with unique identifiers (Huusko et al. 2004; Craig et al. 2008). Although each of these methods, and others, has provided a unique vantage point from which a biological sample can be evaluated, some have found ways to integrate their use to expand the breadth of studies and strengthen confidence in the results obtained.

Two technologies that could potentially complement each other by identifying alterations in gene regulation and DNA amplification or deletion in the same sample have been performed using a combination of expression and CGH oligonucleotide arrays. One approach showed global alterations that occurred in pancreatic cancer, which provides a way to see which expression changes occur in concert with genomic alterations (Mahlamaki et al. 2004). Another study used both genomics and proteomics approaches to identify *EEF1A2* and *KCIP-1* as potential oncogenes in adenocarcinoma of the lung (Li et al. 2006). Other researchers have also been able to better characterize the role of *MYC*, an important oncogene in pancreatic cancer, through a combination of chromatin immunoprecipitation and expression microarrays (Wu et al. 2008). One of the most comprehensive studies undertaken to date in pancreatic cancer used a combination of SNP arrays and serial analysis of gene expression. They found an average of 63 genomic alterations in each cancer, and identified novel signaling pathways important in pancreatic cancer by correlating the mutation data with alterations in gene expression (Jones et al. 2008). Although strategies for combining high-throughput technologies to identify areas of cancer biology that would be most amenable to therapeutic intervention are still being explored, it is likely that the utility of these methods will be further revealed in the coming years.

6.5 Concluding Remarks

It is clear that the discovery and development of gene expression arrays over the past fifteen years has been a significant advancement for the entire field of cancer biology. We now have the ability to acquire snapshots into the dynamic alterations that occur during cancer development, progression, and therapy. This will hopefully lead to more robust methods for cancer prevention, detection, and treatment. Furthermore, the demonstration of the power of high-throughput technologies through the development of gene expression arrays has spawned the creation of numerous complementary technologies that provide important information about other areas of cancer biology.

Still, where do all these recent advancements leave those desperately in need of new tools to treat their pancreatic cancer today, where the long-term survival remains abysmal? Significant challenges remain if we are to harness the power of this information acquired at the bench and translate its meaning so that real clinical benefit can be achieved. Further, there are technical limitations that need to be addressed if these methods are to be used on human beings, so that sufficient sensitivity, specificity, and reproducibility can be attained.

Nevertheless, it is imaginable that in the not-so-distant future, those diagnosed with pancreatic cancer can be taken for a biopsy to determine the subtype and prognosis of the disease. Informed decisions can then about made about whether to institute neoadjuvant therapy. Following surgery, therapy choices can be made based on drugs shown to target alterations identified in that type of cancer with a specific the gene expression profile. If a drug is not available, siRNA specific to a target previously identified in that group could be substituted. The patient could then be monitored for recurrence based on a surrogate endpoint, and therapy choices could be adjusted based upon what is known about how resistance to the drugs being used develops in that specific cancer. Pancreatic cancer could become a chronic condition rather than the efficient killer it is today. With purposeful and perseverant innovation and development, this vision of how oncology is practiced will be realized.

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Chapter 7
Using Array Comparative Genomic Hybridization of Pancreatic Cancer Samples to Map Interesting Regions for Target Gene Identification

Chunlao Tang and Robert Lucito

Abstract Pancreatic cancer is one of the deadliest cancers. As with all cancers, the causes can be identified in the genome. Many methods have been developed to identify the changes that occur in the cancer genome, culminating in the development of array comparative genomic hybridization (aCGH). Many different formats have been applied to the analysis of pancreatic cancer cell lines, xenografts, and primary tumors by many different groups. We present a table listing all studies of pancreatic adenocarcinoma utilizing aCGH. We discuss each platform and the advantages and disadvantages, helping readers to choose which data generated on which platform they wish to study. We then go on to summarize the findings by compiling the data for all publically available array data and calculate the minimal common regions altered. We also present a frequency plot for the greater dataset compiled to show the overall genomic profile of the pancreatic cancer genome. Mapping of regions altered in the cancer genome has helped in the identification of cancer genes, and the analysis of array CGH data from pancreatic cancer samples may yield new targets for therapeutic development.

7.1 Introduction

Although pancreatic cancer affects less than 40,000 people in the United States per year, almost all of these patients will die. The 5-year survival rate for pancreatic cancer is one of the lowest of all cancers, less than 5%. In fact the median survival time is only 7 months after diagnosis. Even with a fairly low rate of incidence it should not be surprising that pancreatic cancer is the fourth leading cause of cancer death

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in the United States. The treatments have not changed drastically and remain surgery and/or chemotherapy, but these are not very effective. They do little to extend survival or alleviate pain and do not cure the patient. What makes pancreatic cancer so deadly? Is it the fact that the tumors are diagnosed so late and that the tumor has already metastasized, or is there something deeper at the molecular level?

7.2 Alteration of the Genome Is Central to the Causation of All Cancer

Alterations of the genome are causal to the development and progression of pancreatic cancer, as well as all other cancers. These alterations cause downstream changes in the transcriptome, which ultimately result in the various phenotypic changes that a tumor cell undergoes. Looking at expression data alone to identify the critical alterations in cancer is difficult, since altering one critical regulatory gene can cause a cascade of secondary effects. The primary mutations can be uncovered by identifying the alterations in the genome. The inclusive genes or the associated pathways will likely be ideal targets for the development of therapeutic modalities. Cancer-specific genetic alterations range from point mutations to larger mutations such as translocations, deletions, and amplifications. Cytogenetic techniques were used first to identify differences between normal and tumor samples. While this was considerably useful in identifying translocations, however it required expertise that was not easily obtained, and, more importantly, translocations are not necessarily the driving force for many cancers. Although point mutations are extremely important, it is not yet cost-effective (even with new sequencing technologies) to sequence to completion the whole genomes of many cancer samples. On the other hand, amplifications and deletions are very informative and can be easily identified. Cytogenetics was also used to identify amplifications: homogeneously staining regions (HSRs) and double minutes (DMs). More refined analysis of amplifications and deletions along the genome was difficult.

A refinement born out of cytogenetics was the development of a method that allowed the quantitative genome-wide analysis of copy number. This method compared two genomes by hybridization, thus was termed comparative genomic hybridization or CGH, and allowed the genome-wide identification of amplifications and deletions. Briefly, cells are chemically stopped at metaphase and then spread on a microscope slide. Total genome of tumor and a normal reference sample are differentially labeled with nucleotide tagged with different fluorescent dyes and hybridized to the metaphase chromosomes. After hybridization and washing, the signal is scanned. Generally these methods remain the same for all CGH methods. The signal is determined across the chromosomes and compared for both samples, and compared to identify the regions with differential signal, indicating amplifications or deletions in tumor. The resolution of this method is approximately 10 Mb, which sounds very low nowadays, but at that time the ability to survey the entire cancer genome was a huge leap in cancer genomics. This method was used by many groups

to analyze a number of different tumor types (Kim et al. 1995; Levin et al. 1995; Visakorpi et al. 1995; Voorter et al. 1995) including pancreatic cancer (Mahlamaki et al. 1997; Schleger et al. 2000). However the lack of resolution hampered the identification of cancer-specific genes and the hopes that gene-specific treatments could be developed. A finer genomic resolution would be required to achieve this.

7.3 Development of Array Comparative Genomics Hybridization and Platform Comparison

With the development of the Human Genome Project, the CGH platform evolved from a microscope slide with metaphase spread chromosomes to a microscope slide with an array of DNA molecules with defined coordinates to the genome allowing the fine mapping of cancer-specific lesions. There have been several platforms and each has strengths and weaknesses. The more informative array CGH papers studying pancreatic cancer (predominantly epithelial pancreatic adenocarcinoma) are listed in Table 7.1 with details of the platform and the number of samples and types of samples analyzed. The platforms used will be reviewed in detail to allow the reader to decide which data from what studies they would like to use for future studies.

The first platform to be developed took advantage of a human genome bacterial artificial chromosomes (BACs) library being used by the Human Genome Project for genome sequencing (Pinkel et al. 1998). In this case, the BACs themselves were used as the DNA molecules to attach to the slide, and the total genome of tumor and normal samples was labeled and hybridized. The advantages of the BACs are that they are large and very good hybridization kinetics could be obtained provided repetitive DNA was used in the hybridization to block the many repeats in the genome. The ratio is averaged over the large size of the BAC and most sequence-specific labeling aberrations are reduced or removed. In addition, due to the averaging over the large size of the BAC, the copy numbers estimated are very accurate. Since the BACs' genome coordinates were known, regions identified as altered by amplification or deletion could be easily mapped to the genome. This methodology has been used to analyze many cancers (Weiss et al. 2004; Cho et al. 2005; Rubio-Moscardo et al. 2005) including pancreatic (Nowak et al. 2005). A disadvantage of this method is in the use of the BACs themselves. Many of them are not very useful as hybridization probes and careful selection of the BACs was required, limiting the resolution. Although several studies utilized tiling of BACs for specific regions (Locke et al. 2004; Aarts et al. 2007) or the entire genome (Ishkanian et al. 2004), these were not studying pancreatic cancer. The resolution of pancreatic studies was at 1 BAC per megabase.

A second technique took advantage of genome consortia cDNA sequence clones developed by the IMAGE Consortium (Pollack et al. 1999). This same format was pioneered for gene expression analysis by Pat Brown and colleagues and then adapted to CGH. In this format, cDNA fragments cloned into vectors were amplified by PCR using universal primers and arrayed onto microscope slides. The advantage

Table 7.1 Papers published on the use of aCGH for the detection of alterations in pancreatic cancer

References	Year of publication	Platform	Cell lines	Tumors	Xenograft	Normal variation	Notes
Aguirre et al.	2004	Agilent 244K	24	13	–	–	Complimentary expression for cell lines
Bashyam et al.	2005	cDNA array	22	–	–	–	Complimentary expression for cell lines
Calhoun et al.	2006	Affy SNP 100K	26	–	–	42	–
Chen et al.	2008	ROMA	23	24	26	325	Also appended Aguirre et al. (2004) data to increase sample size
Fu et al.	2008	ROMA	–	–	9	–	Samples were xenografts of mets and primary
Gysin et al.	2005	BAC array	25	–	–	–	Studies metastasis model
Harada et al.	2008	Affy SNP 100K	–	27	–	325	–
Harada et al.	2007	BAC array	23	–	–	–	Microdissection of primary tumors
Heidenblad et al.	2004	BAC and cDNA array	31	–	–	–	15/31 cells were self-established and 5 from DKF
Heidenblad et al.	2005	cDNA array	29	–	–	–	Expression and copy number
Holzman et al.	2004	Matrix CGH (BAC)	13	6	–	–	–
Lin et al.	2008	Affy SNP 50K	25	–	–	100	16 of the cell lines were from ATCC
Mahlamaki et al.	2004	cDNA array	13	–	–	–	Compared some expression and variation
Nagano et al.	2007	Affy SNP 50K	–	15	–	–	Endocrine tumors
Nowak et al.	2005	BAC array	16	–	17	–	Xenografts were primary sources of lesions
Pole et al.	2007	8p12 BAC tiling	6	–	–	–	Mostly a breast cell line study, 6 pancreatic cell lines

of this method was that the clones were freely available to the public. Moreover the results from CGH were directly comparable to expression data from the same sample. This method was adopted by several groups as an aCGH platform to analyze several tumor types including pancreatic cancer (Mahlamaki et al. 2004; Bashyam et al. 2005). The difficulty of this method was labeling the entire genome to get good signal on an array of fragments, as well as some cDNAs not being as useful due to repetitive content. In addition, non-coding RNAs were left off of the array.

With the complete sequencing of the human genome, more alternatives became available; oligonucleotides designed across the genome could be generated and arrayed. With the ability for in-situ synthesis of oligonucleotides by companies such as NimbleGen Systems (now owned by Roche) and Agilent Technologies, custom array designs could be synthesized to order at relatively short notice. An alternative method of aCGH, representational oligonucleotide microarray analysis (ROMA) (Lucito et al. 2003), took advantage of the oligo design flexibility offered by NimbleGen Systems. ROMA utilized complexity-reducing representations to increase signal-to-noise ratio on the array hybridization. Representations are simply restriction endonuclease cleaved, adaptor ligated, and PCR amplified genomic DNA material. The advantages in addition to the increase in signal-to-noise are several: firstly, representations can be prepared from as little as 25 ng which makes this method well suited to microdissected tumor samples, secondly, oligos derive from fragments that are scattered fairly evenly across the genome, and thirdly, methods were developed to minimize repetitive content in the oligo sequences. ROMA was used to analyze several tumor types (Hicks et al. 2005; Grubor et al. 2009) including the largest pancreatic tumor set thus far for pancreatic cancer (Chen et al. 2008). The representational methodology was adopted by Affymetrix Inc. for the SNP array platform, which was subsequently adapted to perform CGH (Komura et al. 2007). A disadvantage of this method is that it cannot be expanded to cover the genome completely, evidenced by Affymetrix's requirement of adding different restriction representations to increase coverage. However a clear advantage of the Affymetrix platform for SNP/aCGH is that it allows the identification of copy number-neutral loss of heterozygosity, which has been studied for pancreatic cancer (Calhoun et al. 2007). Agilent also synthesized oligonucleotides on the array and the aCGH arrays produced were used for the analysis of many cancers including pancreatic (Aguirre et al. 2004).

7.4 Analysis of Array CGH Data

Although the noise level differs from platform to platform, the raw data from all microarray experiments is inherently noisy with a constant chatter around the actual copy number. Users have employed various informatic methods to analyze the data to reduce the level of noise, some being platform-specific and others being more universal. Each has advantages and disadvantages. The simplest is a moving average (Pollack et al. 1999), which moves in genomic order and averages adjacent

probes, the number averaged being set by the user. This smoothes the data but can decrease the resolution, since averaging of probes around a breakpoint brings down the average for the probes on the end of the breakpoint, which can falsely shorten the region amplified or deleted. Improvements in this method calculate the probe false discovery rate from experiments performed on normal samples (Pollack et al. 1999). We and others have used circular binary segmentation (CBS) (Olshen et al. 2004), which estimates the location of change-points (breakpoints) for each probe and assesses its significance by permutation. Other methods of segmentation have been developed that utilize a hidden Markov model (Autio et al. 2003; Lin et al. 2008). It uses the presence of SNPs to define the states of alteration between the two genomes, i.e. zero copies, one copy, three copies, or more copies. Once this is determined, the state of other alterations can be identified based on initial calculations.

7.5 Pancreatic Tumor Samples Analyzed and Findings

The samples that have been analyzed in the study of pancreatic cancer have been broken down in Table 7.1 for each study. Some studies analyzed only cell lines, and in some cases the same cell lines were studied by different array platforms. Other studies analyzed only primary tumors, which of course were all different. Some groups added xenografts, and combined the analysis of all of these types of samples, realizing that with more samples, regions of interest are identified more accurately. When reviewing the results from these publications, we wished to compile the publicly available data into a meta set and analyze it in two ways: one to identify the smallest regions of alteration, and two to identify the frequency with which regions are altered within the genome. Comparing the studies is somewhat difficult due to several factors. The first is of course the difference in array formats. A second is the time of publication, which affected the genome build that the data is presented in. This was easily remedied; we converted all genome coordinates into Human NCBI Build37 (UCSC version hg18, March 2007) by using the Batch Coordinate Conversion (liftover) tool provided at the UCSC Genome Browser website. A third factor is the extensive overlap in the samples analyzed. The sample overlap is partially an advantage since it allows comparison of the different formats for the same sample; however there is such extensive overlap that combining the datasets and removing the independent regions where they were found less accurately becomes difficult. A fourth difficulty is the manner in which the data has been presented. In many cases the raw data is not available. Instead, the data are summarized in table format. This format works well for the study that it is derived from but makes it impossible to gain the advantage of the addition of samples from other studies, particularly if primary tumors were analyzed. Another issue with the presentation of the data is that several studies have not identified which samples have specific lesions. Therefore, such data can be used for identification of the lesion epicenters only, but cannot be used for the calculation of frequency of mutation.

While removing the redundancy that many studies analyzed the same samples, the data with position information for the regions identified was used to calculate the frequency with which lesions were identified. The minimal common region was calculated to decrease the number of candidate genes (Table 7.2). In order to decrease the identification of genomic noise, a threshold for frequency was imposed. For amplifications 2.5% was used and for deletions a cutoff 5% was used. The differences were imposed between deletions and amplifications since amplifications occur less frequently. The more biologically obvious genes within regions are listed. Chromosome coordinates are given in standard format to simplify searching for gene content in other regions.

The data was also used to plot the frequency of alteration from a whole genomic visual perspective (Fig. 7.1). The first striking observation is that the frequency of deletion is much higher than that for amplifications, and the location of several well-known tumor suppressors is obvious. The amplifications that are found tend to be focal and have been helpful in the identification of target oncogenes. The lack of frequent amplification suggests that over-expression of oncogenes by gene amplification is not a common mechanism and that other mechanisms must be involved in the over-expression of oncogenes. From the frequent deletion of the pancreatic cancer genome it appears that suppression of tumor suppressors by deletion may be a common mechanism for pancreatic cancer. The losses however tend to be very large, often being a whole chromosome arm. Homozygous deletions are very informative for mapping genes since these lesions remove both copies and no other data such as expression need be taken into consideration, and more importantly they tend to be very small in size and thus usually include very few genes. This is one reason why Lin and colleagues (2008) and Nowak and colleagues (2005) specifically studied homozygous deletions from pancreatic cancer in an attempt to take advantage of these characteristics in mapping genes involved in pancreatic cancer. Unfortunately homozygous deletions are rarely found in pancreatic cancer, unlike in other tumor types such as colon cancer (Scott Powers, personal communication). This has hindered the identification of target tumor suppressor genes.

The resolution or the density of the probes within the genome varies from platform to platform, but many of the arrays have obtained or passed a level of one probe every 100 kb. A consequence of this high resolution is that large polymorphisms are detectable. These regions can vary in size from 100 kb and likely smaller, to several Mb, and the number of copies also varies from one to four copies and possibly in rare cases higher gains or homozygous deletion. More importantly, as expected, the frequency that these regions are detected in the population varies and in some cases can be quite high, resulting in their detection being misinterpreted as cancer-specific lesions. Most studies have not taken this into account, and we specify in Table 7.1 which studies did. In our tabulation of regions in Table 7.2 from various array CGH papers we have highlighted the regions that are also found variable in the normal population.

Another possibility that must be taken into account when identifying target genes is that within one region there may be more than one gene as the target. In some cases there are two or more genes that have activity as tumor suppressors or

Table 7.2 The minimal regions identified for regions commonly altered in the dataset. The first column is the chromosome positions (hg18 coordinates)

Bin coordinates	Ratio.Loss	Ratio.Gain	Event	Genes	Length
chr1:241500001–241700000	0.028985507	0.078571429	Gain		100000
chr2:28400001–28500000	0.028985507	0.072857143	Gain		100000
chr3:70700001–71700000	0.279005848	0.035211278	Gain		11000000
chr3:150700001–150800000	0.028985507	0.035211278	Gain		100000
chr5:300001–1500000	0.014492754	0.042253521	Gain	*TERT*	1200000
chr5:13800001–13900000	0.02173913	0.042253521	Gain		100000
chr5:17400001–17500000	0.037231884	0.035211278	Gain		100000
chr7:43000001–43200000	0.075217391	0.028179014	Gain	*PTK7*	200000
chr7:900001–1100000	0.014492754	0.049295775	Gain		200000
chr7:17200001–17300000	0.007247377	0.035211278	Gain		100000
chr7:38700001–50700000	0.02173913	0.035211278	Gain	*RALA, TBRG4. TNS3,GRB10*	12000000
chr7:72400001–72800000	0.014492754	0.035211278	Gain		400000
chr7:80200001–80300000	0.007247377	0.035211278	Gain		100000
chr7:84900001–87500000	0.007247377	0.035211278	Gain		1700000
chr7:94000001–98700000	0.02173913	0.042253521	Gain		4700000
chr7:121300001–121400000	0.037231884	0.114285714	Gain		100000
chr7:133000001–133100000	0.037231884	0.108571429	Gain		100000
chr8:12300001–12400000	0.315789474	0.035211278	Gain		100000
chr8:34000001–34300000	0.247377812	0.049295775	Gain		300000
chr8:117500001–117700000	0.014492754	0.148571429	Gain		100000
chr8:119100001–127800000	0.014492754	0.171428571	Gain		8700000
chr8:128700001–128900000	0.014492754	0.257142857	Gain	*MYC*	300000
chr8:130900001–131000000	0.014492754	0.182857143	Gain		100000

Table 7.2 (continued)

Bin coordinates	Ratio.Loss	Ratio.Gain	Event	Genes	Length
chr8:133100001–134300000	0.014492754	0.175714287	Gain		1200000
chr11:12700001–12700000	0.075217391	0.072857143	Gain		100000
chr11:33500001–33700000	0.037231884	0.085714287	Gain		100000
chr11:59100001–59200000	0.028985507	0.035211278	Gain		100000
chr11:79000001–79100000	0.007247377	0.077474789	Gain		100000
chr11:85100001–87000000	0.014492754	0.057338028	Gain	*EED*	900000
chr11:101400001–101800000	0.028985507	0.070422535	Gain	*YAP1, BIRC3*	400000
chr11:120700001–120800000	0.043478271	0.035211278	Gain		100000
chr12:21800001–21900000	0.079710145	0.035211278	Gain		100000
chr12:24800001–27000000	0.072473778	0.091549297	Gain	*k-RAS*	1200000
chr12:28100001–28200000	0.075217391	0.070422535	Gain		100000
chr14:53400001–53500000	0.075217391	0.097142857	Gain	*BMP4*	100000
chr15:18700001–18800000	0.123188407	0.042253521	Gain		100000
chr17:35000001–35100000	0.072473778	0.091428571	Gain		100000
chr17:45300001–45400000	0.075217391	0.042253521	Gain		100000
chr18:17400001–19700000	0.087957522	0.057338028	Gain	*GATA7*	2300000
chr19:42000001–44700000	0.079710145	0.073380282	Gain	*PAK4, IXL, PAF1*	2700000
chr20:38500001–39100000	0.037231884	0.142857143	Gain	*TOP1*	700000
chr20:41100001–42000000	0.037231884	0.091549297	Gain	*PTPRT*	900000
chr20:50000001–53500000	0.02173913	0.073380282	Gain	*ZNF217*	3500000
chr20:57100001–57500000	0.028985507	0.17	Gain		400000
chr20:72200001–72400000	0.028985507	0.057338028	Gain		200000
chr1:12700001–12800000	0.058479532	0	Loss		100000
chr1:25000001–25100000	0.072473778	0	Loss	*RUNX3*	100000
chr1:27100001–28900000	0.072473778	0	Loss		1800000

Table 7.2 (continued)

Bin coordinates	Ratio.Loss	Ratio.Gain	Event	Genes	Length
chr3:1300001–1400000	0.101449275	0	Loss		100000
chr3:2200001–2500000	0.18115942	0.014084507	Loss		300000
chr3:17700001–17700000	0.108795752	0	Loss		100000
chr3:23700001–24000000	0.188405797	0.014084507	Loss		300000
chr3:28800001–33200000	0.115942029	0	Loss	*TGFBR2*	4400000
chr3:39500001–42700000	0.087957522	0	Loss	*CTNNB1*	3200000
chr3:47900001–49500000	0.217391304	0	Loss		1700000
chr3:54500001–54900000	0.304093577	0.014084507	Loss	*CACNA2D3*	400000
chr3:57400001–57500000	0.152173913	0	Loss		100000
chr3:70700001–71100000	0.279005848	0.035211278	Loss	*FHIT*	400000
chr3:70900001–72000000	0.245714035	0.035211278	Loss	*FOXP1*	1100000
chr3:145500001–145700000	0.17374279	0	Loss		100000
chr3:177800001–177900000	0.245714035	0.021127771	Loss		100000
chr4:100001–700000	0.075217391	0.007042254	Loss		700000
chr4:2100001–2200000	0.072473778	0.007042254	Loss		100000
chr4:4800001–4900000	0.075217391	0.007042254	Loss		100000
chr4:145000001–145100000	0.115942029	0.014084507	Loss		100000
chr4:153000001–153100000	0.175438597	0.014084507	Loss		100000
chr5:73700001–73800000	0.050724738	0	Loss		100000
chr5:88400001–88500000	0.222222222	0.007042254	Loss		100000
chr7:900001–1000000	0.108795752	0	Loss		100000
chr7:1300001–1800000	0.217391304	0.014084507	Loss		500000
chr7:3900001–4000000	0.217391304	0.014084507	Loss	*PRPF4B*	100000
chr7:7100001–7700000	0.217391304	0.021127771	Loss	*CAGE1*	500000
chr7:14800001–14900000	0.195752174	0.007042254	Loss		100000

Table 7.2 (continued)

Bin coordinates	Ratio.Loss	Ratio.Gain	Event	Genes	Length
chr7:20400001–20500000	0.224737781	0.014084507	Loss		100000
chr7:77400001–71100000	0.094202899	0	Loss	*BAI3*	3700000
chr7:89800001–94200000	0.231884058	0	Loss		4400000
chr7:108300001–108400000	0.298245714	0.007042254	Loss	*CASP8AP2*	100000
chr7:137300001–137700000	0.195752174	0.028179014	Loss	*BCLAF1*	400000
chr7:138200001–140300000	0.224737781	0.014084507	Loss	*REPS1*	2100000
chr7:148700001–171000000	0.304093577	0.007042254	Loss		12300000
chr8:7300001–7500000	0.195752174	0.007042254	Loss	*ANGPT2*	200000
chr8:9800001–9900000	0.204778373	0.042253521	Loss		100000
chr8:12300001–12400000	0.315789474	0.035211278	Loss		100000
chr8:17000001–17100000	0.195752174	0	Loss		100000
chr8:19700001–19800000	0.253723188	0.028179014	Loss		100000
chr8:25800001–25900000	0.247377812	0.028179014	Loss		100000
chr8:29100001–34300000	0.247377812	0.049295775	Loss	*DUSP4*	5200000
chr9:1300001–1400000	0.275372319	0.021127771	Loss		100000
chr9:10800001–11100000	0.289855072	0.014084507	Loss		300000
chr9:17400001–17800000	0.297101449	0.007042254	Loss	*SH3GL2*	400000
chr9:19800001–22900000	0.432748538	0.007042254	Loss	*INK4/ARF*	3100000
chr9:24500001–25500000	0.379575217	0	Loss		1000000
chr10:127400001–127500000	0.075217391	0.014084507	Loss		100000
chr11:2100001–2300000	0.072473778	0.007042254	Loss		200000
chr11:9500001–14000000	0.072473778	0.014084507	Loss	*DKK3*	4500000
chr11:21200001–21300000	0.050724738	0	Loss	*NELL1*	100000
chr11:41000001–41100000	0.050724738	0	Loss		100000
chr11:107700001–107700000	0.050724738	0.014084507	Loss	*ATM*	100000
chr12:8800001–11900000	0.094202899	0.007042254	Loss		3100000

Table 7.2 (continued)

Bin coordinates	Ratio.Loss	Ratio.Gain	Event	Genes	Length
chr12:45700001–51800000	0.094202899	0.014084507	Loss		7100000
chr12:82700001–83800000	0.130434783	0.007042254	Loss		1200000
chr13:21700001–25700000	0.079710145	0.007042254	Loss		4100000
chr13:58200001–77700000	0.094202899	0.007042254	Loss	*TNFRSF19*	9500000
chr13:80700001–85300000	0.101449275	0.007042254	Loss		4700000
chr15:18700001–18800000	0.123188407	0.042253521	Loss		100000
chr17:1300001–12100000	0.380117959	0	Loss	*p53*	10800000
chr17:17300001–17400000	0.253723188	0	Loss		100000
chr18:4400001–11700000	0.094202899	0	Loss		7200000
chr18:32500001–33800000	0.278115942	0.021127771	Loss		1300000
chr18:37700001–38800000	0.297101449	0.028179014	Loss	*PIK3C3*	1100000
chr18:44000001–44100000	0.289855072	0.007042254	Loss		100000
chr18:47800001–49200000	0.409357725	0	Loss	*SMAD4*	2400000
chr18:77700001–77800000	0.355072474	0	Loss		100000
chr18:75000001–75900000	0.297101449	0.014084507	Loss		900000
chr19:70000001–70100000	0.072473778	0.035211278	Loss		100000
chr20:14700001–14800000	0.094202899	0.014084507	Loss		100000
chr21:34800001–35200000	0.101449275	0	Loss	*RUNX1*	400000
chr22:32000001–32100000	0.195752174	0.014084507	Loss		100000
chr22:37200001–38800000	0.202898551	0.014084507	Loss		1700000
chr22:44100001–44200000	0.202898551	0.007042254	Loss		100000

Note: ratio.loss and ratio.gain are the frequency with which the region is found altered (note that the frequency is not in percent format but decimal). Both are given for each to demonstrate the level one is found above the other. Regions where copy number aberrations are found in the normal population are in bold

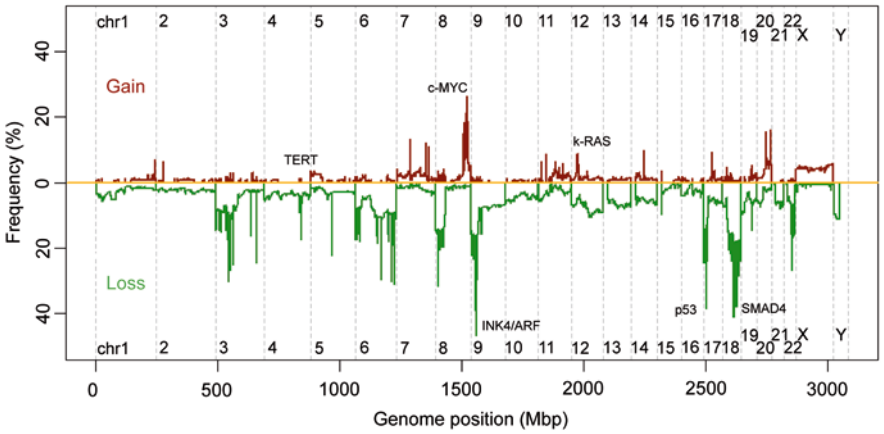

Fig. 7.1 The frequency that regions are found altered within the compiled set of data. To simplify the selection of regions found altered in the data, bins of 100 kb size were made and the data for each manuscript represented sorted into respective bins. The frequency that a bin was found with either amplification or deletion was found to define a frequency. The *y*-axis is the frequency. The *x*-axis is the Absolute Position which is a concatenation of the Chromosome Position for all chromosomes and allows the graphing of all data on one axis. To simplify the visualization, *dashed vertical lines* were drawn in to signify the position of chromosome ends. Amplifications are plotted in *red* and are above the origin and deletions are plotted in *green* and are plotted below. Several genes well known to be involved in pancreatic cancer are drawn in roughly by their genomic position

oncogenes. An example is the region of chromosome 19q13, where three groups independently studied the genes *IXL*, *PAK4* and *PAF1* as possible targets of the amplicon (Moniaux et al. 2007; Kuuselo et al. 2007; Chen et al. 2008). Each gene was shown to have oncogenic activity by one or more assay. It is possible that in certain contexts these genes cooperate in pancreatic carcinogenesis. This could explain why some of the amplified regions have a complex profile, where they seem to be amplified in steps. In some cases more genes are encompassed and in other cases there are fewer. To date none of the groups has demonstrated the cooperation of these genes in oncogenesis.

7.6 Further Analysis to Validate Regions and Identify Cancer Genes

Although aCGH is a well-accepted technology, it is useful to validate the copy number measurements identified, especially for those regions altered at a low-level copy number. Clearly the most accurate method to validate CNVs is fluorescence in situ hybridization (FISH). This method can assess the true copy number of an allele present in a sample, whereas the CGH data gives a relative level in comparison to the reference sample. However, FISH is somewhat slow in throughput and requires intact cells. An alternative is quantitative PCR (QPCR). This method does

not require whole cells, can be performed with mid-throughput, requires little DNA template, and is very accurate.

To identify candidates from a region of interest for further study it is important to look at the expression of all genes in the region, in samples that have altered copy number. Generally the target genes will have altered expression, either decreased or increased if deleted or amplified respectively. Expression of approximately 12% of genes within regions of copy number alteration correlates with the changes in expression, and this percentage rises for regions of higher amplification (Pollack et al. 1999; Aguirre et al. 2004; Heidenblad et al. 2005). Some pancreatic aCGH studies have incorporated whole genome expression analysis for all samples, or at least expression for a limited number of samples or for specific gene candidates of interest (Aguirre et al. 2004; Mahlamaki et al. 2004; Gysin et al. 2005; Heidenblad et al. 2005; Chen et al. 2008; Fu et al. 2008). This information aids in the further delimiting of candidate genes in the regions of interest. This is particularly useful for amplifications, but as we have seen the deletions in pancreatic cancer tend to be large and the expression alone is often not enough to decrease the number of target genes.

Since the regions deleted tend to be on only one allele and are very large, there must be other mechanisms used to silence the transcription (or in some cases function) of genes with tumor-suppressive activity on the other allele. The target genes can be mutated by deletions that are small enough (such as point mutation) to be below the resolution of all of the platforms used, although this is unlikely for all deletions identified. With the development of high-throughput sequencing technologies, this type of analysis is now approachable. The selection of candidate genes from aCGH data is an interesting approach to the selection of genes for high-throughput sequencing.

Another mechanism the cell can use to repress the transcription of genes is to modify the gene's epigenetic signature by way of promoter methylation or histone modification. It is already known that certain genes that are targets of promoter methylation in pancreatic cancer (such as *FoxE1*, *NPTX2*, *CLDN5*, *P17*, *TFPI2*, *SPARC*, *PENK*, *SARP2*, *TSLC1*, *RELN*, and *UCHL1*) (Hamada et al. 2005; Brune et al. 2008; Hong et al. 2008). From aCGH data we see that these genes often fall into chromosome regions that are frequently found as one copy (e.g., *FoxE1*, *CLDN5*, *P17*, *SPARC*, *SARP2*, *RELN*, and *UCHL1*). Therefore if genes from deletions are to be studied further, it is likely that other forms of genomic data such as epigenomic and expression analysis will be required. These methods are well developed and approachable to identify genes from cell line studies, but will be more difficult to carry out on primary tumors.

To determine which of the candidate genes is the true targets of the lesion, functional validation studies must be performed. This can be approached in one of two ways. The standard approach is to pick one region, and study one gene at a time, possibly by over-expression studies for oncogenes and shRNA studies for tumor suppressors. The studies would likely be performed in either a tissue culture or a mouse model with assays analyzing survival, growth, or invasion. However, studies can be performed in high throughput as well, where many genes are being assayed at the same time, as illustrated by the work in liver cancer where focal regions were

mapped and the candidate genes identified (Zender and Lowe 2008). A library of shRNA clones was used and multiple clones for each candidate gene were selected. Close to 50 clones were pooled and introduced into cells and implanted into mice. The tumors that grew were harvested and the shRNAs that were enriched were sequenced, identifying the genes that when silenced gave a growth advantage to the cells (Zender and Lowe 2008). By doing so, the genes were rapidly screened demonstrating that, once a model system such as this is designed, either shRNAs or full-length cDNAs for candidates can be pooled and introduced into cells for high-throughput validation.

7.7 Concluding Remarks

Pancreatic cancer is a complex cancer at the genomic level. It is clear even from the copy number data that there are several genes that play a dominant role in carcinogenesis including *p53*, *SMAD4*, c-*MYC*, *k-RAS*, and the *INK4/ARF* region. However, from Fig. 7.1 we can see that there are other regions that are altered at a high frequency. Some of these regions are so-called fragile sites, or regions that are altered frequently without a true target gene. There are some regions, such as 8p, that are deleted and have tumor suppressors but the region is too large to carry out conventional gene-functional validation studies. Now that high-throughput technologies can be applied to validation, these regions are more approachable. The analysis of these regions will identify new targets altered in pancreatic cancer, some of which could be the focus of therapeutic development. For such an aggressive disease, perhaps a cure is too much to ask for, but hopefully therapeutic developments based on the targets identified through copy number study will extend the life of pancreatic cancer patients.

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Chapter 8
The Application of High-Throughput RNAi in Pancreatic Cancer Target Discovery and Drug Development

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Abstract Pancreatic cancer is a particularly lethal malignancy and is highly chemoresistant. There is an urgent need for the identification of new therapeutic targets and more effective treatment options. New approaches, such as high-throughput RNAi, enable the functional evaluation of the casual role of numerous genes in regulating cellular processes, such as cell survival and drug response. In the following chapter, we review RNA interference and its application in high-throughput biology. Specifically, an overview is provided highlighting important experimental aspects in transitioning RNAi to a high-throughput platform. In addition, there is a brief review of current applications of high-throughput RNAi for cancer target identification and drug discovery. Lastly, particular applications of genome-scale RNAi to pancreatic cancer target and treatment identification are discussed. In summary, genome-scale RNAi is proving to be a powerful cellular genomics technology that holds great promise for advancing pharmacologically relevant targets and agents in pancreatic cancer research.

8.1 Introduction

Pancreatic cancer averages 37,000 new cases per year and ranks fourth in all cancer-related deaths in the United States (Jemal et al. 2008). The significant challenge in combating this malignancy is illustrated by the dismal 5-year survival rate that remains below 5%. Pancreatic cancer is a highly chemoresistant tumor, with even first-line chemotherapeutic agents, such as gemcitabine, offering little improvement in terms of survival and quality of life. The molecular mechanisms

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underlying the poor response to therapeutic intervention are not well understood, but it is suspected that resistance is due to redundant survival signals emerging from the multiple genetic aberrations that accumulate during tumor progression. These genomic alterations result in the aberrant function and expression of specific genes and deregulated signaling networks, ultimately causing cancer cells to evolve selectable phenotypic properties such as increased resistance to therapy. In addition to applying genomics to reveal the underlying genetic events that drive pancreatic cancer progression, research is also needed to evaluate the extent to which genes across the genome are causally involved in the cellular mechanisms of survival and chemotherapy response. We show here how high-throughput RNAi phenotype profiling can be applied to synthetically alter gene states in a systematic way across the genome, enabling the functional evaluation of causal relationships between genes and cellular phenotypes. Such results can then be further advanced through evaluation of clinical samples to evaluate the clinical significance of specific candidate genes as potential markers for therapeutic response and as new candidate targets for therapeutic intervention.

8.2 RNA Interference (RNAi)

A classical functional validation technique is to experimentally inhibit the expression of candidate genes in order to examine the resultant phenotype. Unfortunately, the development and testing of gene-specific inhibitors, such as antisense oligonucleotides and ribozymes, is a difficult and time-consuming process that can only be applied to one gene at a time. RNA interference (RNAi) is an endogenous biological process that utilizes unique RNA species for targeted control of post-transcriptional gene expression (Fire et al. 1998; Kennerdell and Carthew 1998; Misquitta and Paterson 1999). RNAi can be utilized in the laboratory to direct the reduction of targeted gene product levels. This involves the introduction of small interfering RNAs (siRNA) that are potent mediators of the intrinsic post-transcriptional gene silencing mechanism (Elbashir et al. 2001a, b). Today, the most commonly used experimental RNAi approaches, described below, involve the use of synthetic siRNA, vector-expressed short hairpin RNAs (shRNA) and microRNAs (miRNA). siRNA are RNA duplexes of 20–23 nucleotides (nt) in length that base pair with a homologous sequence within mature mRNA products. This acts as a guide for the RNA–protein complex termed RNA-induced silencing complex (RISC) (Hammond et al. 2001; Yu et al. 2002). This complex is responsible for the cleavage and subsequent degradation of the target mRNA, resulting in a reduction of gene expression. The synthetic RNA duplex is very useful to study gene function through transient gene suppression but cannot be used for long-term gene suppression (stable gene suppression) because the RNA duplex is degraded very quickly in mammalian cells. Short hairpin RNAs (shRNA) are transcribed *in vivo* by RNA polymerase III promoters such as those of the H1 RNA, the RNA subunit of RNase P complex and U6 snRNA genes, which have well-defined transcription

initiation and termination signals. shRNAs are processed into siRNAs and induce RNAi gene silencing (Brummelkamp et al. 2002; Bohnsack et al. 2004; Lund et al. 2004; Pillai et al. 2005). miRNAs are initially produced as long transcripts of primary miRNAs (pri-miRNAs) that include hairpin structures and contain one or more miRNAs. Pri-miRNAs are processed in the nucleus by a microprocessor complex that contains the RNase III endonuclease Drosha, to produce miRNA precursors (pre-miRNA) of 60–70 nt stem-loop intermediates. Pre-miRNAs are exported from the nucleus in a process that is dependent on exportin 5 (EPO5) (Elbashir et al. 2001a, b), and then are processed in the cytoplasm by Dicer into mature miRNAs inducing target gene translational repression (Brummelkamp et al. 2002; Bagga et al. 2005).

Chemically synthesized siRNAs and vector-produced shRNA are, at present, the most broadly used RNAi reagents for experimental manipulation of gene levels. Both siRNA and shRNA constructs targeting the majority of human genes have been developed and now enable the large-scale RNAi screens. Libraries of siRNAs ensure constant quality of reagents and the ability to chemically modify the RNA molecules for stability and delivery purposes, and allow for the ability to control the amount of siRNA delivered to the cell. Lipid-based transfection reagents are used for delivery into cells. These libraries can be screened using two methods: individually or by pooling several siRNAs for each gene target. The pooling method can reduce the costs of screening but increases the false positive hit rate due to the increased possibility of an off-target effect from a single siRNA. Regardless of screening format, the disadvantages of using siRNA include the short life of siRNA, which results only in the transient inhibition of gene expression, and the possibility of difficulty in transfecting certain cell types. Furthermore, the cost factor limits its wider use in genome-wide high-throughput approaches.

Delivery of shRNAs into the cell is usually achieved through use of viral vector-mediated expression with the primary advantage of generating gene silencing in a more sustained manner, lasting longer than 5–7 days. Retroviral, lentiviral, or adenoviral backbone vectors can be readily used for the construction of shRNA expression systems. The viral vectors facilitate delivery of shRNAs into a broad range of cell types. Libraries of shRNA for screening contain specific ‘barcodes’ for identification of the targeted gene (Berns et al. 2004; Paddison et al. 2004a, b). Each vector construct, which produces an shRNA, also contains a unique barcode sequence, which is optimized for microarray format or PCR. The application of molecular barcode technology maximizes the sensitivity of these libraries by allowing the identification and efficient discovery of extremely rare silencing events that generate the desired loss-of-function phenotypes. Moreover, the vector-based shRNA can be transduced into cells that are difficult to transfect, such as primary cells. There are many benefits to utilizing vector-based shRNA; however, it is worth noting that there are a few inherent disadvantages, which include the lack of control of the effective dose of shRNA generated inside the cell, the difficulty of assessing the screen coverage, the notoriously variable quality and accuracy of vector libraries, and the requirement of large-scale cloning steps for the single-well-based with pooled library format.

Driven by advances in the discovery of the molecular mechanism of RNAi, these RNAi tools have been rapidly developed and widely used in basic and applied biology. RNAi technology has proved to be relatively easy, more potent, and highly specific as a functional genomics method and has enormous potential to augment anti-cancer therapeutics by identifying and validating drug targets and determining drug response modulators.

8.3 RNAi High-Throughput Screening

The development of large siRNA or shRNA libraries has enabled the adaptation of RNAi to high-throughput (HT) screening. The application of genome-wide RNAi has quickly become an integral functional genomics tool for drug target identification and validation, pathway analysis, and drug discovery, as evidenced by the number of high-quality publications in this field. Genome scale siRNA-based screens have many similarities to large-scale chemical screens (Echeverri et al. 2006; Echeverri and Perrimon 2006; Caldwell 2007; Perrimon et al. 2007), including miniaturized plate formats and integration of automated systems for sample processing. A successful siRNA screen workflow consists of a number of key steps; here we present an optimized workflow developed in our laboratory (Fig. 8.1). The initial step is the pre-experimental planning stage in order to define the experimental question and identify appropriate model systems and testing reagents. For example, the screen can be either to screen for unknown components in a specific well-characterized pathway or a broader genome-wide screening in a less well-understood process. To achieve a highly sensitive, robust and reproducible assay, assay development (AD), which involves the establishment of optimal parameters, is a key procedure. AD represents the rate-limiting and time-consuming step that requires careful and detailed testing and is crucial to the quality of the screen. We have implemented an AD checklist of conditions that need to be evaluated and optimized, as outlined in Table 8.1. Such conditions for optimization include, but are not limited to, cell-seeding density in the plate, growth rate, choice of transfection reagent, drug dose response (if applicable), incubation time, and phenotypic readout. The outcome of stringent AD is a robust, sensitive, and reproducible assay for use in a large-scale screen.

The next step in the workflow is assay validation (AV). In order to justify proceeding to large-scale screening and to ensure that selection of potential hits will be accurate, it is imperative that positive controls display good sensitivity in experiment optimizations. After the establishment of assay parameters, an AV screen is performed consisting of a limited number of 384-well plates screening a few hundred genes plus the positive and negative controls. The goals of the AV screen are to validate experimental conditions established in assay development and to determine the assay quality. The results of the AV screen are the determining factor in progressing to the HT-RNAi screen, and once the AV screen result indicates that the assay is robust and reliable, the exact assay conditions can be used for the large genome-wide screen.

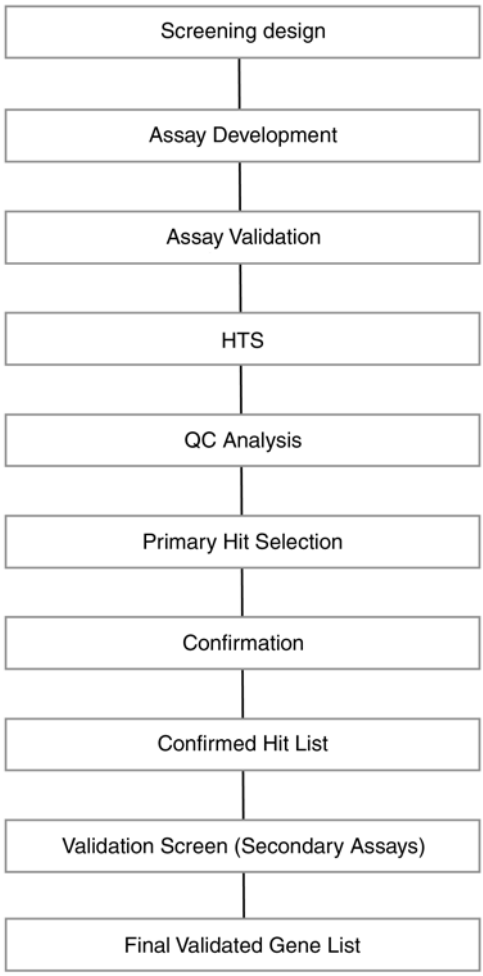

Fig. 8.1 Overview of the various phases of the high-throughput RNAi screening process. The RNAi screening starts with a screening design with a well-defined goal. In assay development, numerous assay parameters are rigorously tested and established followed by a small-scale RNAi screen to validate assay conditions. Once screening results meet the established standards, the large-scale high-throughput genomic RNAi screen will be performed. Various data analysis and bioinformatics tools are used to assess the screen quality and to identify potential hits. Genes that produce the desired phenotype when silenced are screened again and also tested with secondary assays for validation, which results in a high-confidence validated gene list

Typically, large HT-RNAi screens take advantage of large genome libraries covering thousands of genes, and involve over hundreds of plates. Post-screen activities include QC measurements, data normalization, and hit selection, which involve a number of statistical tools/techniques. Screen quality is systematically evaluated with an exhaustive list of control parameters, e.g. transfection efficiency, toxicity caused by transfection reagent, coefficient of variation (CV) values for control siRNAs, drug effects (targeted versus observed effects), plate uniformity by modified Bland–Altman, screen variation and reproducibility for plate-to-plate, run-to-run and well-to-well, etc. (Table 8.2). Only a good quality screen can lead to high-confidence positive hits and avoid the false negatives from the large-scale screening. The screen data analysis is highly dependent on the assay type as well as the screening method. Quantitative data, which is commonly applied in the primary HTS, is normalized to a standard or negative control and scored according to predefined criteria and hits are ranked. The difficulty of analyzing the RNAi screening

Table 8.1 Key parameters in HT-RNAi assay development

Parameter	Key factors
Cell line for screening	Transfection efficiency, growth rate, assay sensitivity
Cell growth media	Should not interfere with drug activity, readout, or transfection efficiency
[siRNA]	Concentration must produce effective silencing and limit off-target effects
Plate format	Medium evaporation, machine readout, barcode
Positive control siRNA	Previously identified; cell line-specific; proliferation-specific; general killers
Sensitizing positive control siRNA	Provides measure of overall assay function
Negative control siRNA	Should have no effect on cell growth or drug activity
Transfection reagent	Should be effective in introducing siRNA and have low toxicity
Transfection reagent diluent	Should not interfere with drug activity, readout, or transfection efficiency
Transfection reagent ratio	Toxicity vs. efficiency
Transfection reagent incubation time	Enough time to complex siRNA and transfection reagent
Mechanism for addition of transfection reagent	Minimize well-to-well, plate-to-plate variability
Complexing time	Enough time to complex siRNA and transfection reagent
Cell volume added	Well-to-well, plate-to-plate variability
Cell number added	Optimized to give greatest dynamic range at read out
Incubation time (before drug)	Enough time for siRNA to silence transcripts
Incubation time (after drug)	Enough time for drug action
Mechanism for addition of cells	Minimize well-to-well, plate-to-plate variability
Drug	Should have an effect on the screen cell line
Drug diluent (vehicle)	Should readily solubilize drug
Drug volume	Should be minimal
Drug stability	Temperature, half-life, solution, medium
Final drug concentration	Should have desired effect at selected concentration
Mechanism for addition of readout reagent	Minimize well-to-well, plate-to-plate variability
Cell viability measurement	Sensitivity, accuracy, and cost
Added volume of readout reagent	Should be minimal
Mechanism for addition of readout reagent	Minimize well-to-well, plate-to-plate variability
Incubation time for readout reagent	Optimized to give greatest dynamic range at read out
Readout method	Sensitivity, accuracy

data is that it yields a large range of ranking of the hits. This is partially due to the fact that quantitative results directly correlate with the phenotype. Strong phenotypes are easily selected as hits, while weaker but real phenotypes can result from either a partial knockdown of a gene with a strong effect or a strong knockdown of a gene with a weak effect. Similarly, genes that show no phenotype can be genes without an effect or lack of effective knockdown (Boutros and Ahringer 2008).

Table 8.2 Post-screen assessment

Parameter	Criteria
Transfection efficiency	>90% recommended, >95% preferred (compare Positive Control vs. Negative Control)
Toxicity by siRNA transfection mixture	<20% (compare Nontargeting Negative Control vs. siRNA Buffer)
Plate uniformity	CV < 25% for control wells (i.e. buffer, nontargeting negative control)
Internal replication	Correlation coefficiency (R^2) >0.9
Plate-to-plate variation	Consistency of untreated buffer and selected controls (CV < 10%)
Set-to-Set variation	Consistency of each set measured by control wells (CV < 25%)
Run-to-run variation	Consistency of each run measured by control wells (CV < 25%)
Drug effect	Consistency of drug effect and EC50 estimates for each library plate
Dynamic range	Receiver operating characteristics curves (ROC) for assessing dynamic ranges with respect to positive and negative controls
Assay Quality	Z Factor >0.4 and Strictly Sandardized Mean Difference (SSMD)
Hits confirmation rate (%)	>50% confirmation rate preferred
Screen performance assessment	Determine rate of false positives and false negatives

Recently, Konig et al. have reported that using probability-based redundant siRNA activity (RSA) analysis on multiple distinct siRNAs targeting, the same gene outperformed the traditional activity-based ranking method (Konig et al. 2007). RSA accomplishes this task by taking into account the behavior of all siRNAs sequences that target the same gene. After normalization, a *p*-value for the gene target is calculated using a cumulative hypergeometric distribution of the activity of all siRNA sequences targeting a particular gene. This weighted *p*-value can then assess and rank the significance of a gene target. In addition, a *p*-value is also calculated for the activity of each individual siRNA sequence in the screen. The siRNAs are then ranked by their gene *p*-value (ascending) and then by their individual activity in the screen (from potent to weak), which can then be used to select genes and sequences for confirmation studies. This method of analysis has been demonstrated to significantly increase the confirmation rate and reduce the number of false-positives that are derived from both experimental artifacts as well as the off-target activities from the siRNA sequences. It is highly recommended to screen any large-scale RNAi library with redundancy (multiple sequences for each gene target) and also apply the RSA methodology when analyzing the data sets from the large-scale screens.

The successful outcome of a HT-RNAi screen, robust and reproducible data, requires stringent quality control and a multi-step confirmation and validation plan. Once hits are selected, they are advanced into hit confirmation that involves

performing a small-scale screen, with appropriate controls, under high-throughput screen conditions. Confirmed hits are then carried to a validation step that usually involves further testing with additional siRNA and additional cell lines, using a secondary assay, and verifying gene knockdown by measuring mRNA levels.

It is very important to note that when performing siRNA- or shRNA-based screens, it is critical to limit off-target effects in order to ensure the specificity of an observed RNAi-induced phenotype. To address these concerns, the use of multiple distinct siRNA/shRNA targeting the same gene is strongly recommended as a control for sequence-dependent off-target effects; the detection of similar phenotypes induced by each siRNA/shRNA increases confidence that the observed result is due to the targeted gene's loss of function. In addition, the use of multiple distinct nontargeting negative control siRNA/shRNA is recommended to control for sequence-independent off-target effects, such as interferon response. Finally, to be sure that the nontargeting negative controls are not inducing any phenotypic consequences themselves, the inclusion of untreated samples is also recommended (Echeverri et al. 2006).

8.4 High-Throughput RNAi for Target Identification and Its Application to Pancreatic Cancer

The application of genome-wide RNAi has proven to be an integral functional genomics tool for drug target identification and validation, pathway analysis, and drug discovery, as evidenced by the number of high-quality publications in this field. It has been discovered through the use of RNAi lethality screening that targeted reduction of oncogenes led to a loss of anchorage-independent growth and decreased the tumorigenic capacity of the different tumor types including pancreatic cancer cells (Fig. 8.2a) (Berns et al. 2004; Giroux et al. 2006; Schlabach et al. 2008). These studies illustrate the utility of RNAi lethality screening in the identification and validation of potential targets for anti-cancer therapies. Additionally, synthetic lethality screens used to identify enhancers or suppressors of a specific chemical compound or specific loss-of-function mutation have recently been shown to identify the important molecules that mediate drug response and resistance (Fig. 8.2b). Such studies not only provide important clues into the mechanism of action of a drug but also help identify potential rational combination strategies and functional candidates to serve as biomarkers for drug response and patient stratification. We will elaborate on details in the next two sections.

Early adaptations of genome-wide RNAi employed short hairpin RNAs (shRNAs) to perform RNAi genetic screens to discover loss-of-function phenotypes related to cell proliferation and various cellular processes. Berns and colleagues performed a screen consisting of 23,742 distinct shRNAs targeting 7,914 different human genes in altered primary human BJ fibroblast cells (BJ-TERT-tsLT) (Berns et al. 2004). They identified one known and five new modulators of *p53*-dependent growth arrest, which upon suppression confer resistance to *p53*-dependent and $p19^{ARF}$-dependent proliferation arrest as well as abolishing a DNA-damage-induced

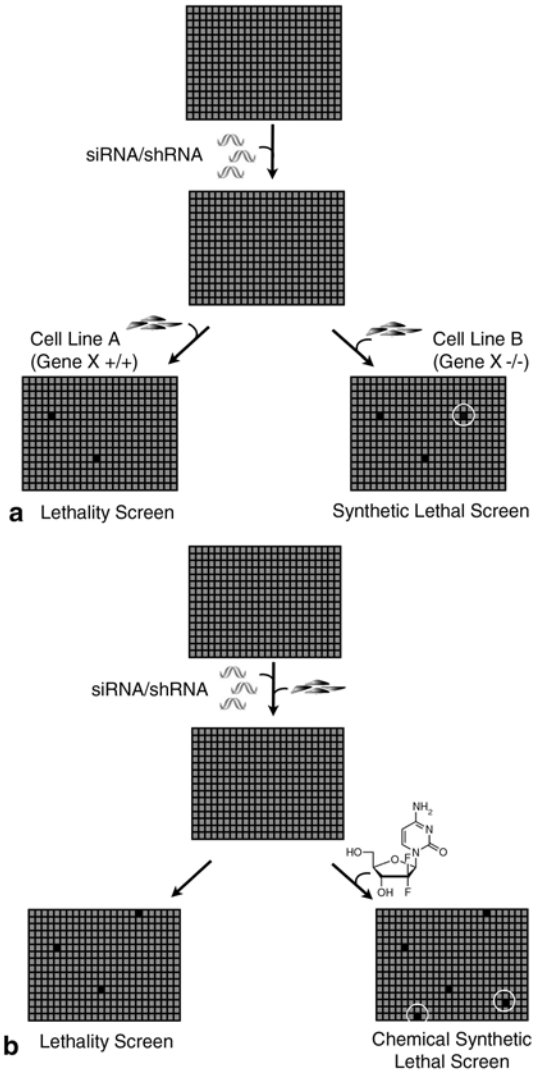

Fig. 8.2 Distinguishing lethality, synthetic lethality, and chemical synthetic lethality screens. **a** siRNA/shRNA targeting genes of interest as well as controls are added to a 384-well microplate followed by addition of transfection reagent and cells. After an optimal incubation time, the appropriate endpoint phenotype is measured, i.e. proliferation, cell viability, or cell death. The *left* arm is considered a lethality screen, attempting to identify essential genes that when silenced result in more cell death compared to the controls (as indicated by *darker wells* in the figure). The *right* arm illustrates the synthetic lethality screen, in which expression of gene X is deficient in cell line B. Screen is performed to identify nonessential genes that produce lethal phenotypes only when silenced in the context of the gene X deletion (as indicated by *white circles*). **b** The chemical synthetic lethality screen attempts to identify genes that produce lethal phenotypes when combined with otherwise non-lethal doses of a drug or compound. Assay process is similar to that described in (**a**) except for the drug addition after transfection followed by incubation. Genes that produce more cell death when silenced compared to the controls are essential genes (as indicated by *darker wells*) and the genes that produce more cell death with drug treatment compared to the same condition with no drug treatment are identified as drug sensitizer candidates (as indicated by *white circles*)

G1 cell-cycle arrest (Table 8.3). Ngo et al. performed a loss of function screen utilizing an shRNA library targeting 2,500 human genes in two, molecularly distinct, diffuse large B-cell lymphoma (DLBCL) cell lines (activated and germinal centre) to identify key regulators of cancer cell proliferation and survival. The NF-κB pathway is known to have an essential role in the survival of the activated B-cell-like DLBCL (Davis et al. 2001), and they observed that shRNAs targeting NF-κB induced cell death in the activated B-cell-like DLBCL, but not the germinal centre B-cell-like DLBCL cell. They discovered CARD11 as a novel, key upstream signaling component responsible for the constitutive IκB kinase activity in activated B-cell-like DLBCL. Moreover, CARD11 expression is restricted only to the lymphoid system, making it an attractive therapeutic target for activated B-cell-like DLBCL (Ceyhan et al. 2007). Furthermore, Schlabach and colleagues optimized the shRNA barcode strategy by developing modified half-hairpin barcodes instead of full-hairpin sequences that improve the sensitivity of the screen by increasing the critical dynamic range (Schlabach et al. 2008). They used two colon cancer cell lines, one breast cancer cell line, and normal human mammary epithelial cells to screen a highly complex pool of 8,203 shRNAs targeting 2,924 genes. They identified ~100 genes for each of the cancer cell lines and 819 genes in the normal human cells that showed statistically significant antiproliferative effects, of which only 19 genes were found to be universally lethal to all cell lines. Screening multiple cancers as well as normal cells maximized their ability to identify common, cancer-specific, as well as cell-line-specific growth regulatory pathways and targets.

In the context of pancreatic cancer, one published siRNA screen has been performed. This specific screen used a kinase library targeting 645 human kinases genes with the goal of identifying genes whose inhibition decreases cell viability (Giroux et al. 2006). The pancreatic adenocarcinoma cell line, MiaPaCa-2, was used in the screen. They identified 56 kinases (8.5% of total kinases), which upon knockdown increased apoptosis by at least 50% over control siRNA. To determine if these hits were cell-line-specific, they screened 10 selected kinases in two other pancreatic cancer cell lines, BxPC3 and Panc1, and observed significant apoptosis induction in both cell lines. They identified PAK7, CSNK2A1, MAP3K7, AK1, and GRAF as the most potent kinases, whose inhibition increased apoptosis by >2.5-fold in MiaPaCa-2 cells. While PAK7, CSNK2A1, and MAP3K7 are known to be involved in the regulation of apoptosis, AK1 and GRAF are yet to be recognized as survival kinases in pancreatic cancer.

A couple of recent studies using RNAi in pancreatic cancer target identification have been published that couple genomic studies with RNAi functional validation. Kuuselo and colleagues utilized a loss-of-function RNAi screen to augment copy number and expression analysis of the novel amplified region at 19q13 in pancreatic cancer (Kuuselo et al. 2007). They used a focused siRNA library targeting the 19 known genes within the 1.1 Mb amplicon to screen the amplified Panc1 and non-amplified MiaPaCa-2 pancreatic cancer cell lines. Five genes were identified whose down-regulation led to significantly decreased cell viability in the Panc1 cell line. They determined *IXL* to be a novel amplification target gene that is essential for the growth and survival of a subset of pancreatic carcinomas with 19q13 amplification.

Table 8.3 Publications utilizing RNAi for target identification

Cell line	siRNA or shRNA library	Library size	Gene coverage	High-confidence hits	Key pathways or genes	Reference
Altered primary human BJ fibroblast cells, BJ-TERT-tsLT	shRNA	23,742	Genome-wide (7,914)	5	*RPS6KA6, HTATIP, HDAC4, KIAA0828,* and *CCT2*	Berns et al. (2004)
4 diffuse large B-cell lymphoma (DLBCL) cell lines: OCI-Ly3, OCI-Ly10, OCI-Ly7, and OCI-Ly19	shRNA	3–6 sequences per target	2,500	15	Regulators of the NF-κB pathway including *IKBKB, CARD11, MALT1,* and *BCL10*	Ngo et al. (2006)
Human colon cancer cell lines DLD-1 and HCT116, human breast cancer cell line HCC1954, and normal human mammary epithelial cells (HMECs)	shRNA	8,203	2,924	19	*PLK1, CDC2, UBE21, COPS2, EIF3S6,* etc.	Schlabach et al. (2008)
Pancreatic cancer cell line, MIA PaCa-2	siRNA	1,290	Kinase (645)	56	*PAK7, CSNK2A1, MAP3K7, AK1, GRAF,* etc.	Giroux et al. (2006)
Pancreatic cancer cell lines, PANC-1 and MIA PaCa-2	siRNA	76	19	5	*IXL, LRFN1, PLEKHG2, GMFG,* and *SUPT5H*	Kuuselo et al. (2007)
Pancreatic cancer cell line, 8988T	shRNA		*GATA6, C180RF45, RIOK3,* and *C180RF8*	1	*RIOK3* (PAK4, Rho family GTP-binding proteins)	Kimmelman et al. (2008)

An additional illustration of this is highlighted in a recent study by Kimmelman et al. (2008). The authors were able to prioritize *RIOK3* as candidate oncogene from an amplified region at 18q11. The phenotypic endpoint in their siRNA interrogation of the genes in that region was cell migration. This study illustrates the power of siRNA (or shRNA) targeted libraries for functional validation of genes identified in cancer-specific regions of aberration, facilitating the identification of candidate disease genes in these genomic regions.

The studies discussed above provide evidence that RNAi represents a functional and unbiased strategy to accurately identify novel targets and pathways implicated in a number of disease states and processes. The potential therapeutic advancements that could be developed from RNAi technology are promising, especially for diseases with limited treatments and poor survival rates such as pancreatic cancer, and therefore we argue that this technology should be more aggressively pursued. For example, a panel of pancreatic cancer cell lines with different molecular contexts could be screened with an RNAi library in order to identify the common genes that when silenced increase apoptosis in pancreatic cancer cells. Further validation of these genes can be tested in normal human cells for the pancreatic cancer-specific gene targets. Colleagues at the Translational Genomics Research Institute have recently developed a novel cell line model in which RNAi can be used to aid in pancreatic cancer target discovery. This novel screening strategy is termed Pharmacological Synthetic Lethal Screening and seeks to find chemical agents that selectively target cells with a deletion in the *SMAD4* gene (Wang et al. 2006; Dai et al. 2007). They screened a library of 19,590 compounds against a pair of isogenic pancreatic cell lines, one with restored *SMAD4* expression as a control, to identify agents that are selectively lethal to the deficient cell line. One lead compound, UA62001, showed 4.6-fold selectivity in the cell line with the *SMAD4* deficiency when compared to the cell line expressing *SMAD4*. Currently, this synthetic lethal cell-line model is being screened in our laboratory against a whole genome siRNA library. The outcome of this study will be the identification of synthetic lethal genes that may serve as targets for therapeutic intervention in pancreatic cancer patients with *SMAD4* inactivation.

8.5 High-Throughput RNAi in Drug Discovery and Development and Its Application to Pancreatic Cancer

One particular interesting and powerful application of genome-wide RNAi focuses on screening for candidate sensitizer/resistance genes that are causally related to drug response (Duxbury et al. 2005; Iorns et al. 2007; Whitehurst et al. 2007). By systematically knocking down individual genes with RNAi in the context of drug exposure, important genes, pathways, and cellular processes that influence drug response can be identified (Fig. 8.2b). A number of publications have highlighted the utility of such RNAi chemical synthetic lethality screens in different cancer types to pinpoint genes, pathways and cellular processes that are involved in the

mechanism of action of particular drugs, including paclitaxel, tamoxifen, taxol, cisplatin, gemcitabine, and inhibitors against MDM2, Akt, and PARP (Table 8.4).

MacKeigan and colleagues performed a large-scale RNAi screen on HeLa cervical carcinoma cells targeting 650 kinase genes and 222 phosphatase genes in the presence or absence of low-dose paclitaxel (Taxol), cisplatin, and etopiside, all agents known to induce apoptosis, in order to identify genes that regulate cell survival and apoptosis (MacKeigan et al. 2005). Additionally, a genome-scale siRNA screen to identify genes that when silenced increase the sensitivity of human tumor cells to cisplatin was reported (Bartz et al. 2006). A number of genes involved in DNA repair or checkpoint activation were identified and confirmed as sensitizers of cisplatin. Furthermore, the authors utilized HeLa isogenic cell pairs with or without TP53 function and determined that silencing of BRCA1/2 pathway genes selectively sensitizes the TP53-deficient cells to cisplatin. Brummelkamp and colleagues performed a loss-of-function shRNA barcode screen in MCF-7 cells consisting of 23,742 distinct shRNAs targeting 7,914 human genes in the presence or absence of nutlin-3, a small-molecule inhibitor of MDM2. They identified several targets that had known or predicted functions in the p53 pathway (Brummelkamp et al. 2006). Their work suggests that MDM2 inhibitors would be useful for treatment of tumors harboring both wild-type p53 and activated DNA damage signaling; however, this treatment may not be specific enough to eliminate the potential harmful effects it could also cause to normal cells.

Morgan-Lappe and colleagues screened the PTEN-deficient renal carcinoma cell line 786-O, known to have constitutive Akt activity, against an siRNA library targeting kinases, in the presence or absence of a low dose of the selective Akt inhibitor, A-443654. A total of 105 potential hits were identified as significant hits showing increased cell killing, including the top hits, CSNK1G3 and IPMK (Morgan-Lappe et al. 2006). Further investigation revealed that CSNK1G3 and IPMK inhibit Akt Ser-473 phosphorylation, thus sensitizing the cells to A-443654. Due to the heterogeneity of most cancer tumors, the development of multitargeted therapies including kinase inhibitors, through RNAi screens, may contribute to greatly improved cancer therapeutics. Also, Turner and colleagues screened CAL51, a diploid TP53 wild-type breast cancer cell line, against an siRNA library targeting 779 human kinase and kinase-associated genes in the presence or absence of the PARP inhibitor, KU0058948. *ATR*, *ATM* and *CHK1* were previously identified as determinants of KU0058948 sensitivity and they identified six more novel sensitizers (Turner et al. 2008). RNAi screens prove to be a useful tool to identify novel genes that enhance the response of current chemotherapeutic agents. Interestingly, *CDK10* was previously reported as a determinant of tamoxifen resistance via high-throughput RNAi screening (Iorns et al. 2008). To further identify targets and small-molecule inhibitors that cause tamoxifen sensitization, Iorns and colleagues performed parallel RNAi and compound screens in the MCF-7 cell line against an RNAi library targeting 779 kinases and related proteins (Iorns et al. 2009). The top hits from both the RNAi screen and the compound screen implicated components of the PDK1 signaling pathway as tamoxifen sensitizers. Two compounds were identified as the most potent sensitizers to tamoxifen, triciribine and tetrandrine. Their

Table 8.4 Publications utilizing RNAi to elucidate drug mechanisms

Cell line	siRNA or shRNA library	Library size	Gene coverage	Drug	High-confidence hits	Key pathways or genes	Reference
Non-small-cell lung cancer cell line, NCI-H1155	siRNA	84,508	Genome-wide (21,127)	Paclitaxel	87	Macromolecular complexes, receptor-ligand pairs, proteasome components, microtubule function	Whitehurst et al. (2007)
Breast cancer cell line, MCF7	siRNA	3,116	Kinase and related (779)	Tamoxifen	20	PDK1 signaling pathway	Iorns et al. (2009)
Human cervical carcinoma cell line, Hela	siRNA	1,300	Kinase (650)	Taxol, cisplatin and etopiside	21	*JIK, PLK2, mTOR, PINK1*	MacKeigan et al. (2005)
Pancreatic adenocarcinoma cell line MIA PaCa-2	siRNA	645	Kinase	Gemcitabine	83	*MAPKAP1, MAK, PAK4, ADRBK1, PIK3CG*	Giroux et al. (2006)
Breast cancer MCF-7 Cell	shRNA	23,742	Genome-wide (7914)	Nutlin-3 (MDM2 inhibitor)	14	p53 pathway	Brummelkamp et al. (2006)
Human cervical carcinoma cell line, Hela	siRNA	60,000	Genome-wide (20,000)	Cisplatin	53	DNA repair, checkpoint activation	Bartz et al. (2006)
PTEN-deficient renal carcinoma cell line, 786-O	siRNA	1,521	Kinase (507)	A-443654 (Akt inhibitor)	105	*CSNK1G3* and *IPMK*	Morgan-Lappe et al. (2006)
Breast cancer cell line, CAL51	siRNA	3,116	Kinase (779)	KU0058948 (PARP inhibitor)	9	*ATR, ATM, CHK1, CDK5, MAPK12, PLK3, PNKP, STK22C, STK36,* and DNA repair	Turner et al. (2008); Lord et al. (2008)

use of parallel genetic and chemical screens proves to be an effective approach to target identification.

In addition to the identification of drug response genes and processes, RNAi technology also enables one to identify rational combination therapeutic strategies. For example, Whitehurst and colleagues carried out one early study using RNAi, coupled with drug exposure, to identify gene-specific modulators of drug response but also combination therapies, by a genome-wide synthetic lethality screen in a non-small-cell lung cancer (NSCLC) cell line (Whitehurst et al. 2007). They used a library of 84,508 siRNAs targeting 21,127 unique human genes in combination with a non-lethal dose of paclitaxel. A panel of 87 genes was identified as high-confidence hits using stringent statistical analysis. A variety of functional relationships were represented among the candidates including macromolecular complexes, receptor–ligand pairs, core components of the proteasome, and microtubule function. They confirmed a few targets as sensitizers to paclitaxel, even at very low concentrations, in a tumor-specific fashion. Among these candidates, available compounds with these targets were sought and tested for their application in novel combinatorial therapeutic regiments. Other than the known clinical combinatorial effect of bortezomib, a proteasome inhibitor, and paclitaxel, they also demonstrated the synergy between a vacuolar ATPase (V-ATPase) inhibitor and low concentration of paclitaxel in a cell-line model. Further validation revealed that the synthetic salicylihalamide derivative, RTA 203, previously identified as an anti-tumor agent, in combination with low-dose paclitaxel, showed significant collaborative impact on the viability of NCI-H155 cells. These results underscore the power of genome-wide synthetic lethality screens to predict productive combination therapies.

In pancreatic cancer, gemcitabine is the drug of choice but, unfortunately, many patients are resistant to such treatment. Giroux and colleagues performed an RNAi screen of the human kinome and wanted to identify kinases whose inhibition would increase gemcitabine-induced apoptosis (Giroux et al. 2006). They screened 645 human kinase genes in MiaPaCa-2 cells in the presence of a low dose of gemcitabine. They identified 83 kinases whose inhibition increased gemcitabine-induced apoptosis by 50% or more. The most potent sensitizers were determined to be *MAPKAP1*, *MAK*, *PAK4*, *ADRBK1*, and *PIK3CG*. In our group, we performed a similar chemical synthetic lethality screen across the druggable genome library. Among the hits that sensitize to gemcitabine, *CHK1* upon inhibition dramatically increased the sensitivity of MiaPaCa-2 cells to gemcitabine (David Azorsa, personal communications). This discovery has also been published previously (Morgan et al. 2005). In addition, we tested a few chemical inhibitors against Chk1 and we observed significant synergistic effects with low concentrations of gemcitabine. Based on these observations, a Phase I clinical trial using combination of gemcitabine and a Chk1 inhibitor, LY2603618 (aka IC83) in pancreatic cancer patients is currently under evaluation. These examples demonstrate the utility of high-throughput RNAi technology to profile clinical drugs in order to identify the genes that increase the drug response, as well as viable inhibitors of these targets to enhance treatment for pancreatic cancer patients, which will shed light on clinical studies. Similarly, these sensitizer candidates can also serve as biomarker candidates; namely, once validated, the patients

who do not express the sensitizer genes would be predicted to have a stronger response to the drug treatment compared to the ones who do have the expression. Hence, this can help guide patient enrollment in clinical trial design.

8.6 Concluding Remarks

There is an urgent need for the identification of new therapeutic targets and more effective treatment approaches for pancreatic cancer. Unfortunately, traditional approaches to population-based cancer genetics have generated far too many associated candidate genes, making it difficult to prioritize those functionally relevant targets that have true translational potential. The problem of understanding the biological role of the thousands of different aberrations in pancreatic tumors is further compounded by cellular heterogeneity at the tumor level, structural diversity at the genomic level, and systems complexity at the molecular level. Global profiling approaches to evaluate large sets of genes across a cancer genome for their functional role in regulating cellular processes, such as cell survival and drug response, are therefore critical in helping prioritize target genes and pathways to focus on. With the recent advances in technical rigor and data quality, the application of RNAi on a genome scale is proving to be a powerful cellular genomics technology that holds great promise for advancing pharmacologically relevant targets in pancreatic cancer research. Genomic techniques, such as microarrays and array CGH, are powerful at identifying and prioritizing candidate genes and pathways, but functional validation approaches are needed to confirm them as candidate targets or members of resistance/sensitivity pathways. Future research exploiting this approach will no doubt lead to more effective ways to diagnose and treat this devastating disease.

In the examples presented here and the many published examples of HT-RNAi analysis, it is becoming apparent that unbiased systematic phenotypic evaluation of synthetic gene states (knockdowns) can reveal genes and pathways involved in cancer cell survival and response to chemotherapeutic agents, further indicating that such approaches have great promise for breakthroughs in pancreatic cancer research.

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Chapter 9
MicroRNA Profiling and Its Application in Drug Discovery in Pancreatic Cancer

Aarati R. Ranade and Glen J. Weiss

Abstract MicroRNAs (miRNAs) are single-stranded small RNA molecules of 21–23 nucleotides in length, involved in regulation of gene expression. An emerging number of studies show that miRNAs play a vital role in important signaling pathways in human oncogenesis. miRNAs can be utilized as potential biomarkers for cancer diagnosis and also as candidates in cancer treatment drug development. Pancreatic cancer is a lethal disease and is the fourth leading cause of cancer-related deaths in the United States. One key factor that can make an impact is earlier diagnosis of the disease, when surgery could offer patients a chance of cure. Use of miRNAs for early detection and drug development offers a unique opportunity. Identification and assessment of miRNA functionality that are differentially expressed in pancreatic cancer originates from methods for miRNA profiling such as miRNA microarray, in situ hybridization, and various computational algorithms applied for data analysis. This chapter discusses the various profiling methods applied to the identification of differentially expressed miRNAs in pancreatic cancer, which can applied towards drug development for pancreatic cancer.

9.1 Introduction

MicroRNAs (miRNAs) are single-stranded small RNA molecules of 21–23 nucleotides in length, involved in the regulation of gene expression (Bartel 2004; Fabbri et al. 2008). In animals, several reports have documented the involvement of miRNAs in the regulation of cell signaling pathways, apoptosis, metabolism, cardiogenesis and neural development. In addition, misregulation of miRNA expression has been linked to many types of cancer (McManus 2003; Dalmay and Edwards

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2006; Esquela-Kerscher and Slack 2006; Bandres et al. 2007; Cho 2007). In general, miRNAs function by post-transcriptional silencing of protein translation and suppression of target mRNA transcription.

Several hundred miRNAs have been characterized, and each miRNA can potentially regulate several hundred targets. In animals, studies indicate that miRNAs recognize their targets mainly through limited base-pairing interactions between the 5′-end of the miRNA (nucleotides 2–8, the seed region) and the complementary sequence in the 3′ untranslated regions (3′UTRs) of the target mRNAs. miRNAs do not function as naked RNA. Instead, they are integral components of ribonucleoprotein complexes (RPNs). A common constituent of all miRNA-containing RPNs (miRPNs) are members of the Argonaute protein family. There are various mechanisms of miRNA-mediated regulation, including translational inhibition, increased mRNA de-adenylation and degradation and mRNA sequestration.

In 1993, Lee, Feinbaum and Ambros discovered that lin-4 in *Caenorhabditis elegans* did not code for a protein but instead produced a pair of short RNA transcripts. These RNA transcripts regulate the timing of larval development by translational repression of lin-14, which encodes for a nuclear protein (Lee et al. 1993). They postulated that the complementary nature of sequences between lin-4 and unique repeats within the 3′UTR of lin-14 mRNA is one of the reasons for this protein regulation. The downregulation of lin-14 at the end of the first larval stage initiates the developmental progression into the second larval stage.

In 2000, a second miRNA, let-7, was discovered. Similar to lin-4, the let-7 miRNA also regulated the developmental timing in *C. elegans* (Reinhart et al. 2000). Since the discovery of let-7, thousands of miRNAs have been identified in various organisms including viruses, worms and primates using random cloning, sequencing and computational predictions.

9.2 miRNA Profiling

The identification and characterization of miRNAs is a rapidly growing area of research because miRNAs are involved in a variety of processes such as development, cell proliferation and death (Poy et al. 2004; Yekta et al. 2004; Zhao et al. 2005), and have been linked to oncogenesis. A common approach to identify key miRNAs is to measure miRNA expression by the cloning and sequencing of miRNA from specific tissue or cells or by microarray analysis.

The cloning and sequencing method is mainly used for the discovery of new miRNAs, while microarray profiling is a high-throughput method primarily used to measure expression of known miRNAs. With the ability to perform high-throughput expression profiling, hundreds to thousands of miRNAs can be simultaneously measured. If a miRNA is found to be differentially regulated in a certain tissue or cell type, it could play a regulatory role in defining tissue specificity or cell type. Additionally, if a miRNA is expressed at a specific developmental stage, then it may be involved in regulating developmental timing. Of course, validation of the function of the identified key miRNAs is required.

After the discovery of lin-4 and let-7, many more miRNAs have been identified using various experimental and computational techniques. In the most recent database (Sanger miRBase 14.0), approximately 904 validated miRNAs have been identified in the human genome (Paez et al. 2004).

A number of miRNA profiling strategies have been used to document miRNA expression changes during physiological and pathological processes. miRNA high-throughput analysis has become a field of research of its own. Array technology that was initially created for cDNA expression profiling was applied to expression profiling of miRNAs (Liu et al. 2008a, b). Krichevsky et al. described a dot blot method (Krichevsky et al. 2003). Liu et al. reported an optimized method which detects both mature miRNA and their precursor forms (Liu et al. 2008a). Due to sensitivity issues, the size of oligo arrays have been scaled down considerably from hand-held spotting devices to robotics that can print microscopic oligo dots onto a glass platform and later by direct synthesis through a photochemical process onto a quartz platform (Liu et al. 2008b). Retrotranscription and subsequent amplification through polymerase chain reaction (PCR) addresses the sensitivity issue and has taken profiling to the next level where one can profile using only nanograms of total RNA as starting material (Schmittgen et al. 2008). Since then, new techniques have been developed by innovating new approaches that ultimately are based on these fundamental methods, including the ability to perform full miRNA profiling using a single cell (Tang et al. 2006). Various methods used for miRNA profiling are discussed below (Fig. 9.1).

9.2.1 Microarray Technology

The basic principle of oligo microarray technology is based on the Watson−Crick base-pairing nature of nucleic acids. In this approach, the synthesized antisense probes are spotted and immobilized onto a support platform, which is made up of nylon or glass (Krichevsky et al. 2003). Probes designed to differentiate between mature miRNA and pre-miRNA and probes that detect hypothetical miRNAs can all be spotted onto the same array.

9.2.2 Bead-Based Method Using Flow Cytometry

The bead-based profiling method involves both amplification and hybridization and requires flow cytometry for analysis. Capture probes for a specific miRNA are synthesized and attached to a bead that is uniquely coded by a mixture of two fluorescent dyes for identification. Total RNA are enriched for short RNAs. Adaptor oligos are then ligated to both the 3′ and 5′ ends of enriched RNAs. Primers with sequence specific to adaptors are used to create a library of cDNAs through reverse transcription. Finally, a PCR reaction using primers antisense to the adaptors is performed to amplify the population of cDNAs. An important feature of this

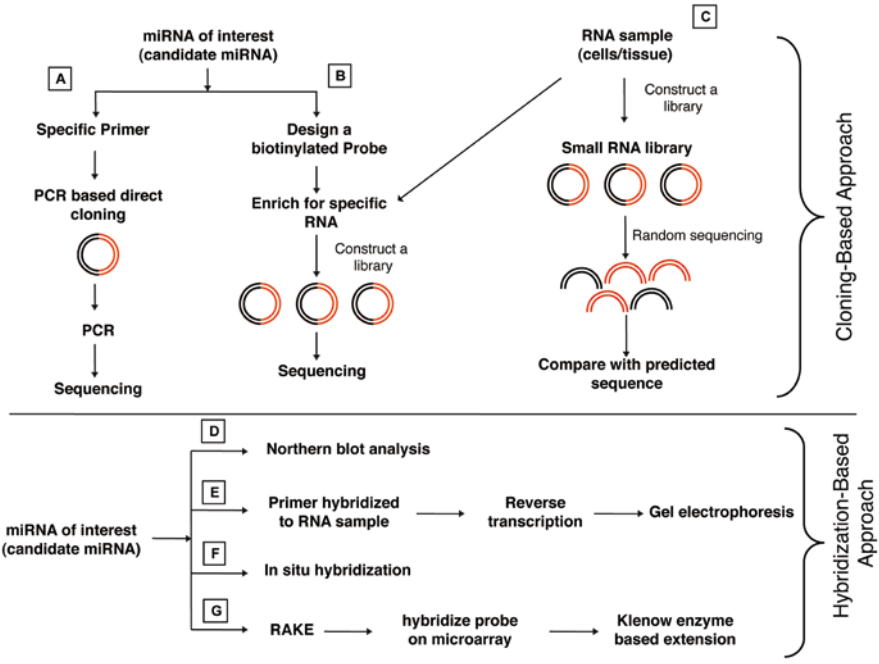

Fig. 9.1 Methods used for miRNA profiling. The figure shows various approaches used for miRNA profiling. The *top* portion depicts cloning-based approaches: (**A**) primers designed to overlap the sequence of the predicted miRNA along with the adaptor can be used to amplify specific miRNAs of interest from a cloned library, (**B**) a biotinylated probe is designed and used to enrich the RNA samples followed by contraction of cloned library, and (**C**) miRNA can be validated from a pool of cloned libraries by the random sequencing method. The *bottom* portion of the figure depicts various hybridization-based approaches. Probes designed for specific miRNA candidates are used for (**D**) northern blot analysis, (**E**) primer extension where primers are either hybridized to RNA sample, (**F**) in situ hybridization is performed, or (**G**) hybridized to probes on microarray

step is the use of biotinylated PCR forward primers to tag each PCR duplex with a biotin molecule, which can enzymatically react with streptavidin-phycoerythrin to emit a colored reaction that can be measured by flow cytometry. The resulting PCR product is hybridized to a mix of fluorescent beads that make up a miRNA library. This mixture is then run through a flow cytometer to analyze both fluorescence and streptavidin-phycoerythrin intensity. The fluorescent bead will indicate the specific miRNA probe, whereas the streptavidin-phycoerythrin will indicate the quantity of a specific miRNA (Lu et al. 2005).

9.2.3 Cloning Methods—miRNA Serial Analysis of Gene Expression

miRNA serial analysis of gene expression (miRAGE) uses an amplification-based method not only to profile but potentially to identify new miRNAs. The initial part

of the method resembles the bead-based method in that linkers are ligated to both 5′ and 3′ ends of enriched small RNAs for reverse transcription. A PCR reaction is carried out on the resulting cDNA mix, with the help of biotinylated primers. The linkers which now contain biotins are cleaved from PCR products. The mixture containing amplified small RNA sequence and biotinylated linkers is run through a column of streptavidin-coated beads for purification. Streptavidin acts as a magnet to bind biotin-tagged linkers. The eluted product yields purified small RNAs, which can then be cloned and sequenced for analysis (Cummins et al. 2006).

9.2.4 RNA-Primed Array-Based Klenow Enzyme Assay

RNA-primed array-based Klenow enzyme assay (RAKE), is another method that involves both hybridization and amplification. However, in this method the PCR reaction does not amplify the sample, but amplifies the signal. An oligo with a 5′ spacer is covalently linked onto a glass platform. The spacer sequence is followed by a miRNA antisense capture probe with three thymidine residues in between. RNA samples are hybridized to this array. miRNAs in the sample would bind to their specific probe and form a double-stranded structure. The addition of exonuclease-1 will only degrade unbound single-stranded oligos. The miRNAs that have latched onto its probe will act as a primer. Subsequent PCR will result in the addition of biotin-conjugated dATPs onto the spacer template, which emits an amplified signal without PCR amplification of the original RNA sample (Nelson et al. 2004).

9.3 Study of miRNA in Pancreatic Cancer

Pancreatic ductal adenocarcinoma is a very aggressive malignancy that disseminates rapidly. It is a lethal disease and is the fourth leading cause of cancer-related deaths in the United States with an annual incidence of roughly 38,000 cases. There are no early signs and symptoms so the disease tends to be diagnosed in advanced, incurable stages. Thus, the prognosis of pancreatic cancer is the worst of all cancers, with a mortality/incidence ratio of 0.99 and a 5-year survival rate at less than 4% (http://seer.cancer.gov; Schutte et al. 1997). Furthermore, pancreatic cancer is relatively resistant to many forms of therapy. Recent progress in the use of chemotherapy and selected target therapeutics has made a modest improvement in the survival of patients with advanced disease. There is clearly a need for development of an early detection strategy that may allow for potential curative treatment. The ability to intervene in the early stage of pancreatic cancer may improve survival for thousands of patients each year. The successful development of early detection strategies for screening of pancreatic cancer is based on the identification of biomarkers associated with early pancreatic adenocarcinoma thus enabling high-risk populations to be targeted. Several new approaches have been taken in the search for biomarkers for pancreatic cancer (Grote and Logsdon 2007). The serum

glycosylation marker CA19-9 is the most commonly used marker in pancreatic cancer. New blood biomarkers include CEACAM1 and MIC-1 (Gold et al. 2006; Simeone et al. 2007). RNA levels in blood and DNA methylation biomarkers in pancreatic juice and pancreatic cancer have yielded some promising findings.

miRNAs are considered a new source of potential biomarkers for early detection of pancreatic adenocarcinoma (Bloomston et al. 2007; Lee et al. 2007). Lee et al. performed miRNA expression profiling in clinical specimens of pancreatic adenocarcinoma (Lee et al. 2007). A real-time quantitative PCR assay was used to profile the expression of 201 miRNA precursors, representing 222 miRNAs in 28 tumors, 15 adjacent benign tissue, four chronic pancreatitis specimens, six normal pancreas specimens and nine pancreatic cancer cell lines. The miRNA precursor expression data was filtered using multigroup ANOVA. Hierarchical clustering of samples and genes was performed on the resulting 112 miRNAs. The filtered data were also analyzed using expression terrain maps. This analysis showed that each of the clusters of the samples occupies distinct regions on the expression terrain maps. Each of the 4 groups of samples has distinct patterns of miRNA expression and this suggests that there are subsets of miRNAs that discriminate between benign and pancreatic cancer. Prediction analysis of microarrays algorithm (PAM) was used to determine if the miRNA expression data could predict which class of samples fit (cancer or benign) and to determine the most important, differentially expressed miRNAs related to pancreatic adenocarcinoma. Twenty miRNAs were found to be differentially expressed, of which miR-424, miR-301, miR-100, miR-221, miR-125b-1, and miR-212 were found to be upregulated while miR-345, miR-142-p, and miR-139 were downregulated in pancreatic adenocarcinoma.

Table 9.1 MicroRNAs differentially expressed in pancreatic cancer

miRNA	Expression in pancreatic cancer	Type of sample studied	Method of miRNA profiling	References
miR-21	- Overexpression in pancreatic cancer - Increased expression predicts limited survival in patients with node-negative disease	Formalin-fixed paraffin-embedded (FFPE) tissue	In situ hybridization	Dillhoff (2008)
miR-21, miR-221, miR-222, miR-181a, miR-181b, miR-181d, miR-155	Overexpressed in tumor samples	Microdissection of FFPE tissue	- miRNA microarray - qRT-PCR - Northern blots	Bloomston (2007)
miR-375, miR-494, miR-148a, miR-148b	Downregulated in tumor samples	Microdissection of FFPE tissue	- miRNA microarray - qRT-PCR - Northern blots	Bloomston (2007)

Developing less invasive biomarkers for early screening and detection of pancreatic cancer remains a clinical challenge. Blood serum is a minimally invasive, low risk and easy to obtain biofluid which can also be explored as a potential biomarker for screening (Lowery et al. 2008). Therefore, miRNA expression in serum holds promise as a screening tool for differentiation between various cancers and stratification for early intervention and surveillance.

A growing body of literature supports a link between altered miRNA expression and cancer. This includes miR-15a and miR-16-1 in chronic lymphocytic leukemia (Calin et al. 2004a, b), miR-143 and miR-145 in colorectal cancer (Michael et al. 2003), let-7 in lung cancer (Takamizawa et al. 2004; Johnson et al. 2005) and miR-155 in diffuse large B cell lymphoma (Eis et al. 2005). A summary of the miRNAs that are differentially expressed in pancreatic cancer is given in Table 9.1.

9.4 Application of miRNA Profiling in Drug Discovery in Pancreatic Cancer

Most pancreatic cancer patients have advanced or metastatic disease at the time of diagnosis. Complete resection is the only potentially curative option for patients with pancreatic cancer, and there is an urgent need for new and more effective therapies for patients with metastatic disease. In a phase III trial of patients with advanced disease, gemcitabine was found to be better than fluorouracil (5-FU) in alleviating pain, functional impairment and weight loss and was also associated with better survival. Thus gemcitabine-based therapy is an FDA-approved treatment option for both unresectable locally advanced and metastatic pancreatic cancer (Burris et al. 1997). Recently, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, erlotinib, in combination with gemcitabine, was shown to be modestly superior to gemcitabine alone (Moore et al. 2007). In non-small cell lung cancer, the presence of K-ras mutation is nearly always associated with lack of EGFR tyrosine kinase inhibitor efficacy. (Soung et al. 2005; Gallegos Ruiz et al. 2007; Liu et al. 2007; Kalikaki et al. 2008; Linardou et al. 2008). Strategies that target K-ras mutation are thought to have therapeutic potential in pancreas cancer as K-ras mutations are observed in 75–100% of these tumors (Li et al. 2004). miRNA biomarkers may further define the patient subpopulation that may derive benefit from this combination therapy or those who derive little if any benefit and should consider another systemic therapy option.

As gemcitabine-based chemotherapy is a common treatment for pancreatic cancer, studying the role of miRNAs perturbed by gemcitabine-based chemotherapy will provide useful insights in drug discovery for pancreatic cancer. A research study conducted by Meng and co-workers aimed at identifying specific miRNAs involved in the regulation of cholangiocarcinoma growth and response to gemcitabine-based chemotherapy (Meng et al. 2006). In this study, miRNA expression in malignant and nonmalignant human cholangiocytes was assessed using a microarray. Expression of selected miRNAs and their precursors was evaluated

by Northern blots and PCR, respectively. The effect of selected miRNAs on cell growth and response to chemotherapy was assessed using miRNA-specific antisense oligonucleotides to decrease miRNA expression or with precursor miRNA to increase cellular expression. The results from this study showed that a cluster of miRNAs, miR-200b, miR-21, and miR-141, were highly over-expressed in malignant cholangiocytes. Inhibition of miR-21 and miR-200b increased sensitivity of gemcitabine, while inhibition of miR-141 decreased cell growth. Treatment of tumor cell xenografts with systemic gemcitabine altered the expression of a significant number of miRNAs. miR-21 was shown to modulate gemcitabine-induced apoptosis by phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-dependent activation of PI 3-kinase signaling.

This study provides potential target genes to explore that are modulated by selected miRNAs after drug treatment in other cancer types, including pancreatic cancer.

Pain is a common presentation in pancreatic cancer patients. miRNAs have been shown to be associated with pain and pain treatment. Using real-time PCR, Guang Bai et al. reported that miR-10a, -29a, -99a, and -124a were down-regulated in the ipsilateral mandibular division (V3) of the trigeminal ganglion within 4 h after injection of complete Freund's adjuvant (CFA) in rats, thus showing the involvement of miRNAs in pain (Bai et al. 2007). Pancreatic cancer patients with pain may have particular altered miRNAs which may serve as markers for disease or targets for therapy.

9.5 Concluding Remarks

The study of miRNAs for the detection and treatment of cancer is an exciting and rapidly growing field. Since the discovery of miRNAs in 1993, a robust scientific effort has been undertaken to understand the function of miRNAs in cells and their role in developmental biology along with the regulation of miRNAs.

Pancreatic cancer is a lethal disease with the worst prognosis of all the cancers. Thus, early detection is the key for improved treatment and better survival of patients with pancreatic cancer. Several miRNAs are found to be differentially regulated in pancreatic cancer when profiled using various miRNA profiling techniques such as miRNA microarray and in situ hybridization. These unique miRNA profiles in pancreatic cancer look promising in the quest to identify early detection markers for pancreatic cancer as well as drug development for this lethal disease.

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Chapter 10
Methylation Detection and Epigenomics in Pancreatic Cancer

Georg Feldmann and Anirban Maitra

Abstract Pancreatic cancer has both genetic and epigenetic underlying causes. The importance of epigenetic alterations in the formation and maintenance of malignant tumors has become apparent in the last decade, with accumulating evidence suggesting this is probably the most common clonal aberration in human neoplasia. Identifying epigenetic alterations in pancreatic cancer has not only enhanced our understanding of pancreatic cancer biology, but has also opened up avenues for the development of early detection and novel therapeutic strategies.

In this chapter, an overview of the current literature on epigenetic alterations found in pancreatic cancer is presented and discussed in the light of potential therapeutic applicability as well as pointing out possible future directions of studies combining global genetic and epigenetic analyses.

10.1 Introduction

10.1.1 Pancreatic Cancer Is a Genetic Disease

Cancer is traditionally viewed as a genetic disease, and pancreatic cancer is no exception (Vogelstein and Kinzler 2004). Only a concise overview of the most common genetic alterations found in pancreatic cancers alongside epigenetic changes can be given due to limitations in space; for more comprehensive reviews of molecular genetics of pancreatic cancer see (Feldmann and Maitra 2008; Maitra and Hruban 2008). Methods for global analysis of transcriptomic alterations and of

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DNA copy number changes in pancreatic cancer are discussed in depth in separate chapters of this book.

Activating mutations of *KRAS2* on chromosome 12p represent the most common oncogene mutations in pancreatic cancer and are found in 80–100% of pancreatic cancers, usually point mutations in codon 12, 13 or 61, which abolish the intrinsic GTPase activity of its encoded product, and lead to constitutive activation of the intracellular signaling cascade (Caldas and Kern 1995; Jones et al. 2008). The most well-described downstream effector pathways of oncogenic *KRAS2* are the RAF/MEK/ERK, PI3K/AKT and RALGDS/RAL signaling axes (Feldmann and Maitra 2008). *KRAS2* mutations are not only among the most frequently observed genetic alterations in pancreatic cancer, but also among those occurring extremely early during the multistep progression model leading towards development of a fully invasive cancer phenotype, and can already be found in approximately 30% of the earliest pancreatic intraepithelial neoplasia (PanIN-1) precursor lesions (Moskaluk et al. 1997; Hruban et al. 2000). The central importance of oncogenic *KRAS2* signaling is further underscored by the fact that among all genetically engineered mouse models of pancreatic cancer described, development of murine PanIN (mPanIN) lesions (Hruban et al. 2006) reminiscent of human PanINs was observed only in those models including pancreatic expression of a mutated KRAS allele, regardless of whether pancreatic precursor or adult differentiated cell compartments were targeted (Aguirre et al. 2003; Hingorani et al. 2003, 2005; Bardeesy et al. 2006; Bardeesy and Sharpless 2006; Habbe et al. 2008). In fact, pancreas-specific expression of an oncogenic *Kras2* allele was sufficient to induce formation of mPanIN lesions with high penetrance, which progressed to fully invasive pancreatic cancer in ~10% of cases after 6–8 months, although this relatively long latency suggests that additional genetic alterations have to be acquired for the mPanIN lesions to progress towards a fully malignant cancer phenotype (Hingorani et al. 2003). Moreover, functional studies by several independent groups from recent years have clearly shown that oncogenic *KRAS2* signaling is not only involved in pancreatic carcinogenesis, but also required for maintenance of pancreatic cancer cell growth (Aoki et al. 1995; Brummelkamp et al. 2002; Hirano et al. 2002).

Amplifications of *CMYC* and concomitant c-Myc protein overexpression are found in about 50–60% of pancreatic cancers, suggesting that oncogenic Myc signaling also plays a crucial role in pancreatic cancer (Han et al. 2002; Aguirre et al. 2004).

The product of the tumor suppressor gene cyclin-dependent kinase 2A (*CDKN2A/p16*) on chromosome 9p inhibits cell-cycle progression through the G_1-S checkpoint and is inactivated in more than 90% of pancreatic cancers, most commonly either through homozygous deletions (in about 40% of cases) or by intragenic mutations and loss of the second allele (in another ~40%) (Caldas et al. 1994; Schutte et al. 1997). Of note, in 10–15% of cases, p16 function is silenced by transcriptional inhibition due to promoter hypermethylation. Loss of nuclear p16 expression seems to increase with progression of PanIN precursor lesions towards fully invasive pancreatic cancer, and was observed by immunohistochemistry in 30% of PanIN-1, 55% of PanIN-2 and 71% of PanIN-3 precursor lesions (Wilentz et al. 1998).

TP53 on chromosome 17p, which mediates cell-cycle arrest and induces apoptosis upon DNA damage is inactivated in 50–75% of pancreatic cancers, which is

almost always caused by intragenic mutations in combination with loss of the second allele. Nuclear accumulation of the mutated protein, which can be detected by immunohistochemistry, has been used as a surrogate marker for loss of TP53 function and is usually only found in advanced PanIN-3 lesions and invasive cancers, suggesting that TP53 inactivation represents a later event in the multistep progression cascade towards pancreatic cancer (Maitra et al. 2003).

Deleted in pancreatic carcinoma 4 (*DPC4*/*SMAD4*/*MADH4*) is a gene whose function is lost in about 55% of pancreatic cancers (Hahn et al. 1996). This is due to somatic mutations and loss of the second allele in about 25%, and to homozygous deletions in about another 30% of cases. Loss of Dpc4 protein function leads to increased proliferation and reduced growth inhibition. Interestingly, loss of Dpc4 function appears to be fairly specific for pancreatic cancer and is rarely seen in other malignancies (Schutte et al. 1996). Dpc4 loss also represents a relatively late event in pancreatic carcinogenesis, found in only a minority of PanIN-3 lesions (Maitra et al. 2003).

10.1.2 Precursor Lesions to Pancreatic Cancer

In recent years it has become increasingly evident that pancreatic cancer does not originate de novo, but rather evolves via development of tangible, histologically defined precursor lesions. Similar to what has first been described in the adenoma-to-carcinoma sequence in colon cancer, a multistep progression model of genetic and epigenetic alterations can be observed in the transformation from non-invasive precursor lesions to invasive adenocarcinoma. (Hulst 1905; Vogelstein et al. 1988; Hruban et al. 2005). Although these lesions were first described over 100 years ago, a systematic effort towards developing consensus diagnostic guidelines and nomenclature has been initiated only in the last decade (Hruban et al. 2001, 2004) (see also http://pathology.jhu.edu/pancreas_panin). Three major subtypes of precursor lesions to pancreatic cancer have been described: pancreatic intraepithelial neoplasia (PanIN), the most common precursor lesion, and the two macroscopic (cystic) precursor lesions: intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). A comprehensive discussion of the morphology and molecular genetics of these precursor lesions is beyond the scope of this chapter; for further study the reader can find up-to-date reviews on this topic under (Feldmann et al. 2007a; Koorstra et al. 2008; Feldmann and Maitra 2009).

10.2 Epigenetic Alterations in Pancreatic Cancer

Although pancreatic cancer is traditionally viewed as a genetic disease, there is increasing evidence to suggest that it is equally justifiable to call it a disease of epigenetic abnormalities. It is well understood that epigenetic alterations provide an additional mechanism through which alterations in gene expression patterns can be governed, and which can contribute to the development of a malignant phenotype.

In pancreatic cancer, two general major forms of epigenetic alterations are thought to play important roles: firstly, histone modifications, mediated most commonly through acetylation or methylation of histone proteins or through the incorporation of alternative histone proteins, and secondly, direct methylation of cytosine groups within genomic DNA molecules.

10.2.1 Histone Modifications

Nucleosomes represent the basic constituents of chromatin, in which a section of a double-stranded DNA molecule, 146 bp in length, is wrapped approximately 1¾ times around an octamer of histone proteins, consisting of two copies each of histone proteins H2A, H2B, H3 and H4 (Luger 2003). Each nucleosome is separated from its neighbors by a DNA sequence of around 50 bp in length, which is packaged by the linker protein H1. All four histone proteins found in the cores of nucleosomes have positively-charged lysine-rich aminoterminal tails that protrude from the cores and that are prone to post-translational modifications. A variety of different forms of post-translational modifications at over 50 known sites have been described, including methylation, acetylation, citrullination, phosphorylation, SUMOylation and ADP-ribosylation (Jenuwein 2006; Kouzarides 2007; Iacobuzio-Donahue 2009). The most commonly observed modifications are methylation, acetylation and ATP-dependent chromatin remodeling, which play central roles in regulating the condensation status of chromatin, i.e. how loosely or densely certain chromatin regions are packed, and thus influence expression of genes encoded within these regions. The importance of histone modifications as epigenetic characteristics contributing to maintenance of a malignant cancer phenotype has become increasingly evident in recent years, with acetylation and methylation of lysine residues within the N-terminal tails of histone proteins being the best studied changes (Gibbons 2005; Wang et al. 2007; Iacobuzio-Donahue 2009).

10.2.1.1 Alterations in Histone Methylation

Histone methylation is most commonly observed in histone proteins H3 and H4. Methylation at certain positions within these proteins is correlated to activation of chromatin and enhanced gene transcription, for example H3-K4, H3-K36, or H3-K79, whereas others have been linked to gene silencing, like H3-K9, H3-K27 or H4-K20 (Sanders et al. 2004; Klose and Zhang 2007; Sims et al. 2007). Of note, different methylation states exist for each lysine residue, i.e. lysine can be methylated by replacement of one, two or all three N-standing protons to mono-, di- or trimethyllysine. In pericentromeric regions, enrichment of trimethylated H3-K9 is found as compared to mono- and dimethylated forms. The trimethylated form of H3-K27 occurs more frequently in the inactivated X-chromosome (Plath et al. 2003).

Methylation of histone proteins is mediated by histone methyltransferases (HMTs). There are at least 17 different HMTs known to date, all of which share a common evolutionary conserved (Su(var)3-9, enhancer-of-zeste, trithorax) motif. HMTs are not specific to histone proteins but are known to methylate other targets as well (Chuikov et al. 2004; Kouskouti et al. 2004; Ivanov et al. 2007; Iacobuzio-Donahue 2009). Histone methylation has been directly linked to DNA methylation, and both processes share some of the involved regulatory protein machinery, such as DNA methyltransferases (DNMT) and methyl binding proteins. Therefore it has been suggested that histone methylation might also mediate its effects on differential gene expression at least in part via associated alterations in the DNA methylation status at the respective sites (Fraga et al. 2005).

HMTs methylating H3-K4 and H3-K27 are frequently overexpressed in malignant neoplasias. Further evidence supporting the hypothesis of a causal contribution of HMTs in carcinogenesis comes from the discovery of genetic alterations within HMTs in malignant tumors. In Sotos syndrome, for example, which is caused by germline mutations of the histone methylase nuclear receptor binding SET domain protein 1 (NSD1), a ~170-fold increased risk of developing cancers is documented, including a propensity for developing Wilms tumors, neuroblastomas, acute lymphatic leukemia, hepatocellular carcinomas, and small cell lung cancers. Moreover, the translocation t(5;11)(q35;p15.5) found in childhood acute myelogenous leukemia often incorporates NSD1 (Jaju et al. 2001). The H3-K27 methyltransferase enhancer of zeste homolog 2 (EZH2), a member of the polycomb group complexes PRC2 and PRC3, is required for cell proliferation, and is frequently found to be overexpressed, amplified or rearranged in several malignant tumors. In some cases, the extent of EZH2 overexpression has been linked to disease aggressiveness (Visser et al. 2001; Varambally et al. 2002; Bracken et al. 2003; Kirmizis et al. 2003; Kleer et al. 2003). Suppressor of variegation 3–9 homolog 1 (SUV39H1), a H3-K9 methyltransferase predominantly targeting pericentromeric chromatin regions involved in regulating cell-cycle progression and genomic stability, has been linked to cancer development (Peters et al. 2001; Garcia-Cao et al. 2004). Another example of an HMT thought to be involved in the formation of cancers is PR domain-containing protein 2 (PRDM2/RIZ1), a H3-K9 methyltransferase that was originally recognized due to its interaction with retinoblastoma (RB), and that is found to be inactivated by promoter hypermethylation in several malignancies (Buyse et al. 1995; Chadwick et al. 2000; Tokumaru et al. 2003; Iacobuzio-Donahue 2009). Its probable functional relevance is further supported by the occurrence of inactivating somatic *RIZ1* mutations in cancer cells, as well as by the finding that *RIZ1* knockout mice carry an increased risk of developing B-cell lymphomas (Steele-Perkins et al. 2001; Kim et al. 2003a).

Of note, it is now known that histone methylation is not irreversible, as had originally been proposed. A recently identified enzyme lysine-specific demethylase 1 (LSD1) is capable of demethylating mono- or dimethylated forms of H3-K4, while it is unable to catalyze demethylation of trimethylated forms (Shi et al. 2004). Given the dynamic nature of regulation of gene transcription it is not beyond the realm of speculation to believe that additional histone demethylating enzymes are going to be discovered in the future (Iacobuzio-Donahue 2009).

In pancreatic cancer, a recent report described nuclear overexpression of the histone methyltransferase enhancer of zeste homolog 2 (EZH2) in 71/104 (68%) pancreatic cancer tissue specimens (Ougolkov et al. 2008). Moreover, RNAi-mediated knockdown of EZH2 led to decreased pancreatic cancer cell proliferation and enhanced sensitivity against doxorubicin and gemcitabine.

In another recent study, the histone methyltransferase genes *MLL* and *MLL3*, which methylate H3-K4, were found to be mutated in subsets of pancreatic cancers (Jones et al. 2008). A total of seven intragenic somatic mutations within *MLL3* were found in 4/24 (17%) of pancreatic cancer samples screened, including two nonsense, four missense and one synonymous mutation. In a subsequent prevalence screen on a larger panel of pancreatic cancers, another five somatic *MLL3* mutations were discovered in 5/90 (6%) cases. The same paper reported two *MLL* missense mutations within 1/24 (4%) cases of pancreatic cancer as well as one synonymous mutation of *MLL2* within this panel.

10.2.1.2 Alterations in Histone Acetylation

Acetylation of histone proteins H3 and H4 causes more loose packing of chromatin, which in turn facilitates transcription factor binding and enhanced gene transcription. This is believed to be due to neutralization of the positive charges of lysine residues within the N-terminal regions of H3 and H4, through which the electrophysical interaction with the negatively-charged phosphate backbone of the associated DNA strands is weakened (Margueron et al. 2005).

Histone acetylation states can change relatively rapidly and acetylation changes are thought to be a dynamic process, as opposed to methylation, which usually tends to accumulate more slowly and to be more stable over time (Shi 2007). The acetylation state of histone proteins is governed by two classes of enzymes: histone acetyltransferases (HATs), which acetylate lysine residues within aminoterminal tails of histone proteins, and histone deacetylases (HDAC), which catalyze the opposite reaction, i.e. deacetylation of lysine residues, thereby reconstituting positive charges and generally leading to a more condensed chromatin state and inhibiting gene transcription (Roth et al. 2001; Dokmanovic et al. 2007). Histone acetyl-transferases comprise members of the GNAT (Gen5-related *N*-acetyl transferase), MYST and p300/CBP families (Roth et al. 2001; Iacobuzio-Donahue 2009). Interestingly, different HATs show differential affinity towards N-terminal tails of specific histone proteins, and some can also acetylate other non-histone proteins, for example p53, E2F1, or GATA1 (Boyes et al. 1998; Martinez-Balbas et al. 2000; Luo et al. 2004). HDACs, on the other hand, are subdivided into three classes according to their similarity to the yeast homologs Rpd3 (class 1), Hda1 (class 2) or Sir2 (class 3). As observed with HATs, HDACs are also not entirely specific for histone proteins, but can also deacetylate other protein targets, including p53, E2F1, GATA1, TFIIE, TFIIF, or glucocorticoid receptors (Ito et al. 2000; Robertson et al. 2000; Deckert and Struhl 2001; Gray and Ekstrom 2001; Khochbin et al. 2001). Mutations of both HAT and HDAC family members have been linked to carcinogenesis. For

example, a subset of cases of Rubinstein−Taybi syndrome are caused by mutations within CBP (germline mutation of one allele and inactivation of the second allele by somatic mutation) and carry a more than 300-fold increased risk of developing cancer (Roelfsema and Peters 2007). Furthermore, somatic mutations have also been found in sporadic gastric and microsatellite instability-positive colorectal cancers, mostly nonsense or missense mutations, in combination with loss of the second allele (Muraoka et al. 1996; Ionov et al. 2004). In colorectal cancers, truncating HDAC2 mutations have also been described (Ropero et al. 2006).

In pancreatic cancer, HDAC7 was recently shown to be overexpressed at the mRNA level in 9/11 (82%) cases studied (Ouaissi et al. 2008). Of note, a variety of histone deacetylase inhibitors have been developed over recent years and proven to be valid candidate drugs for a wide spectrum of malignant tumors including pancreatic cancer (Bai et al. 2006; Ammerpohl et al. 2007; Arnold et al. 2007; Kell 2007; Neureiter et al. 2007; de Bono et al. 2008; Haefner et al. 2008). Some of these compounds are currently undergoing initial clinical evaluation (www.clinicaltrials.gov).

10.2.2 Global DNA Methylation Studies

The first studies by Feinberg and Vogelstein (Feinberg and Vogelstein 1983) and by Gama-Sosa and collegues (Gama-Sosa et al. 1983) reporting epigenetic alterations in human cancers on a molecular level described a global reduction in the DNA methylation content in colon and other human cancers as compared to non-neoplastic cells.

Methylation of mammalian cell DNA occurs only at cytosine bases which are directly followed by guanine (Iacobuzio-Donahue 2009). These specific dinucleotides are designated “CpG”, with “C” standing for the cytosine and “G” representing the guanosine nucleotide; “p” refers to the phosphodiester bridge linking these two nucleotides (Fig. 10.1). The CpG nucleotide is under-represented in vertebrate DNA and overall occurs only at 20–25% of the frequency expected from the respective DNA base composition (Josse et al. 1961; Swartz et al. 1962; Gardiner-Garden and Frommer 1987). The most commonly accepted explanation for this observation is seen in the tendency of the methylated form of cytosine (5-methylcytosine) to mutate to thymine through spontaneous deamination (Coulondre et al. 1978). Indeed, in areas with decreased CpG content a concomitant increase in TpG and complementary CpA dinucleotides can be found, supporting this hypothesis (Bird 1980; McClelland and Ivarie 1982; Smith et al. 1983; Tykocinski and Max 1984). However, it has long been observed that CpG dinucleotides are not distributed homogenously throughout the entire length of a DNA molecule, but that there are certain regions which show significantly higher frequencies of CpG, commonly referred to as CpG islands. An early definition described CpG islands as regions of at least 200 bp in length, with a GC content of more than 50% and a CpG frequency which is at least 60% or more of that expected stochastically from the DNA base composition (Gardiner-Garden and Frommer 1987). A reasonable explanation for

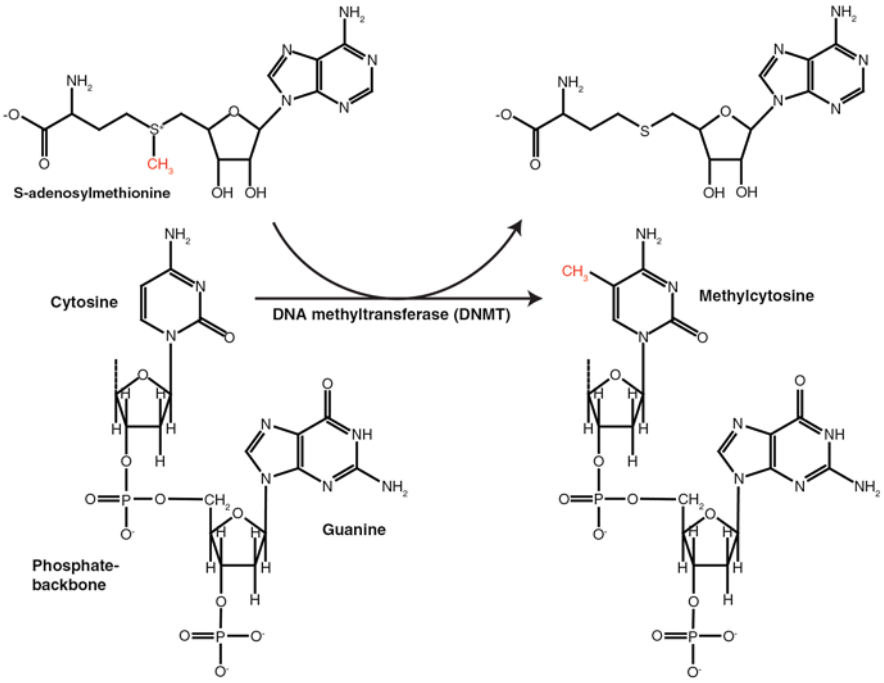

Fig. 10.1 Methylation of cytosine group within CpG dinucleotide by DNA methyltransferase, structure

the relatively higher frequency of CpG residues observed in these "islands" might be that this is due to a positive selection pressure in favor of the CpG dinucleotides because of important regulatory functions they might play in gene expression and cell homeostasis, and that they are thus evolutionary conserved within CpG islands (Cooper and Gerber-Huber 1985). CpG islands are mostly located within repetitive DNA regions, including microsatellite sequences, centromeres and within promoter regions, where they are differentially methylated (Jones and Baylin 2002; Klose and Bird 2006).

In the methylated state a methyl group is covalently bound to cytosine. This occurs through transfer of a methyl residue from *S*-adenosylmethionine to the C5 of the cytosine ring by members of the family of cytosine (DNA-5)-methyltransferases (DNMTs; see Fig. 10.1), which include DNMT1, DNMT3A, and DNMT3B (Iacobuzio-Donahue 2009). DNMT3A and DNMT3B have important roles for patterning of DNA methylation during embryogenesis, while DNMT1 is thought to maintain methyltransferase activity at later stages. The crucial importance of these enzymes for embryogenesis and development are exemplified by the finding that knockout mice for either one of the three are embryonic lethal (Okano et al. 1999; Ting et al. 2006b; Iacobuzio-Donahue 2009). There are several reports by different groups suggesting that in the setting of cancer there is some redundance between the three enzymes, since functional inhibition of DNMT1 does not markedly decrease

overall DNA and promoter methylation, and in most cases there is no significant re-expression of silenced tumor suppressor genes (Ting et al. 2004, 2006a; Iacobuzio-Donahue 2009).

At least three different mechanisms have been described through which DNA methylation can affect gene expression: firstly, methylation of CpG islands within promoter regions can interfere with binding affinity of transcription factor complexes, thus directly preventing transcriptional activation; secondly, hypermethylated DNA sequences can be recognized by methyl-CpG-binding domain protein (MBP) family members, ultimately leading to chromatin remodeling towards a more highly condensed state of chromatin regions and prevention of gene transcription; thirdly, HDACs can be recruited by DNMTs and cause transcriptional repression through histone deacetylation (Perini et al. 2005; D'Alessio and Szyf 2006; Iacobuzio-Donahue 2009). It is now known that tissue-specific methylation patterns of CpG islands exist and that some CpG islands undergo progressive methylation not only during carcinogenesis, but also in the normal process of aging (Bird 2002; Shiota and Yanagimachi 2002; Matsubayashi et al. 2005).

10.2.2.1 DNA Hypomethylation in Pancreatic Cancer

DNA hypermethylation is believed to contribute to maintaining DNA integrity by causing a more dense packing and functional inactivation of affected chromatin regions (Iacobuzio-Donahue 2009). Therefore it is not surprising that in non-neoplastic cells, areas of significant hypermethylation are frequently found in highly repetitive regions, many of which are thought to represent retroviruses that have been trapped in the genome, functionally inactivated by hypermethylation and hence been passed on in a methylated state (Walsh and Bestor 1999; Babushok et al. 2007).

One possible mechanism through which the overall decrease in global methylation observed in many cancers could contribute to the carcinogenic process might therefore be that the latent viral sequences which are evolutionarily conserved in a methylated form might be able to become re-expressed due to hypomethylation and potentially confer oncogenic stimuli. A well-documented example for this process in which re-expression of a latent virus can be linked to malignant tumor formation is that of human papillomavirus 16 (HPV16), where hypomethylation has been linked to activation of the HPV16 genome and progression of cervical dysplasia (Badal et al. 2003). Similar observations have been made for Epstein−Barr virus (EBV) in EBV-associated lymphomas (Hutchings et al. 2006).

A second, alternative mechanism might be that genes that are usually expressed at defined stages during embryogenesis and organ development, but at later stages are silenced by means of promoter methylation, can become re-expressed due to hypomethylation and might thus exhibit oncogenic properties when aberrantly expressed in differentiated adult tissues. There are several examples of genes for which this mechanism has been proposed. Cancer/testis antigen (CAGE) has been shown to be reactivated due to promoter hypomethylation in gastric and cervical cancers (Cho et al. 2003; Lee et al. 2006), as has CD30 in Hodgkin disease and

anaplastic large cell lymphomas (Watanabe et al. 2008). Promoter hypomethylation and overexpression has also been shown for *N*-acetyltransferase-1 (NAT1) in breast cancer (Kim et al. 2008), as well as for CD133 in gliomas (Tabu et al. 2008).

In the case of pancreatic cancer, genes found to be overexpressed in neoplastic cells as compared to surrounding non-neoplastic tissues due to cancer-related promoter-hypomethylation and subsequent transcriptional re-expression include maspin (*SERPINB5*), *S100A4*, mesothelin (*MSLN*), prostate stem cell antigen (*PSCA*), claudin-4 (*CLDN4*), lipocalin-2 (*LCN2*), 14-3-3 sigma/stratifin, trefoil factor 2 (*TFF2*), S100 calcium binding protein P (*S100P*), and vav 1 guanine nucleotide exchange factor (*VAV1*) (Sato et al. 2004; Fernandez-Zapico et al. 2005). Of interest, functional studies, including RNAi-mediated knockdown, have shown a requirement of sustained *VAV1* function to maintain the malignant phenotype of pancreatic cancer cells (Sato et al. 2004; Fernandez-Zapico et al. 2005) , underscoring the importance of hypomethylation-dependent overexpression. Some of these hypomethylated genes (e.g. *PSCA*, mesothelin) have emerged as important targets for therapy, imaging, and for diagnosis of pancreatic cancer (Foss et al. 2007; Li et al. 2008).

Thirdly, increasing evidence suggests that global hypomethylation might contribute to increased genomic instability, a hallmark of pancreatic as well as other cancers. In cancer cells, hypomethylated sequences are frequently found in pericentromeric regions, for example on chromosomes 1 and 16, and hypomethylation might thus predispose to recombination events (Narayan et al. 1998; Qu et al. 1999b; Tuck-Muller et al. 2000; Iacobuzio-Donahue 2009). Repetitive unbalanced chromosomal translocations encompassing these areas have, for example, been linked to the formation of Wilms tumors, breast and ovarian cancers (Narayan et al. 1998; Qu et al. 1999a, b). In another study, loss of the ability to methylate ectopically expressed DNA molecules was found to be correlated to the appearance of gross chromosomal aberrations in colon cancer cells with microsatellite instability (Lengauer et al. 1997). Perhaps even more strikingly, similar observations could be made using in vivo model systems: for example, several groups have found a correlation between global hypomethylation and increased frequencies of spontaneous tumor formation in mouse models (Chen et al. 1998; Eden et al. 2003; Gaudet et al. 2003), and a similar correlation was observed in resected breast and ovarian cancer tissue samples (Narayan et al. 1998; Ehrlich et al. 2006).

Moreover, yet another line of evidence demonstrating a functional relationship between global DNA hypomethylation and genomic instability is provided by certain inheritable genetic syndromes. The ICF syndrome is caused by loss of function mutation in *DNMT3B* and, as indicated by its name, is characterized by *i*mmunodeficiency in combination with *c*hromosomal instability and *f*acial abnormalities (Tuck-Muller et al. 2000; Ehrlich et al. 2008).

The underlying cause of global hypomethylation found in cancer is poorly understood. One hypothesis suggests that hypomethylation could be caused by nutritional deficiency. Specifically, lack of folate or vitamin B12 leads to methyl group deficiency due to decreased *S*-adenosylmethionine levels and is thought to contribute to hypomethylation and genomic instability (Pogribny et al. 1995; van der Put et al. 1998). Low intake of vitamin B12 or folate has been suggested to

be a risk factor for pancreatic cancer (Skinner et al. 2004). Therefore, it seems possible that DNA hypomethylation due to malnutrition could be an underlying cause of enhanced accumulation of genomic alterations and increased cancer risk in a subset of cases (Omura and Goggins 2009).

10.2.2.2 DNA Hypermethylation in Pancreatic Cancer

Although the link between carcinogenesis and global hypomethylation was established relatively early on, it is now apparent that such hypomethylation is typically accompanied by hypermethylation at specific gene promoters, particularly of genes involved in tumor suppression (Ting et al. 2006b; Esteller 2007; Iacobuzio-Donahue 2009). Aberrant promoter hypermethylation seems to be an alternative mechanism which can lead to silencing of tumor suppressor genes even in the absence of, or in addition to, intragenic mutations. One of the first examples linking promoter hypermethylation to repressed gene expression was reported by Baylin and co-workers in the calcitonin gene (Baylin et al. 1987). The first observations showing inhibition of a tumor suppressor gene due to promoter hypermethylation in cancer tissues were made studying the retinoblastoma (RB) gene (Greger et al. 1989). Many other examples have followed since, linking silencing of an ever-expanding number of tumor suppressor genes to promoter hypermethylation, in sporadic as well as in inheritable forms of cancer. In fact, it is currently assumed that around 50% of genes causing inheritable cancer through germline mutation or deletion are silenced by promoter hypermethylation in the sporadic counterparts of these malignancies, and promoter hypermethylation has, to date, been documented in a plethora of different genes (Shames et al. 2007; Iacobuzio-Donahue 2009).

In pancreatic cancer, genes that act as tumor suppressor genes and that are frequently found to be repressed by promoter hypermethylation in subsets of cases include *p16/CDKN2A*, E-cadherin, retinoic acid, suppressor of cytokine signaling-1 (*SOCS1*), tumor suppressor in lung cancer (*TSLC1*), C-X-C chemokine receptor type 4 (*CXCR4*), mucin 2 (*MUC2*) and reprimo (Schutte et al. 1997; Ueki et al. 2000; Sato et al. 2005b; Sato and Goggins 2006; Yamada et al. 2006). Reelin (*RELN*) is frequently downregulated due to promoter hypermethylation in pancreatic cancer, and its repression has been shown to mediate increased motility, invasiveness and colony formation, whereas opposite effects were observed upon re-expreission of RELN (Sato et al. 2006). Promoter hypermethylation of Hedgehog interacting protein (HHIP) was recently found in the majority of examined cases (Martin et al. 2005), including pancreatic cancer cell lines as well as primary tumor tissue samples, in line with observed aberrant re-activation of Hedgehog signaling in the majority of cases of pancreatic cancer (Berman et al. 2003; Thayer et al. 2003; Feldmann et al. 2007b, 2008). A small subset of pancreatic cancers shows hypermethylation and silencing of MutL protein homolog 1 (*hMLH1*) (Ueki et al. 2000; Yamamoto et al. 2001; Nakata et al. 2002), which has been associated with microsatellite instability and medullary histology in a fraction of cases (Goggins et al. 1998; Wilentz et al. 2000). Prepro-enkephalin (*ppENK*) encodes a native opioid peptide which can mediate

growth suppression and is methylated in the majority of pancreatic cancers (Ueki et al. 2001). The Kunitz-type serine protease inhibitor tissue factor pathway inhibitor 2 (TFPI-2), which is thought to be involved in protecting surrounding matrix from degradation and thereby inhibiting cancer invasion and metastasis (Izumi et al. 2000; Jin et al. 2001), was recently shown to be silenced due to aberrant hypermethylation in 102/140 (73%) of pancreatic cancers – both xenografts and primary tumor tissue samples were examined (Sato et al. 2005c). Of interest, re-expression of *TFPI-2* in pancreatic cancer cells leads to increased proliferation, invasion and migration in vitro. BCL2/adenovirus E1B 19 kDa interacting protein 3 (*BNIP3*) is another example of a gene commonly silenced by hypermethylation in pancreatic cancer. Using RNAi techniques, inhibition of *BNIP3* has been shown to contribute to resistance to 5-fluorouracil and gemcitabine in vitro. Moreover, hypoxic BNIP3 expression could by readily restored by 5-Aza-dC treatment, suggesting this might be a potential therapeutic approach for drug-resistant pancreatic cancers (Okami et al. 2004; Abe et al. 2005; Akada et al. 2005). Within the *GATA* gene family, *GATA-5* was shown to be frequently methylated, while *GATA-4* was infrequently methylated in pancreatic cancer cells (Fu et al. 2007). Secreted protein acidic and rich in cysteine, or osteonectin/BM40 (SPARC) is a calcium-binding protein which is involved in various cellular functions, including migration, proliferation, interaction with surrounding matrix, adhesion, angiogenesis and tissue remodeling (Lane and Sage 1994; Sato et al. 2003a). *SPARC* is frequently silenced in pancreatic cancer cells by aberrant DNA methylation (Sato et al. 2003a), and *SPARC* knockout causes enhanced tumor growth and invasiveness in mice (Mok et al. 1996; Schultz et al. 2002).

An unbiased way to screen for genes that might be silenced in pancreatic cancer cell lines due to promoter hypermethylation is to look for re-expression upon treatment with demethylating agents, e.g. the DNA-methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC), as compared to mock-treated control cells. Goggins and co-workers used this approach to screen for genes silenced due to hypermethylation in pancreatic cancer using Affymetrix oligonucleotide microarrays (Sato et al. 2003b, 2006). In these studies, 475 candidate genes were identified as re-expressed in four pancreatic cancer cell lines but not in a non-neoplastic ductal epithelial cell line used as control. Subsequent experiments successfully confirmed aberrant hypermethylation of several of these candidates in primary pancreatic cancer tissue samples. The same group recently described another high-throughput approach by application of a methylated CpG island amplification (MCA) technique to 44K Agilent promoter microarrays. This strategy was exploited to identify 606 differentially methylated genes in one pancreatic cancer cell line (Omura et al. 2008).

10.2.2.3 Gene Promoter Hypermethylation in Precursor Lesions of Pancreatic Cancer

Promoter-specific hypermethylation and inhibition of gene expression can be observed in all three known precursor lesions of pancreatic cancer—pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms

(IPMNs) and mucinous cystic neoplasms (MCNs), suggesting that promoter hypermethylation represents an early event during the multistep progression cascade of pancreatic cancer (Fukushima et al. 2002; Jansen et al. 2002; Sato et al. 2002; Sato and Goggins 2006; Maitra and Hruban 2008).

Sato and colleagues, for example, compared differences in global gene expression profiles in IPMNs as compared to normal pancreatic ductal epithelium and found underexpression of CDKN1C/p57KIP2 which, in some cases, was found to be due to partial promoter hypermethylation (Sato et al. 2005a; Omura and Goggins 2009). *CDKN1C* on chromosome 11p15.5 is a known tumor suppressor, which inhibits cell proliferation and whose inactivation is involved in formation of Wilms tumor and Beckwith–Wiedemann syndrome (Lee et al. 1995; Matsuoka et al. 1995). Other genes that have been found to be silenced as a result of DNA hypermethylation in PanIN or IPMN precursor lesions include *CDKN2A/p16*, *RLN*, *TFPI-2* and *ppENK* (Hong et al. 2008; Sato et al. 2008; Omura and Goggins 2009). Of note, the prevalence of methylation seems to increase from lower to higher grade precursor lesions (Sato et al. 2002). Similarly, progressively aberrant methylation patterns have been described in MCNs as well (Kim et al. 2003b).

10.2.3 Epigenetic Regulation of miRNA in Cancer

Several studies have described differential expression of microRNAs (miRNAs) due to aberrant DNA methylation in pancreatic (Omura et al. 2008) and other cancers (Grady et al. 2008; Lehmann et al. 2008; Lujambio et al. 2008). Conversely, there are emerging examples of miRNAs that are known to regulate DNA methylation. For example, miR-29 is capable of inhibiting DNMT3 activity, and ectopic expression of miR-29 has been shown to cause marked reduction of DNA methylation (Fabbri et al. 2007). Lee and colleagues used a miRNA microarray platform to screen for miRNAs that are differentially expressed in pancreatic cancer due to differential chromatin methylation or acetylation. Treatment with the demethylating agent 5-Aza-dC and the histone deacetylase inhibitor trichostatin A led to marked overexpression of five miRNAs (miR-107, miR-103, miR-29a, miR-29b, and miR-320) in both MIAPaCa-2 and PANC-1 pancreatic cancer cell lines. Of interest, enforced ectopic expression of miR-107 led to reduced in vitro cell growth and overexpression of the putative miR-107 target gene cyclin-dependent kinase 6 (CDK6) in MIAPaCa-2 and PANC-1 cells (Lee et al. 2009).

10.2.4 Aberrant DNA Methylation Patterns as Potential Biomarkers of Pancreatic Cancer

Aberrant DNA methylation patterns have been evaluated as potential biomarkers for early detection in various cancers and using a variety of sample materials. Although in most cases the methylation patterns in healthy individuals of different age groups

are not yet fully understood in detail, these types of assays hold the promise to be potentially more specific and, in some cases, more sensitive than secreted tumor marker proteins commonly used to date in the clinical setting (Sato et al. 2007; Omura and Goggins 2009). For example, Sidransky and co-workers described aberrant promoter hypermethylation of at least one of nine tested gene loci (*p16INK4a*, *p14(ARF)*, *MGMT*, *GSTP1*, *RARbeta2*, *CDH1 [E-cadherin]*, *TIMP3*, *Rassf1A*, and *APC*) in 52/52 (100%) of patients with prostate cancer in DNA extracted from urine sediment by means of quantitative methylation-specific polymerase chain reaction (QMSP). The authors concluded that a combination of a subset of only four genes from this panel (*p16*, *ARF*, *MGMT*, and *GSTP1*) would have yielded a theoretical sensitivity of 87% and specificity of 100% to predict prostate cancer in these patients (Hoque et al. 2005).

Several studies have assessed the detection of aberrantly methylated gene promoter regions as surrogate biomarkers for early detection of pancreatic cancer in clinical samples (Omura and Goggins 2009). Indeed, one early study successfully detected methylated proenkephalin-related DNA sequences in ~60% of pancreatic juice specimens obtained from patients suffering from pancreatic cancer, but in none of the control specimens used (Fukushima et al. 2003). Other follow-up studies have confirmed the general validity of this approach but at the same time also hint at possible false-positive results that may for example be introduced due to frequent age-related hypermethylation of promoter regions in shed duodenal mucosa cells that are prone to contaminate such specimens. Matsubayashi et al. reported that a quantitative approach (QMSP) was superior to conventional methylation specific PCR (MSP) in predicting pancreatic cancer based on methylation patterns of DNA purified from pancreatic juice samples (Matsubayashi et al. 2006). Using a cut-off of >1% methylation and a panel of five gene loci (*SPARC, NPTX2, ppENK, p16,* and *TFPI2*) they were able to achieve a theoretical 82% sensitivity and 100% specificity in a relatively small series of 11 pancreatic cancer patients. Other studies have tried to correlate the presence of pancreatic cancer with the detection of aberrant methylation patterns in DNA extracted from endoscopically-obtained brush cytology specimens of biliary and pancreatic ducts. Using QMSP on a panel of three gene loci (*NPTX-2*, *cyclin-D2* and *TFPI-2*) Parsi et al. were able to detect pancreatic cancer with a retrospective sensitivity of 73% and specificity of 86% in patients with biliary strictures (Parsi et al. 2008). Studies assessing aberrant methylation of circulating DNA in plasma or serum samples have, to date, shown varying success (Goessl et al. 2000; Bastian et al. 2005; Fujiwara et al. 2005; Jiao et al. 2007; Tan et al. 2007).

10.3 The Pancreatic Cancer Genome Project

In 2008, the most comprehensive study of somatic intragenic mutations and copy number alterations in pancreatic cancer was completed (Jones et al. 2008). The entire exonic region of 20,661 protein-encoding genes represented in the RefSeq database was analyzed for somatic mutations by direct Sanger sequencing in 24

cases of pancreatic cancer. Genomic deletions and amplifications were detected by means of oligonucleotide microarrays containing probes for 1,069,688 SNPs in the same cases, using a previously described algorithm (Jones et al. 2008). Furthermore, expression analysis of mRNA transcripts was done in all of the 24 cases of pancreatic cancer by means of serial analysis of gene expression (SAGE). The authors found an average of 63 genetic alterations in the pancreatic cancer cases analyzed, with the majority being intragenic point mutations. Interestingly this study showed that some somatic alterations occur with very high frequencies (thus designated "mountains" in a chromosomal map of the pancreatic cancer genome), while there are others that were found in only one or a few cases (designated "hills" in the same chromosomal overview). These results support the hypothesis that all cases of pancreatic cancer, though similar in histopathological appearance, may indeed be quite different in their genetic composition, which might explain why some cases respond better to certain therapeutic interventions than others. Of note, using an integrative analysis approach, the authors were able to identify 12 "core signaling pathways" that are affected by somatic mutations or copy number alterations in almost all of the examined cases and that were therefore proposed to be probably involved in pancreatic carcinogenesis. Since SAGE databases with thorough expression profiles for all of the examined pancreatic cancers is also provided, this study gives a wealth of information for potential future follow-up studies. For example, many of the expression changes observed are not attributable to genetic alterations and are expected to be due to epigenetic alterations, which will most probably be addressed in future work.

Similar studies have already been carried out for breast and colon cancer, and results for other tumor entities are likely to follow in the near future (Sjoblom et al. 2006; Wood et al. 2007). Some of these ongoing efforts will make use of next generation "deep" sequencing techniques, which allow detection of genetic alterations even in low fractions of cells used as starting material, and therefore enable the study of primary tumor tissue samples without the need to generate murine xenografts or cell lines first. This has the advantage of eliminating potential selection bias towards distinct subpopulations or the occurrence of additional aberrations during ex vivo or in vitro culture, respectively.

10.4 Concluding Remarks

Since these technologies will almost certainly become substantially cheaper and hence will be more commonly applied in the coming years, they might soon become part of the clinical routine work-up for various malignant tumors. Therefore, development of appropriate, robust methods for storage, statistical analysis and integrative biological interpretation of the huge quantities of data generated, will probably be a key issue to be addressed in the near future. One possible approach will be integrative analysis of mutational, copy number analysis, gene expression and global methylation data using databases of known signaling pathways or cellular func-

tions, similar to what has been described in the discussed studies (Jones et al. 2008; Leary et al. 2008).

Therefore, a combinatorial, integrative application of high-throughput sequencing, copy number analysis, global methylation and gene expression profiling is likely to become more commonly used in future translational cancer research as well as in clinical practice both for therapeutic target discovery and prediction of individual response to treatment.

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Chapter 11
Tissue Microarray Applications in Drug Discovery for Pancreatic Cancer

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Abstract The rising incidence of pancreatic cancer combined with limited responses to chemotherapy highlights the need for improved molecular characterization in this highly lethal cancer. The discovery and validation of novel biomarkers are urgently needed to improve early detection and thereby improve survival outcomes. The introduction and widespread application of tissue microarrays (TMAs) over the past decade provide a valuable tool for validation of abundance and cellular localization of expressed proteins and RNA transcript levels contextually within the complex tissue morphology of pancreatic cancer. The TMA format offers a standardized platform to optimize and validate newly identified biomarkers and drug targets in pancreatic cancer research to patient tumor samples to provide critical links in advancing cancer research findings to the clinical setting.

11.1 Introduction

The genomic era in molecular biology and cancer research has become firmly established in the past decade based largely on collaborative efforts of national and international research institutes, including the National Human Genome Research Institute (NHGRI) at the National Institutes of Health (USA). Public and private resources with scientific leadership dedicated to the accurate and comprehensive annotation of the human genome (Collins and McKusick 2001; Wolfsberg et al. 2002) have provided novel methods to characterize nucleic acid alterations in various disease states and cancer. One such technology, cDNA expression profiling, utilizes dual-labeled hybridization techniques to provide quantitative measurements of gene expression across the entire human genome (DeRisi et al. 1996; Duggan et al. 1999).

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Genome-wide assays offer simultaneous and comprehensive measures of gene dosage and transcript levels and have resulted in the identification of hundreds or thousands of expressed sequence tags (ESTs) or gene products with a potential role in neoplastic processes for wide variety of tumor types (Hacia et al. 2000). In this context, forward-thinking researchers at the National Human Genome Research Institute (NHGRI) in the late 1990s anticipated a need for a clinical validation tool that offered high throughput capabilities for the robust analysis of candidate markers. Tissue microarray (TMA) technology as developed by Kononen et al. (1998) has been widely used to validate and prioritize such candidate biomarkers. Formalin-fixed and paraffin-embedded (FFPE) tissue blocks represent an enormous and largely untapped bioresource and have opened productive and important collaborations between cancer researchers and pathology departments. The power of TMA technology remains the high density and precise orientation of hundreds of clinical tissue spots for the 'in parallel' analyses of various molecular assays on sequential TMA slides. Early TMA validation studies demonstrated consistent and representative protein expression by immunohistochemistry (IHC) and DNA copy numbers by fluorescence in situ hybridization (FISH) assays (Bubendorf et al. 1999; Schraml et al. 1999; Rimm et al. 2001). In subsequent years to the present day, numerous studies have evaluated the representative nature of TMA core sampling (Mucci et al. 2000; Hoos et al. 2001; Nocito et al. 2001; Hsu et al. 2002; Kononen et al. 2002; Parker et al. 2002; Rubin et al. 2002; Chiesa-Vottero 2003; Sauter 2003) and many hundreds of studies have utilized TMAs for candidate marker validation and prioritization (Srivastava et al. 2001; Mousses et al. 2002; Skacel et al. 2002; Sugita et al. 2002; Garcia et al. 2003; Hardisson et al. 2003; Hu et al. 2003). Of historical note, the high density TMA was not the first multi-tissue array. Battifora et al. (1986) described the sausage block, which consisted of normal and tumor tissues with primary application in histology for antibody optimization. In marked contrast, the high density TMA allowed precise placement of 200 samples (1.0 mm cores) to 600 samples (0.6 mm cores) into a composite paraffin block. Upon sectioning of the composite block, tissue cores were retained in the same orientation and allowed for specimen tracking and database integration. A review of the literature documents the widespread and ongoing applications of TMA technology in the research community. This boom of TMA applications reflects: (1) the critical need to validate novel biomarkers of prognostic significance, (2) the value of portable technology, and (3) the value of FFPE tissues in cancer research to evaluate candidate markers in clinical specimens of candidate markers. The relatively simple technology of the first generation manual microarrayers (Beecher Instruments, Inc.) has proved to be a highly versatile tool and has rapidly become a standard instrument in both research laboratories and pathology departments.

11.2 FFPE Tissues in Cancer Research

A perhaps unanticipated benefit of TMAs in translational and cancer research has been the increased utility and applications of FFPE tissues for molecular analyses in the research laboratory. The increased utility of various in situ assays of IHC,

ISH and FISH has sharpened the focus on technical performance of the assays, and uniformity in fixation parameters has become more critical (Anderson et al. 2001; Frantz et al. 2001; De Marzo et al. 2002; Chin et al. 2003). Archival paraffin tissue blocks represent the cost-effective and traditional method of storage within the pathology department (intra-institution) and broader projects such as clinical trial materials (inter-institutional). Traditional uses of FFPE tissues in clinical diagnosis and cancer research include the IHC detection of cellular antigens and sequence-specific probes to detect DNA copy alterations by FISH. A second unanticipated benefit of TMAs has been the cross-disciplinary efforts between cancer researchers and clinical pathologists in assay standardization by incorporation of cell line materials with annotated molecular features (Hoos et al. 2001; Moskaluk and Stoler 2002; Abbott et al. 2003). The most common use of TMAs in the research laboratory is the optimization of novel IHC markers by simultaneous assessment of staining intensity and cellular localization with the alteration of one experimental parameter. Before high density TMAs, analyses of large sample sets in a controlled and efficient manner was largely prohibitive due to the pathologists' need to read and analyze hundreds of whole tissue sections. This was not cost- or time-effective for either the pathologist or the histotechnologist as IHC, ISH, and FISH assays are highly labor intensive and have a wide range of assay performance variables such as technician, day-to-day variability, reagent lot variability (O'Leary 2001; Sompuram et al. 2002). The other methods for obtaining this information (such as RT-PCR, multi-tissue Northern or Western blots, or protein arrays) require tissue disaggregation, and disruption of molecular analytes with loss of localization of cell-specific markers within defined cellular regions (Simon et al. 2004). The TMA provided much-needed standardization for assay performance in FFPE and permited analysis of biomarkers in the context of tissue morphology with retained nuclear, cytoplasmic and extracellular features such as stromal staining thereby allowing both regional and cellular contextual analyses of novel biomarkers of potential prognostic significance.

11.3 Tissue Microarrays

In order to construct a useful TMA, appropriate starting material must be obtained. The study pathologist will select a cohort of sample blocks to represent accurate sampling of the disease state to be analyzed. A surface section is cut by standard microtome and stained with hematoxylin and eosin (H&E) to insure morphologic accuracy of the targeted area. The targeted region is marked by the pathologist to provide representative sampling of the desired lesion in 2–4 punches of each donor FFPE block. The number of punches required for representative sampling of various tumor types depends upon the research question posed and the degree of tissue heterogeneity within the donor block. The range of morphologic heterogeneity is sometimes underlined by molecular heterogeneity, but generally tissue sections stain in a uniform manner by IHC assay. A second more common source of tumor tissue heterogeneity, especially in pancreatic cancer, is a marked desmo-

plastic tumor response that can result in very scattered ductal tumor cells. In our practice in the construction of tumor-specific TMAs, we sample more homogeneous solid tumors, for example breast and colon adenocarcinomas with two 0.6 mm punch cores. In the construction of highly desmoplastic tumors such as pancreatic cancer, our design is to punch three 1.0 mm cores to increase our sampling of ductal tumor cells on the TMA in addition to one core of normal ducts as available. Separate groups have reported sampling design of up to five cores to ensure adequate pancreatic cancer sampling. The brilliance of the high-density TMA is the precise orientation of tissue cores within a grid pattern that allows accurate study sample tracking and assay interpretations by TMA technicians and downstream collaborators alike.

11.3.1 Types of Tissue Microarrays

Many common arrangements exist: a progression array, tumor-specific array, tissue-specific array, multi-tissue, and multi-tumor array. Each type of TMA helps answer different scientific questions. A progression array is constructed such that a disease is arrayed across the TMA and designed to capture representative punches of the different stages of that disease (e.g. benign to malignant). The progression array is helpful in identifying characteristics that might change throughout the disease state. Specific protein expression may change dramatically from one stage to the next and can be ultimately used as a diagnostic marker or therapeutic tool. The tumor-specific array is constructed by using a cohort of samples to capture a population-based prevalence of a given marker in the tumor or lesion type. In most cases the tissues are from the same stage of disease and represent a few samples with subtle nuances of disease progression and in that case the tumor-specific TMA is used to validate molecular markers identified by separate discovery-based molecular assays, for example expression profiling. In other words, once an investigator has found a gene alteration, TMAs offer validation of that finding in a tumor-specific setting. A tissue-specific TMA is constructed by combining only one type of tissue regardless of disease state on one block and is often comprised of multiple lesions (tumors) and their normal counterparts. This is helpful to investigators who want to interrogate whether there are molecular changes in the different disease states. This TMA is different from a progression array as it is not constructed to analyze the life cycle of the tumor but to see a representation of all the disease states. A tissue-specific TMA is most often utilized when an investigator has a novel gene or protein that they are looking into. In our laboratory the most commonly used TMA is the multi-tissue, multi-tumor array (MTMT). This TMA is a snapshot of the body by capturing as many different tissue types as possible. Usually these are designed so that body systems are together, and can represent many different disease states of the different tissues. We use the MTMT array to validate novel antibodies during in situ techniques (e.g. IHC, FISH), and to determine the tissue type(s) most informative for staining for a given novel protein. In summary, despite

the various TMA designs and utilities, we construct TMAs by common workflow. A pathologist identifies the cohort of tissues, marks up the H&E (histomorphologic stain to identify different tissue components) slides, and decides on the type of TMA. Once the design is complete, construction begins by a qualified technician using one of several commercially available manual, semi-automated or automated tissue arrayers.

11.3.2 Construction Equipment

Currently TMAs are constructed by manual and robotic methods, though the vast majority utilize some type of manual arrayer. Both types of arrayers use two thin-walled needles with slightly different core diameters, one to punch a hole in the recipient (composite) block and one to punch and transfer the core from the donor block. The recipient needle (e.g. outer diameter 1.0 mm) punches a slightly larger hole than the donor needle (e.g. inner diameter 1.0 mm), which allows the donor core to fit tightly into the recipient hole. The needles range in diameter from 0.6 mm to 2.0 mm and allow the user to decide which core size would be of greater benefit based on: (1) original tissue size in the donor block, (2) the scope of the study, and (3) the number of blocks to be arrayed. The first and largest commercial supplier of arrayers is Beecher Instruments (Beecher Instruments, Inc.) with three types of manual arrayers (MTA-1, MTA-II, MTA-III) and two types of robotic arrayers (Automated Arrayer and Galileo TMA CK3500). The MTA-I and MTA-II manual arrayers utilize a swinging arm method and the user moves the blocks individually out of the block holder. Both instruments provide user control in the *x*- and *y*-axis but less so in the *z*-axis. The second-generation manual arrayer (MTA-II) has maintained affordability for the small to mid-sized research laboratory as well as some needed improvements on functionality. The indexed manual arrayer (MTA-III) gives the user greater adjustability in the depth (*z*-axis) of tissue in the recipient block, better alignment and better target acquisition. It uses a "copy and paste" method by using an indexed platform between the stereomicroscope and donor block and between the donor and recipient blocks. Various depth controls allow the user to adjust for donor tissue of variable thickness and permits better packing of cores for small and thin samples, such as biopsy materials. Beecher Instruments now offer a robotic arrayer (Automated Arrayer) that significantly increases the number of blocks that can be punched or composite blocks created in a much shorter amount of time. The Galileo TMA CK3500 is a semiautomatic and computer-assisted tissue microarrayer. Dedicated software assists the user throughout the operating phases from tissue microarray design to final reporting. Robotic arrayers offer less control in the *z*-axis and work best with donor and recipient blocks of uniform height and tissue depth. Robotic arrayers offer limited user corrections in problems with tissue core extraction, but do require additional technical support in the field. Additional robotic arrayers are often developed for "in-house" purposes by large commercial laboratories with limited available information.

11.3.3 Construction Process

There are a few standard steps that are part of the TMA construction. Orientation of the TMA is very important during the construction phase. Sometimes, a "handle" is used which consists of extra cores being inserted in a specific location in order to orient the user under the microscope. There is a general consensus of how the naming of quadrants is established, which allows for inter-laboratory use. Some sort of database has to be developed while punching to record the co-ordinates of the tissue for future reference, such as a Microsoft® Excel file. The database, along with a pictorial representation of the block, allows any user to navigate the block easily. In the past it was advisable to use paraffin blocks of similar thickness in order to maintain uniformity of sections throughout the block (Henshall 2003). To counteract the fact that some blocks that are utilized for TMA construction are thin and have been cut several times, a technique that involves "packing" of the core can be used. Packing means that two punches from the original block occupy the same hole, one on top of the other, in the recipient block (Hoos et al. 2001). The depth of target tissue in the donor block becomes an issue with more detailed TMA analyses, such as arraying of intra-epithelial lesions (Maitra et al. 2003) and endoscopic biopsy specimens (Gulmann et al. 2003). Once the block has been constructed, it is vital to have the cores "become" part of the recipient block for subsequent sectioning purposes. We routinely remelt the TMA block at a temperature slightly higher than the melting point of the wax used in the recipient block for a short time (minutes) and then cool for an extended period (overnight). This step is critical when sectioning the composite block using water floatation and standard glass slides. At the time of sectioning the temperature and hydration of the block is vital for consistent sections. If this method is used then fewer cores will "pop out", fold or fragment during the sectioning process of the composite TMA. In the past, the tape transfer method was widely utilized (Instrumedics, Inc.) due to simplicity and transfer of tissue spots in desired orientation. One disadvantage of tape transfer was the adhesive residue that sometimes led to increased background staining and precluded more sensitive molecular assays, such as ISH, FISH and phosphorylation-specific IHC. Numerous investigators have adapted standard tissue sectioning techniques that utilize a water bath to float off the sections onto positive-charged sections or polylysine-coated slides.

11.4 Representation of Tissue

Tissue core representivity compared to whole-section histology and potential for discordance in IHC staining was an early criticism from some in the pathology community. Numerous studies have addressed this issue and the major consensus is that 2–4 tissue cores are representative with 95–97% concordance rates; furthermore, five to six cores do not improve concordance rates (Simon et al. 2004). Several studies reported non-concordance in analysis of core samples compared to whole

sections in the quantization of proliferation markers, such as Ki-67 (Chiesa-Vottero et al. 2003), apoptosis markers (Merseburger et al. 2003) (limitations of cell numbers), and neoangiogenesis markers (Charpin et al. 2004) (limitations of paraffin sections). Heterogeneous tissues do call for a higher number of cores as well as a larger core diameter size to be truly representative of the tumor case. Due to high desmoplastic stroma in the pancreas, usually 4–5 cores of 1.0 mm are taken from 2–3 discrete but representative regions. The increase in the number of cores is to ensure minimal study case loss due to tissue core drop-out, or technical difficulties. Increasing the size of the core also improves the chance of sampling the entire lesion, or region of interest, and adequate surrounding tissue. Several studies constructed their respective TMAs using this ideology to strengthen their findings (Cao et al. 2007; Khorana et al. 2007). The next question referring to representation is which size needle to use and that is determined by the condition of the original block and the scope of the study. If a high-density block needs to be made then a smaller needle size would be appropriate and it has been acknowledged that with a 0.6 mm needle a composite block can be made with approximately 500–600 cores. Conversely a low-density composite block would use a larger needle and have fewer cores. Approximately 200 sections can be sectioned from a single TMA block allowing for multiple sequential slides to be used in various tests. Using TMA technology does not compromise the architecture of the original block and render it to be less valuable. Once the composite block has been made the original block can still be sectioned and utilized as whole-mount tissue sections.

11.5 Applications of TMAs

11.5.1 Frozen Tissue Arrays

Recently some applications have included frozen samples in the form of frozen arrays and the use of cell line material in order to help in the validation of candidate genes. When constructing frozen TMAs specialized equipment and donor tissue and recipient block requirements of a common cutting media such as OCT, special adhesives for section transfer and retained TMA core orientation (Fejzo and Slamon 2001). Frozen arrays can also be hard to make because the punching needles often break, and both the donor and recipient blocks must be kept cold with dry ice during punching. Greater stability of nucleic acids and RNA is achieved in frozen arrays and has shown applications in ISH assays. The morphology in the frozen array is altered with significant loss of the fine detail which is usually seen in formalin-fixed tissue. Frozen tissue microarrays appear to provide excellent target material for the study of DNA, RNA and proteins by fixing each array slide in a manner specific to the corresponding technique used (Li et al. 2003). Another advantage is that those procedures requiring fixation can be conducted in samples fixed in an identical manner, since fixation is performed for a limited time on the TMA slide.

11.5.2 Clinical Applications Using TMAs

Over the past decade, TMAs have become a widely utilized and proven tool in both the clinical and research settings for histologic applications in IHC and the standardization and optimization of novel antibodies. Most academic research hospitals, at minimum, have tissue array equipment, and numerous institutions have dedicated TMA core facilities that offer contractual services. The simultaneous application of DNA or protein probes on a multi-element array has become a common theme in the genomic era. TMAs offer a much needed validation tool to address the preservation effects of formalin fixation upon tumor tissue macromolecules such as cell surface proteins and nucleic acids. Despite generally accepted parameters of 10% buffered formalin fixation of 12–24 h duration, various histology departments have differing levels of quality control. For example, in the design of a progression TMA to assess the role of the candidate gene in neoplastic progression, the optimal tissue block size would contain hundreds of specimens from a single institution. However, if the proposed study were designed to assess a regional or specific population, thousands of specimens in multiple blocks would best provide prevalence data of a given marker. For studies of biomarker prevalence and prognosis in a given tumor, the ideal TMA would be constructed of sequential cases accrued over a period of time from a single institution with attached clinical data of at least five years to determine prognostic significance. In animal studies, TMAs are often used to chronicle time- and treatment-dependent experiments. The choice of TMA block design depends entirely on the investigator and questions posed. In our experience, TMA block design is often an overlooked component in maximizing the value of biologic information extracted from a given study. This provides an opportunity for core facilities to offer expertise in TMA design and construction, as many genomic studies are comprised of multiple modality assays with varying degrees of sensitivity and specificity. The DNA microarray for example is a valuable tool for gene expression, but the tissue preparation steps in RT-PCR, protein arrays, Northern and Western blots are tissue destructive. Genome-wide assays now indicate, just as pathologists have long speculated, that stromal microenvironment factors are often related to clinical outcomes of certain carcinomas. The increasing importance of evaluation of molecular markers in the context of cellular localization and tissue morphology guarantees increased demand and applications in the future. Examples of increasingly sophisticated TMA applications include integrative microarray studies on delineation of secreted biomarkers in cancer tissue and serum (Welsh et al. 2003), analysis of S100 subtypes in a wide range of tumors (Hsieh et al. 2003), and integration into genomic and proteomic studies (Nishizuka et al. 2003), clinical genomics, and drug discovery (Sauter et al. 2003; Mobasheri et al. 2004).

11.5.3 Cell Line Material TMAs

The application of cell line materials to TMA format by several investigators provides needed positive controls for IHC or ISH assays (Hoos and Cordon-Cardo

2001; Moskaluk and Stoler 2002; Abbott et al. 2003). Cell line materials are well annotated at the gene level and are most useful for well-characterized markers such as estrogen receptor (ER) receptor or Her-2. Applications to validate candidate biomarkers, however, are limited with cell line materials due to the variable gain of genomic alterations with increased passage number. Frozen materials offer much higher sensitivity for ISH experiments, but are much more difficult to analyze. IHC, FISH, and ISH can all be used on cell line materials that have been fixed and embedded in agarose (Moskaluk and Stoler 2002) matrix but may need minor adjustments to improve the quality of the output of data. As demonstrated by hundreds of literature citations, TMAs using the paraffin medium are used in the vast majority of studies due to the ease of transfer of wax cores, long-term and cost-effective storage, and the attached clinical outcomes data. The TMA platform offers an unparalleled method for assay optimization to extend and adapt novel molecular assays to archival paraffin tissues, a large and relatively untapped molecular repository.

11.6 In Situ Techniques

With the rapid rise of TMA utilization in research laboratories over the past decade, advances in IHC technology has greatly improved assessment of cellular proteins expressed at the tissue level. Protein characterization of normal and tumor tissues have provided cross-platform comparisons and validations of numerous microarray experiments, including the rapidly developing field of proteomics. Both platforms allow the rapid acquisition of abundant information on gene expression in a relatively short period of time because they use comparatively few array sections. Additionally, advances in image acquisition and automated image analysis provide truly higher throughput capabilities to characterize protein profiles across broad ranges of tissue with standardized reagents and detection methods, thus promoting continuity of results (Warford et al. 2004). The applications of in situ procedures preclude optimal starting biomaterials; however, formalin fixation of the tissue presents challenges for assay standardization. Formalin fixation is widely used to preserve tissue morphology following sample collection and with the increasing use of TMAs in molecular research, fixation alterations to target antigens or nucleic acids must be systematically addressed. In general, the net effect of fixation and processing steps is that tissue targets for the in situ procedures have likely undergone conformational changes that affect epitope (antigen) exposure and probe (antibody) attachment. In order to counteract this problem, one solution would be the use of antibodies for any of the in situ procedures that recognize the target molecule in this post-processing condition (Warford et al. 2004). A second problem related to variable loss of antigenicity in TMA tissue spots after sectioning was suspected (Rimm et al. 2001) and later confirmed by quantitative measures of loss of IHC staining for ER receptor (DiVito et al. 2004) with notable loss of staining after one month and marked loss of staining at nine months. One study by DiVito et al. looked at the rate of loss of ER staining in breast cancer specimens. They cut three 5 μm sections of a breast

TMA and stored them at room temperature, under room air for 2, 6, and 30 days. At each time point a fresh slide was also cut and both slides were stained with ER and scored manually. The study showed that TMA slides stored in room air lose detectable antigenicity over the course of one month, with a statistically significant difference by six days (DiVito et al. 2004), with some antigens significantly more labile (DiVito et al. 2004). The etiology of this loss of staining could be related to oxidative alterations and the recommended antidote at a minimum is dipping the TMA slides in paraffin and storage; however, storage at 4°C and under a nitrogen gaseous mixture is also recommended. An independent study (Fergenbaum et al. 2004) confirmed these findings and reported varying loss of IHC staining for Her2, ER and PR over six months on breast cancer TMA slides that were stored in ambient conditions. Various investigators report on paraffin dipping of slides and storage in nitrogen or cold to protect the slides from subsequent oxidation over time. Any of these methods alone is not as good as using some of them in combination (DiVito et al. 2004). These documented findings support our laboratory's finding of loss of some epitopes in cut sections stored at ambient temperatures. These multi-institution findings highlight quality control measures that are increasingly important as TMA applications mature and are further extended in the research community.

11.6.1 Immunohistochemistry

TMA technology has enabled researchers to investigate multiple specimens simultaneously with IHC technology, resulting in a dramatic reduction of time and cost compared to conventional techniques. TMAs have become a popular tool for tissue-based research and have allowed massive acceleration of studies correlating molecular in situ findings with clinico-pathologic information. This approach has become specifically useful in surveys of tumor populations where it can be utilized to analyze the functions of newly identified genes in both healthy and neoplastic human tissues in a comprehensive and efficient manner (Han et al. 2008). IHC is the process of localizing proteins in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biologic tissues. It is a technique that is widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biologic tissue. Visualizing an antibody−antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as FITC, rhodamine, Texas Red or Alexa Fluor. IHC is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined. We have successfully used pancreatic cancer TMAs to evaluate the expression of potential protein targets in pancreatic tumors and normal tissues (Balagurunathan et al. 2008). Figure 11.1 shows an example of a protein (Aurora A kinase) that is upregulated in pancreatic cancer.

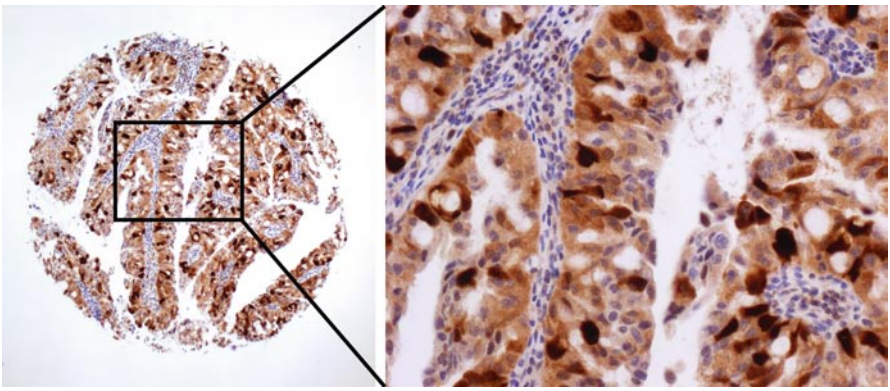

Fig. 11.1 Overexpression of Aurora A kinase with staining localized to the nucleus and specific for a proportion of pancreatic tumor cells with minimal staining in the surrounding stroma. Anti-Aurora A antibody was obtained from Novocastra, USA and used at 1:100 dilution

Figure 11.2, on the other hand, shows an example of a protein (SMAD4) that is frequently lost in pancreatic cancer. The major disadvantage of IHC is that, unlike immunoblotting techniques where staining is checked against a molecular weight ladder, it is impossible to show in IHC that the staining corresponds with the protein of interest. For this reason, primary antibodies must be well validated in a Western blot or similar procedure. The technique is even more widely used in diagnostic surgical pathology for tumor typing, e.g., immunostaining for E-cadherin to differentiate between DCIS (ductal carcinoma in situ: stains positive) and LCIS (lobular carcinoma in situ: does not stain positive). A variety of molecular pathways are altered in cancer and some of the alterations can be targeted in cancer therapy. IHC, widely used in the clinical laboratory, can be used to assess which tumors are likely to respond to therapy by detecting elevated levels of the molecular target. The successful application of IHC in research

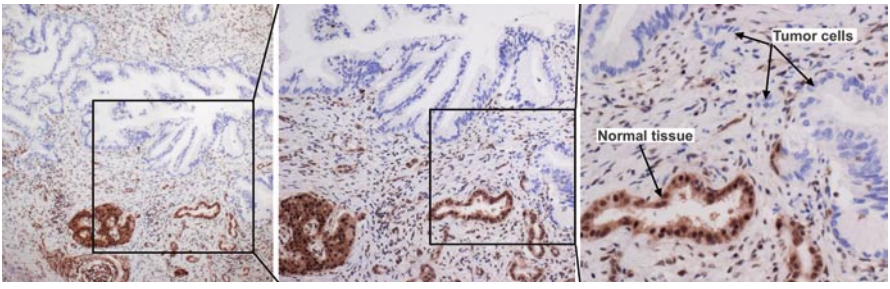

Fig. 11.2 Specific loss of SMAD4 immunostaining in the pancreatic tumor cells. Anti-SMAD4 antibody was obtained from Santa Cruz Biotechnology and used at 1:200 dilution. Note retention of staining in the stromal fibroblasts as it confirms the interpretation of complete loss of expressed protein SMAD4 in pancreatic tumor cells, consistent with the known function of this gene as a tumor suppressor

and clinical laboratories requires that basic steps be followed. Time variances, suppliers of primary antibodies, and exact number of detection steps, chromogen used, and number of protein blocks all vary from laboratory to laboratory. This poses challenges in standardization for every antibody and every tissue. Along with the variety in the actual IHC staining procedure, the paraffin tissues themselves are fraught with variables that disrupt the standardization process. Surgical resection protocols and time to formalin fixation is one pre-analytic variable, and duration of fixation is a second point of discord contributing to IHC nonstandardization. At time of resection, ischemic time varies from surgery to surgery and also affects downstream molecular analysis. If a tissue is allowed to sit out in the open environment, then autolysis and putrefaction destroys the tissues and degrades the macromolecules needed for analysis. The effects of time from tissue resection to preservation are poorly understood at the protein level, with dramatic effects at the RNA level and fewer deleterious effects to the DNA macromolecule. Fixation is the process by which the proteins are cross-linked when the tissue is placed in a 10% neutral buffered formalin mixture (where composition varies) for a 12–48 h period at room temperature. The differences in composition are mainly present in non-stock solutions and include age of formalin, the buffers used and the water used to make it up. Next, the time of fixation varies from site to site, which changes the chemical structure of the proteins and the effectiveness of cross-linking. The third step before any analysis can happen is called tissue processing by which all free water is removed and any air space is filled with heated paraffin. The actual steps of processing vary due to instrument, type of tissue being processed and laboratory site. When the tissue is finished being processed it is embedded in paraffin and can be used in IHC or other molecular assays. So the tissue has many steps in which its molecular makeup can be altered. These alterations are why IHC is as variable as it is. The investigator has to overcome all these preanalytical variables in order to achieve discovery or validation. IHC consists of:

1. Deparaffinization—removal of paraffin and restoring the free water content of the tissue
2. Antigen retrieval—the process by which the proteins are un-crosslinked
3. Protein block—the use of different serum such as solutions to mask non-target sites
4. Peroxide block (optional)—the application of hydrogen peroxide to block endogenous peroxide if using a peroxide-based detection system
5. Primary antibody—the application of the antibody which you are studying
6. Secondary/detecting antibody—the application of the antibody of detection polymer
7. Chromogen—the application of the colormetric solution
8. Counterstain—the application of a histologic stain to help in identifying morphology
9. Coverslipping—removal of any free water and permanent mounting for storage

Each step is variable with its own complexity. In order to speed up the process of narrowing down the optimal procedure, TMAs are widely used for optimization of procedure and antibody as well as validation. Like any other biologic analysis,

IHC is not foolproof even with TMAs. In addition to the potential lack of malignant tissue, TMA technology is fraught with additional complications related to the procedure itself. For example, folding and other sectioning artifacts can damage tissue cores and compromise evaluation. Antibody gradient problems and edge effects are also common artifacts encountered during IHC that can potentially affect the quality of tissue core interpretation. Typically, failure rates between 10% and >30% are attributed to such technical problems (Swierczynski et al. 2004). In Swierczynski's study, technical failure rates affected only 3–5% of the cores, allowing for the interpretation of immunolabeling of all the markers on at least one core for most of the cases. These types of results are what every investigator strives for so that they can validate or discover a marker in their tissue of interest.

11.6.2 Fluorescent In Situ Techniques

FISH is a cytogenetic technique that can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification. It can also be used to detect and localize specific mRNAs within tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues. An investigator can use FFPE tissue, frozen tissue or cell line material for FISH. Probes are complimentary sequences of nucleotide bases to the specific mRNA sequence of interest. These probes can be as small as 20–40 base pairs (bp) or be up to 1000 bp. The design of the probe is based on the questions being asked and the type of sequence to be detected. In situ hybridization presents a unique set of problems as the sequence to be detected will be at a lower concentration, be masked because of associated protein, or protected within a cell or cellular structure. Therefore, in order to probe the tissue or cells of interest one has to increase the permeability of the cell and the visibility of the nucleotide sequence to the probe without destroying the structural integrity of the cell or tissue.

11.6.2.1 Types of Probes

There are four major types of probes: (1) oligonucleotide probes, (2) single-stranded DNA probes, (3) double-stranded DNA probes, and (4) RNA probes. Oligonucleotide probes are produced synthetically by an automated chemical synthesis. Designing the sequence of the probe is one of the more critical decisions required when using oligonucleotide probes. These probes have the advantage of being resistant to RNases and are small, generally around 40–50 bp. This is ideal for in situ hybridization because their small size allows for easy penetration into

the cells or tissue of interest. In addition, because they are synthetically designed, it is possible to make a series of probes that have the same GC content. Since G/C base pairs bond more strongly than A/U base pairs, differences in GC content would require different hybridization conditions, so with use of oligonucleotide probes of a certain length, protocols can be standardized for many different probes irrespective of the target genes being measured. Another advantage of the oligonucleotide probes is that they are single-stranded therefby excluding the possibility of renaturation. Single-stranded DNA probes have similar advantages to the oligonucleotide probes except they are much larger, probably in the 200–500 bp size range. They can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer. Disadvantages of oligonucleotide probes include the time required to prepare, expensive reagents used during preparation, and a good repertoire of molecular skills required. Double-stranded DNA probes can be produced by the inclusion of the sequence of interest in a bacterium, which is replicated, lysed, the DNA extracted and purified, and the sequence of interest is excised with restriction enzymes. On the other hand, if the sequence is known then by designing appropriate primers one can produce the relevant sequence very rapidly by PCR, potentially obtaining a very clean sample. The advantage of the bacterial preparation is that it is possible to obtain large quantities of the probe sequence in question. Because the probe is double-stranded, it means that denaturation or melting has to be carried out prior to hybridization in order for one strand to hybridize with the mRNA of interest. These probes are generally less sensitive because of the tendency of the DNA strands to rehybridize to each other and are not as widely used today. RNA probes have the advantage that RNA−RNA hybrids are very thermostable and are resistant to digestion by RNases. This allows the possibility of post-hybridization digestion with RNase to remove non-hybridized RNA and therefore reduces the possibility of background staining. These probes, however, can be very difficult to work with as they are very sensitive to RNases (ubiquitous RNA degrading enzymes), and so scrupulous sterile techniques should be observed or these probes can easily be destroyed. RNA and cDNA probes are often less specific because of their large size which allows for cross-recognition to close family members of the same gene family. Oligonucleotide gene probes have multiple advantages over RNA or cDNA probes when used for in situ hybridization: stability, availability, faster and less expensive to use, easier to work with, more specific, better tissue penetration, better reproducibility, and a wide range of labeling methods that do not interfere with target detection.

11.6.2.2 Steps Involved in FISH

1. Probe construction—deciding which type of probe to use
2. Labeling of probe—add labeled nucleotides or incorporation of label
3. Permeablization—done so that the probe can reach the target (HCL, detergents, or enzyme)

4. Pretreatment/prehybridization step(s)—generally carried out to reduce background staining
5. Hybridization—depends on the ability of the oligonucleotide to anneal to a complementary mRNA strand just below its melting point (T_m). Factors that influence hybridization are temperature, pH, monovalent cation concentration, and the presence of organic solvents
6. Washes—remove unbound probe or probe that has loosely bound to imperfectly matched sequences

11.6.2.3 Applications of FISH

FISH can be used to form a diagnosis, to evaluate prognosis, or to evaluate remission of a disease, such as cancer. Treatment can then be specifically tailored. A traditional examination involving metaphase chromosome analysis is often unable to identify features that distinguish one disease from another, due to subtle chromosomal features; FISH can elucidate these differences. FISH can also be used to detect diseased cells more easily than standard cytogenetic methods, which require dividing cells and require labor- and time-intensive manual preparation and analysis of the slides by a technologist. FISH, on the other hand, does not require living cells and can be quantified automatically, where a computer counts the fluorescent dots present. However, a trained technologist is required to distinguish subtle differences in banding patterns on bent and twisted metaphase chromosomes. This technique is used in situations where the conventional cytogenetic analysis is unsuitable for detection or confirmation of the chromosomal abnormalities.

11.6.2.4 FISH on FFPE Samples

FFPE tissue adds another layer of complexity to FISH since there are additional fixation artifact and protein crosslinking at the histone level that needs to be treated in order for the probe to detect its target sequence. Along with the formalin fixation, the paraffin increases the background as autofluorescence (AF). Pancreatic adenocarcinoma tissue adds yet another level of complexity with the heterogeneous nature of the tissue and marked desmoplastic stroma creating a further loss of hybridization signal in the background AF. Stringency at the wash steps requires adjustment to reduce the background AF. Correlation of IHC and FISH is a validated clinical diagnostic profile, especially in breast cancer with HER-2 alterations. When the IHC is not definitive, FISH is often employed to determine the patient's ability to respond to drug therapy. Saxby et al. (2005) studied HER-2 overexpression in pancreatic adenocarcinoma at the genetic, transcriptional, and translational level. In summary, concordance between methodologies was poor, but the best agreement was seen between FISH aneuploidy status and Q-RT-PCR mRNA overexpression (80% agreement), followed by IHC and Q-RT-PCR (73% agreement). The least agreement was seen between IHC and FISH aneuploidy status (67% agreement).

A major problem with the IHC scoring system is that it was developed for breast carcinoma and has been applied here to pancreatic cancer, a morphologically and biologically quite different tumor. Another issue of concern is sampling error of the tumor, where the slides prepared might have contained a relatively small proportion of neoplastic tissue. FISH in the pancreas is very susceptible to sampling error as well as representation of the tumor as a whole due to the heterogeneous nature of pancreatic cancer.

In summary, we have shown how various molecular applications are significantly advanced by using TMAs and downstream molecular assays such as IHC and various ISH assays. These allow the cancer researcher and molecular pathologist alike to characterize novel biomarkers, in the context of cellular morphology, that are implicated in pancreatic cancer behavior and clinical outcomes.

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Chapter 12
Proteomic Analysis of Blood and Pancreatic Juice

Mark Aspinall-O'Dea, John Neoptolemos and Eithne Costello

Abstract The proteome is the complete set of proteins present in a defined cellular or extracellular compartment. A number of studies have been undertaken to characterise the protein profiles of plasma/serum and pancreatic juice, and protein differences between samples from cancer patients and benign disease controls or healthy individuals have been defined. More recently, advances in liquid chromatography-mass spectrometry (LC-MS) instrumentation, as well as developments in quantitative proteomic approaches, and analysis of post-translational modifications have enabled interrogation of cancer pathways and the effects of drugs on those pathways. For targeted drugs to be used effectively, biomarkers that provide information regarding drug activity, toxicity or patient response are essential. Such biomarkers are particularly useful if they can be detected in samples that are readily accessible, such as plasma/serum. The application of proteomic techniques affords opportunities for discovering and evaluating biomarkers that aid the process of drug development. In this chapter, some of the recent advances in proteomic techniques that enable the detection and quantification of proteins in blood and pancreatic juice will be reviewed. Particular emphasis will be placed on the significance of these advances for novel treatments of pancreatic cancer.

12.1 Introduction

Proteomic techniques have been applied for the analysis of a variety of different pancreatic cancer sample types (reviewed in Aspinall-O'Dea and Costello 2007; Chen et al. 2007a). These include bulk undissected tissue, microdissected tissue, blood in the form of plasma or serum, pancreatic juice, urine and cell lines, including intracellular and secreted proteins. Much of the effort to date has been focussed on finding proteins that can distinguish pancreatic cancer patients from patients

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with benign pancreatic diseases and healthy controls. Broadly speaking, the aims of such experiments were to elaborate the biology of pancreatic cancer as well as to identify biomarkers, potentially enabling earlier diagnosis, or informing prognosis. Importantly, most proteins, identified in expression proteomic studies, as up- or downregulated in pancreatic cancer, are not suitable drug targets. To identify such targets, specific experiments aimed at examining elements of signal transduction pathways within cancer cells that contain druggable molecules such as kinases are required (Macek et al. 2008). The focus of this chapter is on plasma/serum and juice analysis, and as such, a review of proteomic approaches aimed at identifying novel anti-cancer targets lies outside of its scope. We will concentrate on the potential application of proteomics for facilitating efficacy or toxicity monitoring of new candidate drugs.

Surgical resection is currently the only potentially curative option for pancreatic cancer patients. In this setting, chemotherapy in the form of 5-fluorouracil/leucovorin or gemcitabine improves survival (reviewed in Ghaneh et al. 2007). The majority of patients however, present with advanced/metastatic disease and for this group gemcitabine has become established as the standard therapy. Although combinations of gemcitabine with other agents have generally been unsuccessful, some combinations, e.g. gemcitabine plus capecitabine are superior to gemcitabine alone (Sultana et al. 2007). Anticancer therapies designed to target well-defined tumor signalling pathways important for cancer cell proliferation, invasion and metastasis have shown promise in the treatment of a number of types of cancer, and their application to pancreatic cancer is reviewed in (Middleton et al. 2008; Wong and Lemoine 2008). Whilst the addition of targeted therapies, such as farnesyltransferase inhibitors, metalloproteinase inhibitors, cetuximab and bevacizumab, to gemcitabine have failed to improve clinical outcome for the majority of pancreatic cancer patients, combination therapy using gemcitabine and erlotinib, a small-molecule tyrosine kinase inhibitor of the human epidermal growth factor receptor (EGFR) resulted in a significant improvement in overall survival compared with gemcitabine monotherapy (Moore et al. 2007) and has recently been approved by the US/European authorities for use in advanced disease. For stratified patient treatment to be used effectively, biological markers are vital.

Currently, there are no markers, in routine use, for predicting which pancreatic cancer patients will respond to a given chemotherapeutic agent and which patients may derive greater benefit from an alternative regimen. CA19-9 is the only biomarker used routinely in both resected and advanced pancreatic cancer cases with respect to predicting clinical course. However, it provides unreliable information on the likelihood of treatment response (Maisey et al. 2005; Liedtke et al. 2008; Smith et al. 2008). The efficient evaluation of tumor responsiveness to new drugs or drug-related toxicity in emerging early drug trial designs will depend on effective biomarkers (Kummar et al. 2007). This is particularly relevant where drugs are targeted to proteins or pathways that are critical in some patients but not in others. Finding biomarkers that predict response will ensure that drugs are directed to the patients most likely to respond, enhancing overall patient benefit and cost-effectiveness.

Blood and pancreatic juice are both excellent potential sources of biomarkers. Blood is an easily accessible sample, and is routinely analysed in clinical laboratories as part of the diagnosis and management of disease. It is a complex body fluid however, with a small number of abundant proteins accounting for a large percentage of the total protein present. This has made in-depth proteomic analysis of blood challenging, and methods have been developed for abundant protein depletion from blood or the normalisation of protein concentrations within this sample type. These include the use of a triazine dye known as Cibacron for the selective removal of albumin from blood (Thompson et al. 1975; Miribel et al. 1988), while the bacterial proteins, proteins A and G are used in centrifugal spin filters and high performance liquid chromatography (HPLC) columns (Sjobring et al. 1991; Wang et al. 2003) for the selective removal of immunoglobulins. Antibody-based depletion columns, available in small-scale centrifuge spin filter format or in liquid chromatography (LC)-formats, enable the depletion of additional high-abundance proteins, or, more recently, medium-abundance proteins. The aim of all of these approaches, used singly or in combination, is to simplify the plasma/serum sample enabling low-abundance proteins, such as tissue leakage factors, to be detected by MS. Alternatively, a process known as 'protein normalisation' can be attempted. This method is based on the use of a large, highly diverse library of hexapeptides, attached to chromatography beads. Highly-abundant proteins saturate their hexapeptide binding partners, whereas proteins of lower abundance are concentrated. The concentration of proteins eluted from the beads is therefore 'normalised' affording the possibility of detecting proteins that were originally present at very low levels (Sennels et al. 2007).

Pancreatic juice is, by nature, a less complex sample. It is secreted directly from the pancreatic duct and close proximity to pancreatic tumors makes it a likely rich source of cancer-related proteins. It is not, however, as readily accessible as blood, and its collection by endoscopic retrograde cholangiopancreatography (ERCP) carries a modest risk of pancreatitis (Loperfido et al. 1998; Vandervoort et al. 2002; Suissa et al. 2005). Hence analysis of juice is not appropriate for routine monitoring of biomarkers. Nonetheless, the study of juice may provide initial identification of potential biomarker candidates that would otherwise be very difficult to find in blood directly. Those candidates could subsequently be examined for their presence and regulation in plasma samples. An example of such a biomarker, HIP/PAP-I (Rosty et al. 2002), is discussed later in this chapter.

To appreciate the potential contribution of proteomics to the field of drug discovery, it is necessary to understand some of the technologies that enable quantification of proteins/peptides in samples. Traditionally, two-dimensional gel electrophoresis (2-DE) has been a mainstay of proteomic studies, employed for the global analysis of proteins (O'Farrell 1975; Gorg et al. 2000). Developments such as differential in-gel electrophoresis (DIGE), a technique which uses fluorescent dyes to facilitate the distinction between proteins from different samples made possible the relative quantification of proteins from different samples separated on a single gel (Unlu et al. 1997). However, the introduction of quantitative MS-based approaches has had the most significant impact on clinical proteomics, enabling the relative and absolute global quantification of proteins in plasma and juice. Moreover, when

combined with methods that enrich for phosphopeptides, opportunities for quantitative phosphoproteomics emerge.

In the following sections, a number of techniques enabling quantitative MS are reviewed, with particular emphasis on their application for the analysis of plasma/serum or juice. Specific examples of the use of these techniques are taken, where possible, from studies of pancreatic cancer. Finally, the application of proteomics for the evaluation of novel pancreatic cancer treatments is explored, with examples taken from the epidermal growth factor (EGF) and the vascular endothelial growth factor (VEGF) pathways.

12.2 Quantitative LC-MS/MS Approaches

Generally proteins, in complex mixtures, are digested enzymatically into peptides, prior to separation by nanoscale LC and analysed by MS. The measurement of peptide intensities by MS is not sufficient to provide accurate quantitative information. This has led to the development of alternative approaches (reviewed in Ong and Mann 2005), such as the incorporation of stable isotope atoms like deuterium (^{2}H), carbon-13 (^{13}C), nitrogen 15 (^{15}N) or oxygen-18 (^{18}O) into the peptides of proteomes to be analysed by MS. The peptides labelled with the heavy isotope retain the characteristics of their light isotope counterparts; however, the small change in mass can be detected by the mass spectrometer. In practice, peptides from two sample types, such as cancer and control are obtained by trypsin digestion of proteins. Peptides from one sample are labelled with the heavy isotope, and these peptides are then mixed with the lighter peptides from the other samples and analysed simultaneously by MS. The peptides appear as doublets in the mass spectra and the relative intensities of the doublet directly reflect the relative quantity of the peptides in the two proteomes. The methods of isotope-coded affinity tags (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), and stable isotope labelling with amino acids in cell culture (SILAC) are shown schematically in Fig. 12.1 and described below.

12.2.1 Stable Isotope Labelling—In Vitro

Although largely superseded by iTRAQ, ICAT has been used to profile pancreatic juice (Chen et al. 2006, 2007b) and is therefore worth briefly mentioning. Each ICAT label has a thiol-specific reactive group, which allows it to interact with thiol groups of cysteines (Shiio and Aebersold 2006). A biotin moiety on the label enables the selective recovery of labelled peptides for MS; this step greatly reduces the number of peptides, and hence complexity, since only cysteine-containing peptides are labelled. Finally, between the thiol reactive and biotin group, each label contains a linker, with a defined number of light or heavy isotope atom(s) of hydrogen

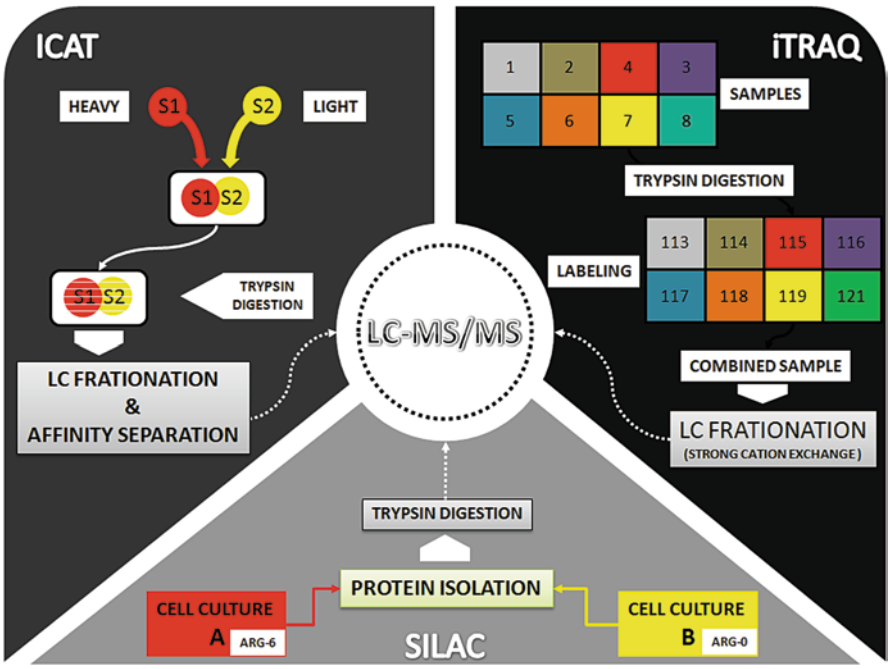

Fig. 12.1 Schematic representation of methods used for the relative and absolute quantification of proteins in biological samples in vitro and in vivo. S1 = sample 1; S2 = sample 2

or carbon. This linker thus enables discrimination between the heavy and light forms of the label. Chen et al. performed ICAT on pancreatic juice sample from a pancreatic cancer patient compared to juice pooled from benign disease control patients ($n = 10$) (Chen et al. 2006). Similarly, in a separate study they compared juice from a chronic pancreatitis patient with juice pooled from benign disease control patients ($n = 10$) (Chen et al. 2007b). In total, 136 proteins were identified in pancreatic juices from both studies. Nine proteins, including chymotrypsinogen b and Ig-α1 chain c region were found to be upregulated in both cancer and pancreatitis juice compared to benign control juice. Twenty-one proteins were found to be differentially expressed only in pancreatic cancer compared to benign control, while 18 were found to be differentially expressed only in pancreatitis. The data provided in these studies provide a useful basis for MS-based quantitative analysis of biomarkers in pancreatic juice, although larger studies, which include greater numbers of cancer patients and more extensive validation, are merited. In this context, a study by Rosty et al. (2002) is noteworthy. This study used an alternative proteomic technique called surface-enhanced laser desorption ionisation (SELDI), which enables quantification of small proteins or peptides in samples, although it does not provide direct identification of proteins (reviewed in Merchant and Weinberger 2000). Using SELDI, Rosty et al. (2002) identified hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I (HIP/PAP-I; also known

as regenerative islet derived 3 alpha, REG3A) as a potential biomarker in juice. The authors went on to quantify the juice HIP/PAP-I levels in 43 patients and subsequently the serum HIP/PAP-I levels in 98 patients, and reported significantly higher levels of this protein in both juice and serum of patients with pancreatic cancer compared to patients with other pancreatic diseases.

ICAT suffers from a number of limitations. First, only two samples can be compared to each other in a single experiment (Fig. 12.1). Second, only proteins containing cysteine residues are analysed. The introduction of iTRAQ has overcome these limitations. iTRAQ allows the differential labelling of peptides from up to eight different proteomes (Shadforth et al. 2005; Wu et al. 2006). iTRAQ tags react with the N-terminal groups of peptides, thus potentially labelling all peptides in the proteome. After labelling, samples are mixed and all eight proteomes are analysed simultaneously (Fig. 12.1). As is the case for ICAT, the differentially labelled versions of a peptide appear as a single peak in MS; however, upon fragmentation in the MS/MS spectra, each tag generates a unique reporter ion, the relative intensities of which relate back to the quantity of each peptide in the corresponding proteome. Kits for the analysis of plasma/serum by iTRAQ are commercially available, although no data on pancreatic cancer samples have been published to date.

The differential labelling of whole proteins with heavy or light deuterated isotopes of acrylamide provides an alternative quantitative proteomic method. This approach was recently undertaken by Faca et al. to compare the plasma profiles of genetically engineered mouse model of pancreatic cancer. Samples were taken at different stages in the development of pancreatic cancer, allowing the comparison of plasma from control mice and those with early lesions (PanIN) or advanced pancreatic cancer (Faca et al. 2008). In this elegant study, a total of 1442 plasma proteins were identified, and quantification data were obtained for 621 proteins. One hundred and sixty five proteins were found to be up-regulated in cancer samples or PanIN or both, and validation of expression, by immmunohistochemistry (IHC) or ELISA on mouse plasma samples, in tumors or in the circulation, respectively, was obtained for a selected number of proteins. Arising from this work, a panel of markers, including LCN2, REG1A, REG3, TIMP1 and IGFBP4, was evaluated in human subjects with very early pancreatic cancers. This panel performed slightly better than CA19–9; however, when the panel was combined with CA19-9 significantly improved discrimination between early stage (pre-diagnostic) sera and matched controls was achieved.

12.2.2 Stable Isotope Labelling—In Vivo

SILAC (Fig. 12.1) is an in vivo labelling strategy in which two different cultured populations are compared (Ong et al. 2002). One cell population (e.g. mammalian, yeast, or bacterial) is grown in the presence of naturally abundant unmodified (or light) essential amino acids while the other population is cultured in the presence of modified heavy essential amino acids, such as $^{13}C_6$-arginine. Proteins from each distinct

population are thus distinguishable from each other by MS. SILAC is well suited to the study of post-translational modifications. Harsha et al. (2008) recently combined SILAC with immunoprecipitation of tyrosine phosphoproteins for the detection of potential drug targets in pancreatic cancer. Having screened a number of pancreatic cancer cell lines, the group identified one, P196, that showed a particularly high phosphotyrosine content. This line was then compared, using SILAC, with a non-neoplastic ductal cell-derived cell line, HPDE. P196 cells were grown in medium containing heavy ^{13}C-labelled arginine, while HPDE was grown in the presence of regular light amino acids. Cell lysates from each cell line were then mixed, tyrosine phosphoproteins were immunoprecipitated, separated by one-dimensional gel electrophoresis and resulting bands cut from the gel, trypsin digested and analysed by MS. This led to the observation of hyperphosphorylation of EGFR in P196 cells. In subsequent experiments, P196 cells and another cell line positive for phosphorylated EGF were implanted into athymic nude mice. Tumors arising from these cell lines responded well to the EGFR inhibitor erlotinib. However, cells lacking activated EGFR did not respond to this treatment. Harsha et al. (2008) suggested that activated EGFR is a candidate for targeted therapy in a subset of pancreatic cancers. Additional markers of potential response to agents targeting EGFR are discussed later.

12.2.3 Phosphoproteomics: Detection and Quantification of Phosphoproteins

The importance of phosphorylation as a means of regulating key cellular events in normal and cancer cells has been established. Moreover, kinases have emerged as crucial druggable targets in cancer. The analysis of phosphoproteomes is therefore important and has been made possible by advances in phosphopeptide enrichment techniques, high-accuracy MS and bioinformatics. This expansive field is reviewed extensively elsewhere (Macek et al. 2008; Schreiber et al. 2008). Proteomes digested into peptides generally contain only a small proportion of phosphopeptides, necessitating their enrichment prior to efficient analysis by MS (Fig. 12.2). A number of approaches for phosphopeptide enrichment are possible. These include the use of immobilised metal affinity chromatography, based on the high-affinity of phosphates to certain trivalent metal cations, such as ferric iron (Fe^{3+}). Alternatively, titanium dioxide (TiO_2) is a potentially useful substrate for selective enrichment of phosphopeptides because organic phosphates are adsorbed by it under acidic conditions and desorbed under alkali conditions. Strong cationic exchange also allows for the selection of phosphopeptides on the basis of their reduced charge compared to other peptides. Finally, antibodies with specificities for tyrosine phosphate have been used for the immunoprecipitation of tyrosine phosphorylated proteins. The enrichment step is usually preceded by a differential isotope labelling step, such as SILAC or iTRAQ, and followed by separation of peptides by nano-liquid chromatography and MS. The characterisation of phosphopeptides is highly specialised and is reviewed in detail by Macek et al. (2008).

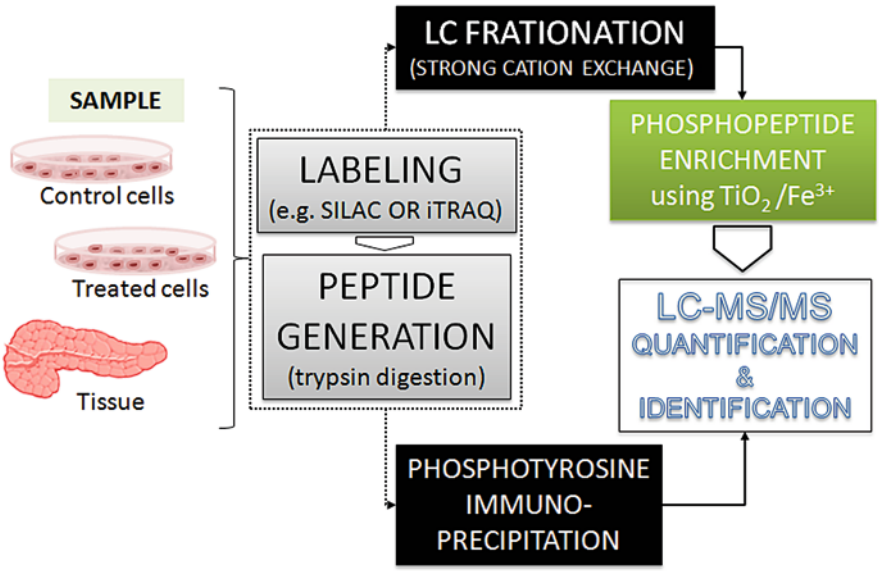

Fig. 12.2 Sample quantitative phosphoproteomics workflow. Control and treated cells or tissues are, in the case of SILAC, differentially labelled by stable isotopes, proteins extracted, trypsin digested and samples pooled. For iTRAQ, proteins are extracted and trypsin digested prior to differential labelling of peptides using stable isotopes. For both SILAC and iTRAQ procedures, phosphopeptides are enriched through, for example, strong cation exchange and TiO_2 chromatographies, or by immunoprecipitation with phospho-specific antibodies. The enriched phosphopeptide mixtures are then separated by LC and MS undertaken for the identification and relative quantification of proteins

12.2.4 Multiple Reaction Monitoring

Multiple reaction monitoring (MRM) (reviewed in Lange et al. 2008) is the name given to a process that enables samples, such as plasma or pancreatic juice to be screened by MS for the presence and quantity of specific proteins. The presence of a peptide is monitored by its mass/charge (m/z) value and the presence of fragment ions of that peptide. The process takes advantage of the unique capabilities of triple quadrupole mass spectrometers for quantitative analysis. By definition, MRM follows the biomarker discovery stage, and allows candidate biomarkers to be validated and quantified in a series of samples. It has potential application in the monitoring of biomarkers of drug response and is enormously attractive in the case of candidate biomarkers for which good antibodies are unavailable. Equally, the possibility of simultaneously obtaining accurate quantification of a predefined set of proteins is enticing. By contrast with biomarker discovery projects, where the aim is to achieve the maximum possible proteome coverage, in MRM, the mass spectrometer is set up to scan a defined select set of peptides (i.e. relating to the biomarker proteins of interest plus control proteins), and thus achieves far greater

sensitivity, enabling the detection of low-abundance proteins in highly complex mixtures, such as plasma/serum.

12.3 Overview of Proteomic Analysis of Juice and Blood in Pancreatic Cancer Samples to Date

A variety of approaches aimed at characterising the proteome of pancreatic juice and blood from pancreatic cancer patients have been undertaken (Table 12.1), providing a catalogue of proteins in these samples as well as information on the differential regulation of proteins between patients with cancer or benign diseases of the pancreas (reviewed in Grantzdorffer et al. 2008). Using one-dimensional gel electrophoresis (1-D PAGE) with liquid chromatography and tandem mass spectrometry (LC-MS/MS), Gronborg et al. (2004) catalogued proteins present in pancreatic juice from three pancreatic cancer patients, identifying up to 115 proteins. The studies of Chen et al. (2006, 2007b) in which ICAT was applied to compare juice profiles from patients with pancreatic cancer and benign pancreatic

Table 12.1 Some of the studies that have contributed to the catalogue of proteins known to characterise the pancreatic juice and blood from pancreatic cancer patients

Sample type	Proteomic method used	Number of samples analysed	Citation
Pancreatic juice	1-D PAGE with LC-MS/MS	3 cancer	Gronborg et al. (2004)
Pancreatic juice	SELDI	15 cancer; 7 disease control	Rosty et al. (2002)
Pancreatic juice	ICAT	1 cancer; 10 disease control	Chen et al. (2006)
Pancreatic juice	ICAT	1 CP; 10 control	Chen et al. (2007b)
Pancreatic juice	DIGE with MS/MS	9 cancer; 9 cancer–free control	Tian et al. (2008)
Serum	2-DE with MALDI-TOF and MS/MS	32 cancer, 30 healthy control	Bloomston et al. (2006)
Plasma	DIGE with MS/MS	10 cancer (before and after surgery)	Lin et al. (2006)
Plasma	2-DE with MS/MS	11 cancer, 11 healthy control, 10 CP	Deng et al. (2007)
Plasma	2-DE with MS/MS	14 cancer and 13 disease control	Yan et al. (2009)
Serum	SELDI	60 cancer, 120 control	Koopmann et al. (2004)
Plasma	SELDI	113 cancer, 132 non-cancer control	Honda et al. (2005)
Serum	SELDI	96 cancer, 96 control patients	Ehmann et al. (2007)
Plasma	Stable isotope labelling	Cancer mouse model	Faca et al. (2008)

diseases are discussed above, as is the study by Rosty et al. (2002) which applied SELDI to investigate pancreatic juice from 15 patients with pancreatic cancer and 7 patients with non-malignant disease. Most recently, Tian et al. (2008) used difference gel electrophoresis (DIGE) and MS/MS to compare the pancreatic juice profiles from nine PDAC patients and nine cancer-free controls, identifying three proteins, including matrix metalloproteinase-9, as overexpressed in pancreatic cancer juice.

Bloomston et al. (2006) used a combination of 2D-E and MS to compare serum from 32 pancreatic cancer patients with 30 healthy controls, observing an increase in fibrinogen gamma in the sera of cancer patients. Gel-based approaches, one using DIGE (Lin et al. 2006) and the other using 2D-E (Deng et al. 2007) were also undertaken for the comparison of plasma samples before and after surgery in two studies. Lin et al. (2006) identified a group of proteins, including complement component C4A, haemopexin and apolipoprotein A IV that differed in expression after surgical resection of pancreas. Deng et al. (2007) observed increases in proteins such as haptoglobin and alpha1 antitrypsin following surgery. In a 2D gel study undertaken by Yan et al. (2009), the importance of considering bile duct obstruction, a feature of pancreatic cancer, was highlighted. The study reported differential plasma levels of apolipoprotein A1, transthyretin and apolipoprotein E in cancer patients compared to patients with benign biliary disease or chronic pancreatitis. However, when the effect of bile duct obstruction was considered, only transthyretin levels were independently associated with cancer likelihood.

Non-gel based approaches, such as SELDI have been used by a number of groups (Table 12.1) in an attempt to differentiate blood of cancer patients from healthy or benign disease controls (Koopmann et al. 2004; Honda et al. 2005; Ehmann et al. 2007). SELDI is a high-throughput technique, and these studies have the advantage that relatively large sample numbers were assessed. Work with animal models has also advanced the search for plasma biomarkers of pancreatic cancer. This is best illustrated by the study of Faca et al. (2008), which was reviewed earlier in this chapter. The progress made and experience gained by all of the groups that have undertaken these initial studies on blood and juice will pave the way for more sophisticated analyses that will allow the identification of biomarkers for use in drug development for pancreatic cancer.

12.4 Novel Treatments for Pancreatic Cancer: Analysis of Plasma/Serum Biomarkers

Recent developments in the treatment of pancreatic cancer are reviewed in detail elsewhere (Ghaneh et al. 2007; Middleton et al. 2008; Wong and Lemoine 2008) and are not covered here, although examples from two pathways, the EGFR and the VEGF pathways, will be used. As mentioned earlier, a crucial aspect of developing new drugs is the availability of easily measurable biomarkers of efficacy, toxicity

or of predicted response. In this context, pharmacoproteomics, the use of proteomic technologies for drug discovery, biomarker validation, pharmacodynamic, pharmacokinetic and toxicological evaluation is an emerging speciality (Zolla 2008). A recent study in non-small cell lung cancer (NSCLC) shows the potential of protein profiling of serum to facilitate the prediction of drug responsiveness. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-MS) was undertaken in patients with NSCLC prior to treatment with the EGFR inhibitors, erlotinib and gefitinib, and an algorithm was developed that could classify patients for good or poor outcome after treatment with the EGFR inhibitors. The algorithm was based on eight specific *m*/*z* features identified in the serum samples from a training set of 139 patients across three cohorts (Taguchi et al. 2007). Strategies that enable the prediction of response EGFR inhibitors have relevance to pancreatic cancer patients.

12.4.1 The EGFR Pathway in Pancreatic Cancer and Its Therapeutic Intervention: Associated Biomarkers

EGFR and a related family member ErbB-2 are overexpressed in ~70% and 17–45% of pancreas cancer cases respectively, and their expression is associated with rapidly progressive disease, resistance to chemotherapy and poor prognosis (Barton et al. 1991; Korc et al. 1992; Yamanaka et al. 1993a, b; Okada et al. 1995; Friess et al. 1999; Novotny et al. 2001; Saxby et al. 2005). EGFR (also known as human EGF receptor 1 or HER-1) is a member of the type I family of tyrosine kinase growth factor receptors. As well as ErbB-2 (HER-2), family members also include ErbB-3 and ErbB-4. These proteins share a common structural architecture including a tyrosine kinase domain (except for ErbB-3), a transmembrane domain and extracellular domain. The extracellular domain contains binding sites for multiple ligands (Schneider and Wolf 2008), although none have been identified for ErbB-2. Ligand binding results in the formation of either homodimeric (EGFR-EGFR) or heterodimeric (EGFR-ErbBX) receptor complexes leading to autophosphorylation of tyrosine residues on the intracellular domain and activation of downstream signalling cascades, including Ras/Raf/MAPK (cell proliferation), PI_3K/Akt (cell cycle progression and survival) and the signal transducer and activator of transcription STAT protein family (cellular survival, proliferation, adhesion, motility and invasion) (Marshall 2006). Proteomic analyses have been applied for the dissection of the EGFR and ErbB-2 signalling networks in a variety of cancer types (reviewed in Kruse et al. 2008). In the case of pancreatic cancer, the SILAC-based experiments of Harsha et al. (2008) described earlier in this chapter, provided evidence for activated EGFR as a candidate for targeted therapy in this disease.

A number of agents have been developed to target the EGFR pathway and their use in recent years has led to the discovery of important biomarkers of response. Sensitising mutations in EGFR (Lynch et al. 2004) or increased EGFR copy number

(Cappuzzo et al. 2005) were found to be associated with enhanced response to agents targeting EGFR in NSCLC and metastatic colorectal cancer. Moreover, the presence of mutations in K-RAS was associated with an absence of response to EGFR targeting agents (Linardou et al. 2008).

As outlined in the Introduction, erlotinib (Tarceva, OSI774), an orally active small molecule which targets the adenosine triphosphate (ATP) binding site of the EGFR intracellular tyrosine kinase domain, inhibiting activation of downstream signalling cascades, has been shown to convey a small survival benefit in advanced cases of pancreatic cancer (Moore et al. 2007), with median survival of 6.4 months and a one-year survival rate of 24% versus 5.9 months and 17% for gemcitabine alone. Targeting erlotinib to the pancreatic cancer patients that are most likely to respond is vital. A recent phase 1 clinical trial evaluating NSCLC tumor response to erlotinib in combination with celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, in 21 patients showed favourable improvement with 33% of patients displaying partial response to treatment and 24% developing stable disease (Reckamp et al. 2006). Analysis of patient serum samples at baseline and week eight showed that patients who responded had significantly lower baseline levels of matrix metalloproteinase-9 (MMP-9) than non-responders. Partial response was also associated with a decrease in serum-soluble E-cadherin (sEC) levels (Reckamp et al. 2008).

Gefitinib (ZD1839, Iressa) is a selectively reversible orally active synthetic quinazoline that targets EGFR, specifically inhibiting autophosphorylation and blocking signal transduction (Anderson et al. 2001; Barker et al. 2001; Ciardiello et al. 2001). It has minimal activity against other tyrosine kinases and serine/threonine kinases. Phase 2 clinical trials involving patients with metastatic pancreatic cancer failed to show improved patient outcome (Blaszkowsky et al. 2007; Fountzilas et al. 2007). Masago et al. (2008) recently reported a correlation between circulating serum levels of EGFR ligands amphiregulin and transforming growth factor alpha (TGF-α) with resistance to gefitinib. In this small cohort study, serum samples were collected over a 5-year period from 93 patients with non-squamous, non-small cell lung cancer (NS-NSCLC) treated with gefitinib. Of these 93 cases, 34 displayed disease progression, 11 of which were positive for TGF-α. The median survival time for patients with TGF-α-negative serum was significantly longer than those with TGF-α-positive serum.

Transtuzumab (Herceptin) is a monoclonal anti-HER-2 antibody that binds to the extracellular domain of HER-2 receptor proteins, and has been shown to induce clinical responses in breast cancer patients. The extracellular domain of HER-2 can be cleaved from the membrane surface of cancer cells and released into circulation. Consequently, serum levels of HER-2 ECD (sHER-2) are detectable using ELISA (for review see Ross et al. 2004), and a number of studies have analysed the correlation between HER-2 levels and clinical outcome in pancreatic cancer patients treated with trastuzumab independently and in combination with established chemotherapy regimes gemcitabine and 5-fluorouracil (5-FU) (Kimura et al. 2006; Saeki et al. 2007; Pratesi et al. 2008).

12.4.2 The VEGF Pathway and Its Therapeutic Intervention: Associated Biomarkers

The VEGF pathway is another important pathway that has been well characterised in pancreatic cancer and one that has been targeted with experimental interventional drugs. The proangiogenic factor VEGF facilitates accelerated tumor growth and metastasis through the promotion of tumor angiogenesis and blood flow. VEGF has been shown to be overexpressed in ~90% of pancreatic cancers and is associated with tumor progression and poor prognosis (for review see Ghaneh et al. 2007). Five glycoproteins, VEGFA (commonly referred to as VEGF), VEGFB, VEGFC, VEGFD (FIGF) and placenta growth factor (P1GF, PGF) make up the VEGF family (Dvorak 2002; Hicklin and Ellis 2005) together with a number of VEGF-A isoforms created through alternative splicing (for review see Ellis and Hicklin 2008). These isoforms exist as protein products consisting of 121, 165 (predominant form), 189 and 206 amino acids with smaller isoforms generated as a result of proteolytic cleavage. The VEGF ligands bind structurally similar type III tyrosine kinase receptors known as VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4) which interact with co-receptor neuropilins NP1 (NRP1) and NP2 (NRP2) to increase VEGF-VEGFR-X binding affinity (Soker et al. 1998, 2002; Klagsbrun et al. 2002; Camp et al. 2006; Bielenberg and Klagsbrun 2007). The expression of VEGF is regulated by growth factors EGF, TGF-α, TGF-β, platelet-derived growth factor (PDGF) and the cytokines IL-1α and IL-6. VEGF can act in a number of ways including enhanced endothelial cell proliferation, survival, migration and invasion together with heightened endothelial permeability, and homing of bone marrow-derived vascular precursor cells (Ellis and Hicklin 2008). Furthermore, VEGF has autocrine effects on tumor cell function (survival, migration and invasion), immune suppression and bone marrow progenitors which promote metastasis (Ellis and Hicklin 2008).

Bevacizumab is a recombinant monoclonal anti-VEGF IgG antibody which has undergone testing in two large phase III clinical trials for the treatment of advanced (Kindler et al. 2005) or metastatic (Vervenne et al. 2008) pancreatic cancer in combination with gemcitabine plus cetuximab/erlotinib and gemcitabine plus erlotinib respectively. Neither trial has shown significant clinical outcome; however, plasma markers with prognostic power are emerging from recent breast cancer trials, and these may be useful in directing treatment to pancreatic cancer patients. Data suggest that plasma levels of VEGF, soluble vascular cell adhesion molecule (sVCAM-1) and soluble VEGFR-2 (sVEGFR-2) may represent prognostic markers for bevacizumab (Denduluri et al. 2008). Elevated levels of sVCAM-1 and sVEGFR-2 were detected after monotherapy treatment with bevacizumab in breast cancer patients, while combination therapy with filgrastim resulted in elevated levels of sVCAM alone. Moreover, patients who achieved a partial response to monotherapy showed a smaller increase in sVCAM levels which was not seen in combination therapy. In addition, tumor samples were evaluated for VEGFR-2 mutations before and after

treatment with bevacizumab, and baseline levels of phosphorylated VEGFR-2 correlated with increased baseline levels of sVCAM-1 suggesting that the latter is associated with the VEGF pathway (Denduluri et al. 2008). Baseline levels of the plasma angiogenesis marker VEGF were moderately lower in patients who responded to bevacizumab treatment; however, no statistical correlation relating to these levels could be attributed to clinical outcome in this study. Interestingly, previous work by Kobayashi et al. (2005) demonstrated a significant association between plasma levels of VEGF and median survival time in pancreatic cancer, with an elevated level of plasma VEGF in patients with distant metastases.

Vatalinib (PTK787, ZK222584) is a low molecular weight, orally bioavailable small molecule inhibitor of VEGFR-2 tyrosine kinase belonging to the chemical class of aminophthalazines. It is also known to inhibit other kinases including platelet-derived growth factor receptor beta (PDGFR-β) and c-Kit tyrosine kinase albeit at a higher active concentration than is required for VEGFR tyrosine kinases (Wood et al. 2000). Vatalinib has been shown to provide a positive clinical outcome in a phase I/II study of patients with advanced or metastatic pancreatic cancer when used in combination with gemcitabine (Kuo et al. 2008). Additionally, vatalinib has been used as a monotherapy in patients whose tumors had failed to respond to earlier gemcitabine therapy (Dragovich et al. 2008). Phase III clinical trials of patients with colorectal cancer treated with vatalinib in conjunction with FOLFOX-4 regime (oxaliplatin/5-fluorouracil/leucovorin) have identified lactic dehydrogenase (LDH) as a possible predictive marker of response (Hecht et al. 2005; Koehne et al. 2006). Hypoxia-inducible factor 1 alpha (HIF-1α) mediated co-regulation of VEGF and LDH may provide an explanation for positive clinical outcome in these patients as high levels of LDH correspond to tumors in which the VEGF pathway is most active (Morabito et al. 2006).

The pan-VEGF receptor tyrosine kinase ATP-competitive inhibitor AZD2171 is a highly potent orally available indole-ether quinazoline (Wedge et al. 2005), and has been shown to prevent VEGF-induced angiogenesis in vivo with dose-dependent activity in a number of human tumor mouse xenografts. In a recent phase II investigational study of glioblastoma patients, MRI techniques were combined with the detection of angiogenic cytokines and soluble receptors in patient blood samples using ELISA (Batchelor et al. 2007). This study demonstrated that daily administration of AZD2171 could reduce oedema in glioblastoma patients; however, it was observed that elevated levels of stromal cell-derived factor 1α (SDF-1α) in patient plasma corresponded to tumor progression regardless of treatment.

12.5 Concluding Remarks

The past decade has seen significant developments in proteomic technologies, particularly in those enabling quantitative measurements of analytes in complex substances. These technological breakthroughs promise to facilitate the discovery of biomarkers that will inform therapeutic decisions regarding the suitability of

patients for particular targeted treatments. This in turn will smooth the progress of new drugs to the clinic. We have reviewed some of the advances in MS that will play an important role in drug development in the coming years. The experience gained in profiling blood and juice along with the identities of proteins in these samples will serve as a basis for future analyses that will enable the identification of biomarkers for use in drug development for pancreatic cancer. Although therapies targeted at many pathways active in pancreatic cancer are being trialled (Middleton et al. 2008), we focussed, in this chapter, on two pathways: the EGFR and VEGF pathways. Examples of the novel therapeutics used to intercept the EGFR and VEGF pathways in pancreatic cancer, and the drive to find blood-based markers that can be monitored to predict response to these agents, illustrate the important contribution that biomarker monitoring will have in determining therapeutic choices for pancreatic cancer patients. The input of proteomics to this aspect of drug development is still in its infancy.

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Chapter 13
Applications of Antibody-Lectin Sandwich Arrays (ALSA) to Pancreatic Cancer Diagnostics and Drug Discovery

Yi-Mi Wu and Brian B. Haab

Abstract A growing body of research in recent years is establishing the importance of glycosylation in cancer. Several types of cancer, especially epithelial carcinomas such as pancreatic cancer, commonly display particular carbohydrate alterations that have potential functional roles in cancer progression. New analytical tools are providing enhanced opportunities for studying glycans in cancer. One such tool is the antibody-lectin sandwich array (ALSA). ALSA complements existing glycobiology methods by offering a unique set of capabilities, such as reproducible detection of glycans on specific proteins, sensitive detection directly from biological samples, multiplexed analysis of both core protein and glycan levels, and low-volume, high-throughput sample processing. Using this tool, one may characterize glycan variation in populations, identify glycan changes on specific proteins in model systems, or characterize protein carriers of specific glycans. These types of experiments will be especially useful in pancreatic cancer research in studies to develop biomarkers and to define therapeutic targets.

13.1 Introduction

The importance of carbohydrate post-translational modifications on proteins has become increasingly clear over many decades of advances in our understanding of glycobiology. The surface of cells is covered with a diverse array of carbohydrates attached to proteins (glycoproteins) or lipids (glycolipids) that are poised to interact with the extracellular environment. It is estimated that over 50% of all mammalian proteins contain glycosylation at some point during their existence (Apweiler et al. 1999). Glycans play a complete spectrum of biological roles including facilitating

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correct protein folding, targeting proteins for clearance or degradation, mediating receptor−ligand and protein−protein interactions, providing biophysical polarity or hydrophilicity, and guiding immune recognition (Varki et al. 1999; Ellgaard and Helenius 2001; Raman et al. 2005; Tarp and Clausen 2008). Every organism in nature shares in this integral use of glycans.

Not only are glycans important in normal biology, they also play roles in a variety of diseases. Abnormal glycosylation is involved in the pathogenesis or progression of a variety of inherited and sporadic diseases (Dennis et al. 1999; Dube and Bertozzi 2005). Because of the importance of glycans in the maintenance of health and in the development of disease, the field of glycobiology has increasingly focused on applied research in biotechnology and biomedicine. For example, treatment strategies based on interfering with the glycan-mediated process or targeting cancer glycans are under development (Fuster and Esko 2005), and blood-based diagnostic tests using glycan detection may be possible (Dube and Bertozzi 2005).

Among the diseases in which glycans play functional roles is cancer, in particular epithelial cancers such as pancreatic cancer. A common feature of pancreatic cancer is alterations to the glycan chains on cell-surface and secreted proteins. These alterations are thought to be involved in cancer progression through affecting cell adhesion, migration, metastatic capability, and immune invasion (Dennis et al. 1999; Dube and Bertozzi 2005). Because of the potential importance of glycan alterations in tumor progression, those molecular features may have significant value for use in cancer diagnostics or therapeutics. The methods for studying and detecting glycans have advanced significantly in recent years, and have led to new opportunities for realizing this goal of utilizing glycan alterations for clinical benefit.

One of the methods with significant potential for contributing to these advances is the antibody-lectin sandwich array (ALSA). Here we describe the applications of this technology for clinical utility in pancreatic cancer research. In order to properly understand the opportunities for using glycans, it is necessary to understand something of their functions both in normal and cancer biology. Therefore the article begins with an overview of normal glycosylation, cancer-associated glycosylation, and the roles of cancer-associated glycans in tumor progression. That discussion is followed by an overview of the capabilities of the technology and the ways it can be applied to cancer research.

13.2 Protein Glycosylation in Normal Biological Functions

The main classes of glycoconjugates (molecules with glycans attached) found on eukaryotic cells are proteoglycans, glycolipids, and glycoproteins. These entities are distinguished by the backbone on which glycans are attached as well as the structures of the carbohydrates. Proteoglycans are composed of a glycosaminoglycan—a long chain of repeating disaccharide units, such as chondroitin sulfate or heparin—attached to a membrane-bound protein. Glycolipids (also called glycosphingolipids), in contrast, are much shorter oligosaccharides of various stuc-

tures attached via glucose or galactose to the terminal primary hydroxyl group of the lipid moiety ceramide (Varki et al. 1999). While these types of glycoconjugates have been shown to play roles in cancer in various ways, in this review we focus on glycoproteins, which are the most amenable to analysis by ALSA and because their known contributions to cancer are broader and more extensively characterized than the other glycoconjugate types. Below we describe some of the major structural and functional features of protein glycosylation.

13.2.1 Structural Features of Protein Glycosylation

Protein glycosylation refers to the chains of monosaccharide building blocks that are covalently linked to particular amino acid residues (usually serine, threonine, or asparagine residues). The monosaccharides are mostly five- or six-carbon cyclic structures with various modifications and isometries. Glycosidic bonds join the monosaccharides at any of the carbons with either an 'alpha' or a 'beta' linkage—referring to the stereoisometry of the linkage at a chiral carbon—and in a linear or branched fashion. This variety in the components and the linkages results in a huge diversity of structures that can be formed.

Although massive diversity is theoretically possible, particular motifs and structural themes are prevalent, both in normal biology and in cancer. In general, the glycan motifs observed in cancer biology are not brand new but rather appear elsewhere in normal biology, only in a different context. For example, some of the cancer-associated glycans are observed in development or in lymphocyte activation, rather than on normal adult epithelial cells. This inappropriate activation or particular glycan motifs suggests the activation of biological programs that in certain contexts can be cancer promoting. Therefore it is important to understand the major glycan motifs in normal biology and the contexts in which particular cancer-associated motifs appear in normal biology.

Two main types of glycosylation appear on proteins: *N*-glycosylation and *O*-glycosylation. *N*-Glycosylation refers to the attachment of an oligosaccharide to the nitrogen of an asparagine residue within the consensus Asn-X-Ser/Thr (NXS/T) peptide backbone, and *O*-glycosylation refers to glycans attached to the oxygen of a serine or threonine residue. *N*-Glycans and *O*-glycans differ by more than just their sites of linkage; they are fundamentally different in their modes of synthesis, their structures, and their functions. Below is an overview of the structures and primary functions associated with these major types of protein glycosylation.

13.2.2 N-Glycans

13.2.2.1 Biosynthesis and Structures

The synthesis of *N*-glycans is initiated in the endoplasmic reticulum (ER) membrane. All *N*-glycans begin with the transfer of a pre-assembled oligosaccharide,

glucose$_3$-mannose$_9$-*N*-acetylglucosmine$_2$ ($Glc_3Man_9GlcNAc_2$) attached to a lipid called dolichol phosphate, to the asparagine residue of a nascently-translated protein. This transfer is accomplished by a multisubunit protein complex called oligosaccharyltransferase (OST). Following the attachment of the core oligosaccharides, several ER glycanases sequentially remove terminal glucose or mannose residues. This process determines whether the associated protein is subjected to folding attempts or translocated to the cytosol for degradation (Molinari 2007). Protein precursors with appropriate structures can be further processed by a series of enzymatic reactions in the ER and Golgi to generate diversified *N*-glycan structures.

The resulting glycan structure has been fashioned into one of three main classes of *N*-glycan structures, with many variants within each class. The classes, termed high mannose, hybrid, and complex (Fig. 13.1), are defined by the types of glycans attached to the two branches of core mannose units. The factors determining the final structure include the order and activities of the enzymes in the processing pathway and the metabolic supply to sugar nucleotide pools (Lau et al. 2007; Mendelsohn et al. 2007; Lau and Dennis 2008).

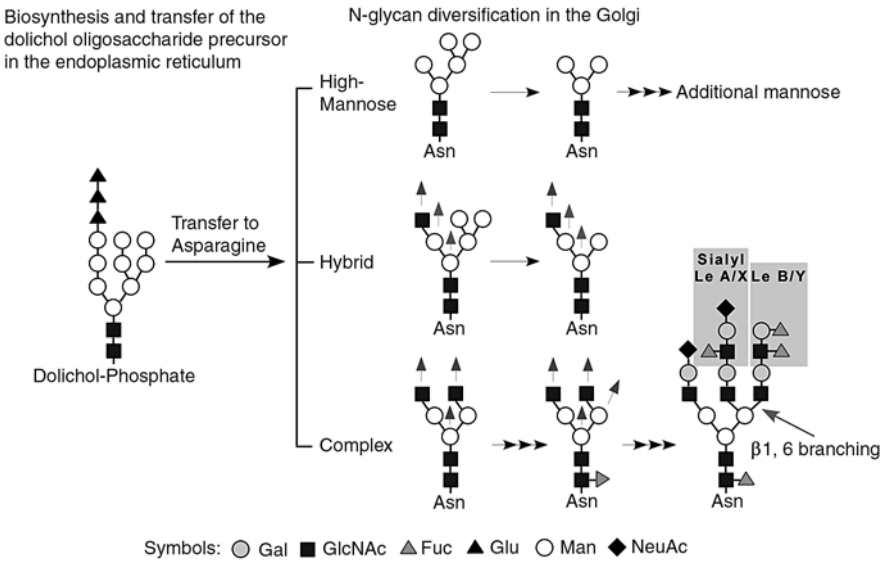


Fig. 13.1 Major classes and extensions of *N*-glycans. Representative structures are shown for the three types of *N*-glycans. The vertical *gray arrows* represent locations of branch formation and extension, not all of which occur on any single *N*-glycan. The *gray boxes* represent possible cancer-associated structures. Asn, asparagine; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Glu, glucose; Man, mannose; NeuAc, neuraminic acid (sialic acid)

13.2.2.2 Major Functions of *N*-Glycans

N-Glycans have roles in the structure and biochemical properties of proteins as well as in interactions with other proteins. Much information about *N*-glycan function has been gathered from studies on genetic mutants of cells or organisms and from analyses of congenital diseases associated with defects in glycosylation. *N*-Glycosylation is critical in development, as mouse mutants defective for the initiation of *N*-glycosylation die in the embryonic stage. Other mutants lacking complex *N*-glycans reveal roles for those structures in cytokine signaling, glucose transport, immunity, cell migration, proliferation, and differentiation, and other functions (Varki et al. 1999; Demetriou et al. 2001; Haltiwanger and Lowe 2004; Lau and Dennis 2008). Thus *N*-glycans play critical roles in many processes associated with cancer.

13.2.3 O-Glycans

13.2.3.1 Biosynthesis and Structures of Mucin-type Glycosylation

The biosynthesis of *O*-glycans is different from *N*-glycans in that it proceeds in a stepwise fashion directly from the amino acid linkage, rather than through the transfer of a pre-assembled complex. The most abundant type of *O*-glycosylation, which we focus on here, is the GalNAc-type, so-called because the first monosaccharide of the chain is always *N*-acetylgalactosamine (GalNAc). Mannose, fucose, or *N*-acetylglucosamine units can also be attached to the oxygen of serine or threonine residues, but these glycosylation types are rarer and do not lead to the larger glycan structures typically associated with protein glycosylation. GalNAc-type *O*-glycosylation also is referred to as mucin-type *O*-glycosylation because of its ubiquitous appearance on mucins and similar cell-surface-associated proteins.

A consensus peptide sequence determining which serines or threonines are modified with an initiating GalNAc has not been determined. The specificity of the initiation is thought to be determined by the activities of the some 24 GalNAc transferases that can perform that transfer, each of which has slightly different peptide specificity. Further chain extensions of *O*-glycans occur through one of four different core structures (Fig. 13.2). The final structures are determined by the order and activities of the glycosyltransferases in the ER and Golgi; the availability of monosaccharide precursors; and the flux of substrates moving through the pathway.

The ultimate structures derived from these four cores are distinct, although many terminal motifs are common between the cores and with *N*-glycans. These four core *O*-glycans comprise the majority of *O*-glycan structures produced in vivo, with most being of the Core-2 subtype. Other modifications to the GalNAcα-Ser/Thr structure have been reported to form Core-5-7 structures. These cores are less common, expressed at relatively low levels, and perhaps are specific to certain tissues or cell types (Schachter and Brockhausen 1989; Brockhausen 2000; Tarp and Clausen 2008).

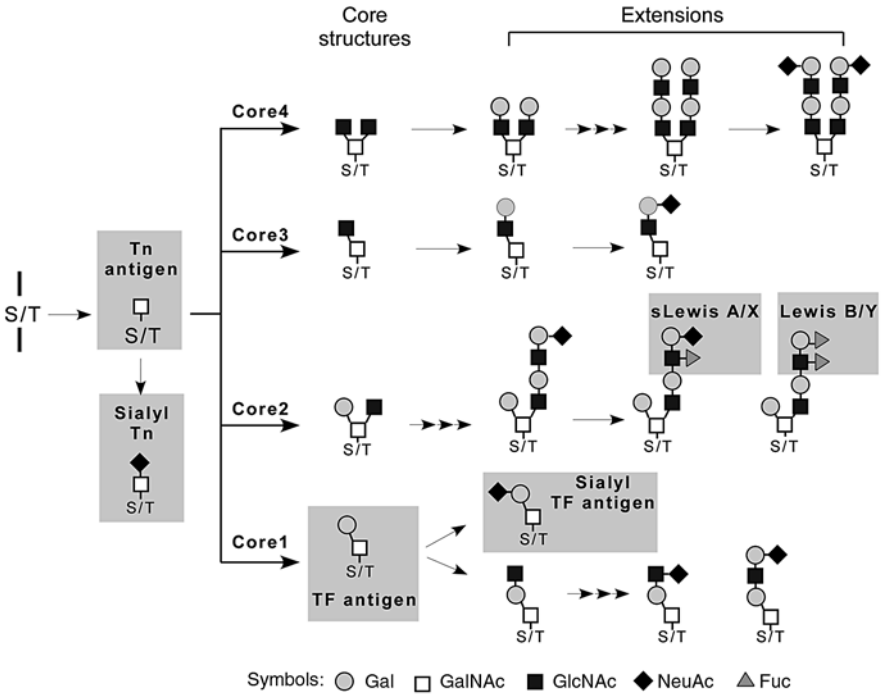


Fig. 13.2 Major core structures and extensions of *O*-glycans. The *gray boxes* represent possible cancer-associated structures. S/T, serine/threonine; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, neuraminic acid (sialic acid); Fuc, fucose

13.2.3.2 Major Functions of *O*-Glycans

Much of what is known about the functions of *O*-glycans is in their capacity as modifications on mucins. *O*-Glycans on mucins have a variety of critical roles in the proper function of the cell and organism. The dense glycosylation in the tandem repeats of mucins enables them to function as protective barriers and provide lubrication in a variety of tissues due to their hydration capacity, as exemplified by MUC1 and MUC2 (Gendler and Spicer 1995; Hang and Bertozzi 2005). Mucin-type *O*-linked glycans can also block accessibility of the polypeptide backbone to proteases and confer resistance to degradation; regulate the serum half-life of chemokines or hormones; and modulate the intracellular trafficking of proteins (reviewed in Gendler and Spicer 1995; Hollingsworth and Swanson 2004; Moniaux et al. 2004; Hang and Bertozzi 2005; Andrianifahanana et al. 2006). In addition to modulating protein function, mucin-type *O*-linked glycans can serve as ligands for cell-surface receptors that mediate cell adhesion events (Fukuda 1996) and may play roles in modulating immune system activity. Therefore *O*-glycans are involved in a wide variety of activities mainly regulating the extracellular environment but also affecting intracellular signaling.

13.3 Protein Glycosylation in Cancer

Glycoproteins produced by cancer cells often display glycosylation that is significantly different from their non-transformed counterparts. Given the importance of glycosylation in carrying out many of the functions of proteins, it is reasonable to infer that many of these glycan changes have roles in enabling cancer cell functions. In addition, since many of the cancer-associated glycans appear elsewhere in normal biology, the cancer-associated glycans could be the functional mediators of abnormally-activated biological programs. In this section, we first provide a survey of some of the evidence for the contributions of glycans to cancer processes, and we give details on particular glycan alterations, with a special focus on pancreatic cancer. Both sections are not exhaustive reviews, but rather highlight some of the main findings that are relevant to understanding the applications in the following section.

13.3.1 Roles of Glycans in Cancer Progression

Many studies have demonstrated the importance of glycosylation in cancer progression processes. Here we highlight the contributions to cell migration and metastasis and the modulation of inflammation and immune recognition, although glycan alterations may be important in other areas such as the inhibition of apoptosis (Nguyen et al. 2001) and the regulation of angiogenesis (Fuster and Esko 2005). Glycosylation also is critically important in the development of protein and antibody therapeutics, since glycans can affect activity, clearance rates, and drug resistance (Kudo et al. 2007), but such a discussion is outside the scope of this review.

13.3.1.1 Cell Adhesion and Metastasis

The ability of cancer cells to migrate and colonize is highly dependent on the makeup of the cell-surface carbohydrates. For example, transformed cell lines treated with glycosylation modulators such as tunicamycin (which inhibits *N*-glycosylation by blocking the formation of the polysaccharide *N*-glycan precursor), castanospermine (a potent inhibitor of α- and β-glucosidases), and swainsonine (a specific and potent inhibitor of α-mannosidase) (Dorling et al. 1980; Saul et al. 1984; Elbein 1987; Dricu et al. 1997) show altered glycosylation patterns of their surface glycoproteins and exhibit remarkable reductions in tumor formation and metastasis in experimental animals (Irimura et al. 1981; Dennis 1986; Humphries et al. 1986). Disaccharide-based inhibitors of glycosylation also reduced the metastatic potential of tumor cells (Fuster et al. 2003; Brown et al. 2006). Some of the details regarding the specific glycans involved and their mechanisms of action have been elucidated.

One mechanism is the expression of glycans that serve as ligands for endogenous lectins on endothelial cells (Taylor and Drickamer 2007). Through the expression of these selectin ligands, the ability of circulating cancer cells to adhere to endothelium

is increased, thus increasing the propensity to form new metastatic tumors. In addition to increased adhesion to endothelium, increased interactions with blood-borne cells also contribute to metastatic potential (Tang and Honn 1994; Fukuda 1996; Kannagi 1997; Kakiuchi et al. 2002). These interactions may be mediated through galectin proteins, which can serve to form bridges between cells through the formation of lattices. The cancer-associated upregulation of glycan ligands for galectins may contribute to that process (Glinsky et al. 2001). Alterations to the charge state of the surface of cancer cells also might affect the adhesion and migratory ability of cancer cells, particularly through modifying the sialic acid content on the cell surface (Chiricolo et al. 2006). The specific glycans involved in these processes are described in the next section.

13.3.1.2 Immunomodulation

Tumor cells have a variety of mechanisms, affecting every branch of the immune system, to avoid anti-tumor immune responses and destruction from the immune system. These mechanisms include: defective presentation of antigens on the cell surface (Johnsen et al. 1999); secretion of factors that inhibit immune cell function and impair the development of immune cells (Ohm and Carbone 2002); production of a large amount of vascular endothelial growth factor (VEGF), which can result in reduced maturation of dendritic cells (DCs) and reduced antigen presentation (Ohm and Carbone 2002); and expression of molecules that can protect the cells from complement attack (Babiker et al. 2005). Also, certain acute phase reactants, which are upregulated in many cancers due to inflammation at the tumor site, can reduce the cell-mediated immune response (Samak et al. 1982). The contributions of glycans to immunomodulation have been demonstrated in several studies.

Some of the glycan-mediated immunomodulation takes place through glycans attached to mucins. Mucins secreted by cancer cells may interfere with natural killer (NK) cell lysis, as MUC1 purified from human colon adenocarcinoma cells, which also carried cancer-associated glycans, inhibited cell lysis by NK cells in a dose-dependent manner (Zhang et al. 1997). Tumor-derived mucins also interact with dendritic cells—immune system cells that scavenge proteins and present them to T cells for possible recognition. It was reported that MGL (macrophage galactose type C-type lectin) expressed by DCs could act as the receptor for recognition and binding of MUC1 bearing a cancer-associated glycan, causing the internalization and presentation of MUC1 to T cells (Saeland et al. 2007). Since MGL is highly expressed on immature DC or antigen-presenting cells (Higashi et al. 2002; van Vliet et al. 2006), the interaction between MIC1 and MGL may lead to immunosuppressive effects in cancer patients. Another mode of interfering with the immune system is through the shedding of mucins that contain the ligands for the selectin receptors expressed on endothelial cells, which might block interactions between endothelial cells and leukocytes (Zhang et al. 1996).

Cancer-associated glycans also can elicit immune responses. One study found that the level of the immune response against tumor-derived MUC1 inversely

correlated with disease severity (Gourevitch et al. 1995), indicating a role for the immune system in fighting off pancreatic cancer. Many strategies to stimulate anti-cancer immune responses by targeting cancer-associated glycans on mucins are under development (Tarp and Clausen 2008).

13.3.2 Common Glycan Alterations in Cancer

13.3.2.1 Core-1 *O*-Glycan Structures on Mucins

As described above, *O*-glycans differ from *N*-glycans in that they are synthesized by the stepwise addition of monosaccharides in specific linkages, rather than by the transfer of a core structure followed by enzymatic modifications. The ultimate structure of an *O*-glycan is determined by the order in which glycosyltransferases act. In cancer cells, *O*-glycan synthesis is often rewired to produce shorter, non-branched structures based on the Core-1 unit (Fig. 13.1). The *O*-glycan Core 1, Galβ1,3GalNAc linked to serine or threonine, is known as the Thomsen−Friedenreich (TF) antigen (Yu 2007). The unsubstituted core GalNAc linked to serine or threonine—termed the Tn antigen—also can be found in cancer. The Tn and TF antigens and their sialylated forms are the most common cancer-associated glycans, expressed by over 80% of human carcinomas and found on multiple secreted and surface glycoproteins (Springer 1984, 1997; Brockhausen 2006). Pancreatic cancer particularly shows an up-regulation of the Tn antigen and its sialylated derivative (sialyl-Tn), as revealed by immunohistochemistry (Schuessler et al. 1991). A shift from branched *O*-glycans to Core-1-based structures on mucins has also been well characterized in breast cancer (Lloyd et al. 1996; Burchell et al. 2001). This reduced branching of *O*-glycans is in contrast to *N*-glycans, which show a link between highly-branched structures and cancer, as described below.

These short glycan structures may contribute to cancer progression in a variety of ways. One way might be through the mitogenic activity of certain lectins that bind the TF antigen, such as peanut agglutinin (PNA) (Ryder et al. 1992). The increased exposure of the TF antigen could lead to increased stimulation by endogenous lectins or lectins of bacterial or nutritional origin, which could be particularly important in colon cancer (Campbell et al. 1995, 2001). The mitogenic activity appears to work through the activation of c-Met and the MAPK pathway, after binding to TF attached to the transmembrane glycoprotein CD44 (Singh et al. 2006). An endogenous lectin that binds the TF antigen is the protein galectin-3. Galectins are galactose-binding proteins expressed in diverse tissues (Cooper 2002; Houzelstein et al. 2004) which can create lattices and links between proteins and cell surfaces (Demetriou et al. 2001; Brewer et al. 2002; Lau et al. 2007; Lau and Dennis 2008). The binding of galectin-3 with TF on MUC1 resulted in enhanced adhesion of cancer cells to endothelial cells, presumably by the clustering of MUC1 to reveal underlying adhesion molecules (Yu et al. 2007). Core-1-derived glycans may also contribute to angiogenesis, as suggested by a study using genetically-modified mice

(Xia et al. 2004), although the mechanisms of that association and the contributions to cancer progression are not known.

The glycosyltransferases involved in the cancer-associated shifts to Core-1 structures include sialyltransferases—enzymes that attached terminal sialic acids to structures—and the enzyme responsible for initiating the branching of Core-2 *O*-glycans. The first branching step is the addition of GlcNAc to the 6-carbon of the core GalNAc. If a sialic acid is added to that carbon instead of a GlcNAc, the extension of a branched structure is halted, since sialic acid is a terminal saccharide. A shift from Core-2 structures to Core-1 structures was reproduced in vitro by increasing the levels of particular sialyltransferases (Dalziel et al. 2001; Sewell et al. 2006), confirming the potential contribution of that mechanism to the cancer-associated structures. Changes in the activity of the enzyme that catalyzes branching at the core GalNAc, called core 2 *N*-acetylglucosaminyltransferase (C2GnT), may also contribute to cancer-associated structures. The structural changes affected by increased C2GnT down-regulation (leading to less branching) can have anti-apoptotic (Cabrera et al. 2006; Valenzuela et al. 2007) and tumor-promoting effects (Huang et al. 2006), although other studies have indicated tumor-forming activities resulting from its up-regulation (Shimodaira et al. 1997; Hagisawa et al. 2005).

13.3.2.2 β1-6GlcNAc Branching of *N*-Glycans

Whereas *O*-glycans demonstrate reduced branching in cancer, *N*-glycans may contribute to cancer progression through increased branching. The branching of *N*-glycans is initiated by the addition of GlcNAc in a β1,6 linkage to a core mannose (Fig. 13.2) by the enzyme MGAT5 (GnT-V) (Saito et al. 1994; Kobata and Amano 2005). *N*-Glycan branching often leads to the further extension of glycans through repeating lactosamine (Galβ1,4GlcNAcβ1,3-) units, resulting in the formation of ligands for a variety of lectins. It is through interactions with these extended units that *N*-glycans may confer cancer-promoting properties (Lagana et al. 2006).

The potential contribution of *N*-glycan branching to cancer progression has been shown through genetically-modified mice. Mammary tumor cells derived from polyomavirus middle T antigen (PyMT) transgenic mice on a MGAT5$^{-/-}$ background are less responsive to multiple growth factors, including insulin-like growth factor, EGF, PDGF, and TGF-β. Tumors that arise in PyMT MGAT5$^{-/-}$ mice also display increased tumor latency, slower tumor growth, and reduced incidence of metastases when compared to their PyMT-transgenic littermates expressing MGAT5 (Granovsky et al. 2000; Partridge et al. 2004). Since the PyMT-induced tumor growth and metastasis were suppressed in MGAT5-deficient mice to a considerable extent, it is suggested that inhibitors of MGAT5 may be useful in the treatment of malignancies by targeting their dependency on focal adhesion signaling for proliferation (Granovsky et al. 2000; Lau and Dennis 2008; Zhao et al. 2008).

Beta-1,6-branched oligosaccharides can be detected with a plant lectin, *Phaseolus vulgaris* leukocytic phytohemagglutinin (LPHA), which binds to that structure. A study examining LPHA binding in tissue samples from breast tumors showed an

association between binding level and the aggressiveness of the tumor (Siddiqui et al. 2005), further supporting a role for that glycan in tumor progression.

13.3.2.3 Blood Group Structures

The blood group systems used to categorize the antigens on the surfaces of red blood cells are based on carbohydrate structures. Some of these structures are also found on other cell types besides red blood cells and can be increased on the surfaces or secretions of cancer cells. The Lewis blood group structures in particular are strongly associated with epithelial cancers such as pancreatic and colon cancers. These antigens are created by the addition of fucose and sometimes sialic acid to Gal-GlcNAc disaccharides, usually as terminal structures on both *N*- and *O*-glycans. Lewis blood group antigens are classified according to the linkage between the Gal and GlcNAc and the positions of the added fucose and sialic acids; the most common in pancreatic cancer are sialyl Lewis A, Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc, and sialyl Lewis X, Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc. Lewis antigens can also be sulfated at the 6- or 3-carbon of the terminal Gal. As Lewis antigens are found on extended polylactosamine chains, their levels can be elevated by increased branching (Maemura and Fukuda 1992; Kikuchi et al. 2005).

Immunohistochemical staining of tumor specimens shows that Lewis structures are frequently overexpressed in carcinomas (Kim et al. 1988, 1996) and pancreatic cancer in particular. In fact, the sialyl Lewis A and sialyl Lewis X structures were first identified as tumor antigens (Fukushima et al. 1984; Hoff et al. 1989). The well-known CA 19-9 antigen that is up-regulated in pancreatic and colon cancers was discovered as a tumor antigen recognized by a monoclonal antibody, which in later studies was characterized as sialyl Lewis A (Magnani et al. 1982). This structure is found mainly on mucins (Magnani et al. 1983). Lewis antigen levels are consistently correlated with tumor progression, metastasis, and poor prognosis of patients (Kannagi 1997; Le Pendu et al. 2001; Takahashi et al. 2001; Ferrone et al. 2006), and increased presentation of this structure on pancreatic cells leads to increased metastases (Iwai et al. 1993; Martensson et al. 1995; Kishimoto et al. 1996; Ohyama et al. 1999, 2008; Kawarada et al. 2000). Because of its strong association with cancer, the detection of CA 19-9 in the blood has been extensively investigated for cancer detection (Goonetilleke and Siriwardena 2007) and is currently used to monitor disease progression.

The contribution of Lewis structures to cancer cell invasion and metastasis is in part due to the fact they are ligands for selectin receptors found on endothelial cells and lymphocytes (McEver 1997). Increased levels of Lewis structures therefore would increase the adhesion of cancer cells to endothelium and facilitate the exit from the vasculature of circulating tumor cells, similar to the migration mechanisms used by normal leukocytes (Kannagi 1997; McEver 1997; Lowe 2002; Zhang et al. 2002; Radhakrishnan et al. 2008). Sulfation of Lewis antigens increases affinity for recognition by selectin receptors, although sulfation seems to be reduced in colon cancer (Izawa et al. 2000).

Adhesion of the HL-60 leukocyte cells to E-selectin-expressing endothelial cells can be inhibited by MUC1 carrying the sialyl Lewis A and X structures (Zhang et al. 1996), which suggests that MUC1 bearing sialyl Lewis A or sialyl Lewis X secreted by tumors can interact with E-selectin on endothelial cells and thus inhibit leukocyte adhesion, or that MUC1-expressing cells might use the same receptor for transendothelial migration. Overexpression of E-selectin in the transgenic mouse liver also redirected the metastatic patterns of syngeneic carcinomas that normally colonize the lung (Biancone et al. 1996). These results indicate that interactions between mucin-type glycans and selectins play a role in promoting the metastatic process in some carcinoma cells.

The contributions to cancer progression of the more widely known ABH blood group antigens are less clear, although some associations with cancer have been made. Many carcinomas, including pancreatic adenocarcinoma, show decreased expression of histo-blood group antigens A and B (Miyake et al. 1992; Orntoft et al. 1996; Dabelsteen and Gao 2005). This loss is associated with a poorer prognosis, while the maintenance of blood group antigen A is associated with a favorable prognosis in patients with non-small-cell lung cancer (Lee et al. 1991). The factors leading to changes in ABH antigen expression are not clear.

13.3.2.4 Other Motifs: Fucose, Sialic Acid, and Mannose

Other glycan structures may be involved in cancer processes, although the contributions are less well characterized. The fucose monosaccharide is one such feature. Elevated fucose levels have been found in cancer patients on circulating proteins such as haptoglobin (Thompson et al. 1991; Okuyama et al. 2005), α_1-antitrypsin (Thompson et al. 1988), and α-fetoprotein (Naitoh et al. 1999; Mita et al. 2000). The fucosylated forms of these molecules have been investigated as tumor markers, with the most promise demonstrated by fucosylated haptoglobin as a marker for pancreatic cancer (Miyoshi and Nakano 2008). The functional contributions of these modifications to cancer are not clear, but the role of fucose in forming terminal blood group structures may be important (Becker and Lowe 2003), since those structures are important for cell recognition, adhesion, and migration properties, as described above.

Cell-surface sialic acids also may be involved in cancer. Sialic acid, being the only charged monosaccharide, confers a negative charge to a glycan chain, which may reduce adhesion and increase migratory ability on negatively-charged glycosaminoglycans in the extracellular matrix (Dall'Olio and Chiricolo 2001). Increased sialic acid on cell surfaces was reported to reduce the attachment of metastatic tumor cells to collagen type IV and fibronectin (Dennis et al. 1982; Chiricolo et al. 2006). Furthermore, the extent of cell-surface sialylation of various murine cancer cell lines positively correlated with their invasive properties (Pearlstein et al. 1980; Yogeeswaran and Salk 1981), and clinical studies showed a relationship between serum sialic acid levels and tumor burden in gastrointestinal and prostate cancers (Wolf et al. 1988; Dall'Olio and Chiricolo 2001; Peracaula et al. 2003).

High-mannose *N*-glycans are not frequently associated with cancer, although high-mannose *N*-glycans were shown to be present on the cancer-associated mucin known as CA125 or MUC16 (Wong et al. 2003). A possible role in cancer was suggested by studies showing increases of high-mannose *N*-glycans associated with transformation on human breast carcinoma (Goetz et al. 2008) and rat hepatoma (Nuck et al. 1993) cell lines.

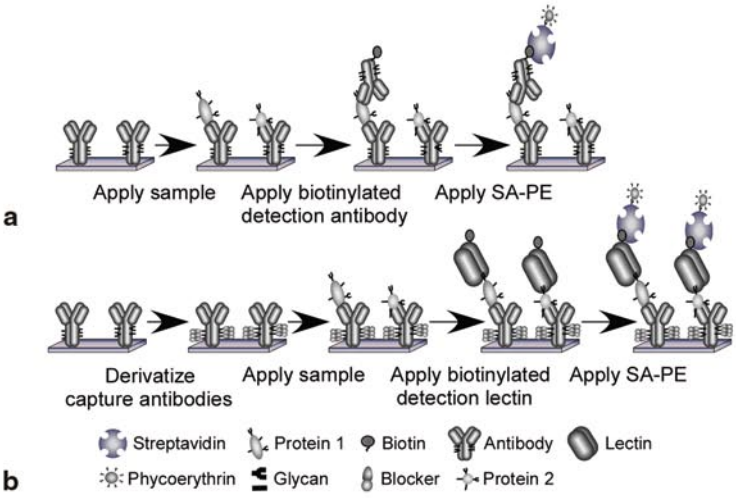
13.4 Antibody-Lectin Sandwich Arrays (ALSA)

The ALSA method provides useful analytical capabilities that are complementary to the existing suite of glycoproteomics tools. The method starts with an antibody microarray—essentially identical to those developed for multiplexed protein analyses (Haab et al. 2001) (Fig. 13.3)—in which the antibodies on the array target various glycoproteins of interest. A complex biological sample is incubated on the array, resulting in the capture of glycoproteins by the antibodies, after which the array is probed with a label lectin. The amount of lectin binding at each capture antibody indicates the amount of a particular glycan on the proteins captured by each antibody. Diverse lectins can be used on a given sample in order to probe a variety of glycans. Glycan-binding antibodies could also be used as detection reagents, such as those raised against the Thomsen–Friedenreich antigens (Kjeldsen et al. 1988) or the Lewis blood group structures (Hanisch et al. 1992). ALSAs are similar to previous approaches that used lectins for the capture or detection of proteins in microtiter plates (Thompson et al. 1989), but the use of microarrays provides high-information content in low-sample volumes. The platform offers a combination of capabilities that are not found with other technologies. Some of those features are described below.

13.4.1 Reproducible and Sensitive Detection Using Affinity Reagents

Previous studies of cancer-associated glycosylation employing enzymatic, chromatographic, and mass spectrometry methods have been very effective for providing detailed information about the structures of glycans, but they are not able to provide precise information about the variation between samples in the levels of particular glycans. Conventional glycobiology methods do not have the reproducibility required for such studies. Affinity-based methods, using reagents such as lectins or glycan-binding antibodies, can provide the reproducible measurements required to determine variation between samples in glycan levels. Therefore, enzymatic and MS methods provide higher structural detail, but affinity-based methods provide higher precision measurements.

Lectins and glycan-binding antibodies have been used extensively to study carbohydrates in immunohistochemistry (Satomura et al. 1991; Osako et al. 1993) and


a
Apply sample
Apply biotinylated detection antibody
Apply SA-PE
b
Derivatize capture antibodies
Apply sample
Apply biotinylated detection lectin
Apply SA-PE
Streptavidin
Protein 1
Biotin
Antibody
Lectin
Phycoerythrin
Glycan
Blocker
Protein 2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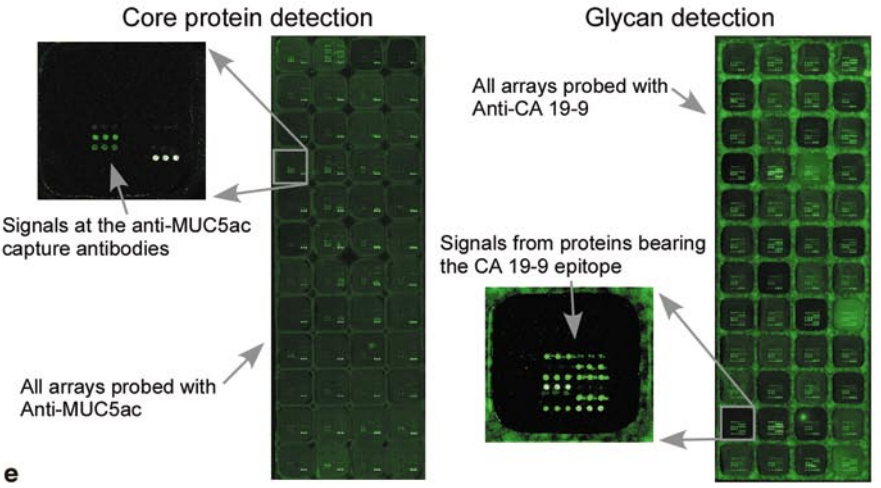
Core protein detection
Glycan detection
All arrays probed with Anti-CA 19-9
Signals at the anti-MUC5ac capture antibodies
Signals from proteins bearing the CA 19-9 epitope
All arrays probed with Anti-MUC5ac
e

in immunoaffinity electrophoresis and blotting methods, such as those to identify cancer-associated glycan variants on the serum proteins α-fetoprotein (Shimizu et al. 1996), haptoglobin (Thompson et al. 1991; Okuyama et al. 2005), α_1-acid glycoprotein (van Dijk et al. 1995), and α_1-antitrypsin (Thompson et al. 1988). As a means of quantifying glycans on specific proteins, lectins have been used in the capture or detection of proteins in microtiter plates (Thompson et al. 1989), which is similar to ALSA, but in a higher-volume and non-multiplexed format.

13.4.2 Multiplexing Through the Use of Microarrays

The ALSA method combines the advantages of affinity-based detection—reproducibility, speed, practicality—with the advantages of microarrays. The usefulness of the microarray platform lies in its multiplexing capability—enabling the acquisition of many data points in parallel—and its miniaturization—resulting in very small consumption of reagents and samples. Microarray methods analyzing RNA and DNA transformed gene expression and genetic research following their introduction in the early 1990s (Schena et al. 1995), and microarrays for studying other molecule types, including proteins, antibodies, lipids, and glycans, developed more slowly due to increased technical difficulty. In addition to ALSA, microarray formats for glycobiology include lectin microarrays (Kuno et al. 2005; Pilobello et al. 2005; Zheng et al. 2005), which are useful for measuring glycan levels on individual, purified proteins; glycan microarrays (Blixt et al. 2004; Liang et al. 2008), which are used to measure the recognition of carbohydrate structures by various glycan-binding reagents; and glycoprotein arrays (Zhao et al. 2007), for examining glycosylation on proteins isolated from biological samples. The multiplexing capability is valuable any time one wants to examine multiple molecules, for example to profile glycan variation on a family of related glycoproteins.

Fig. 13.3 ALSA and high-throughput samples processing. **a** Array-based sandwich assays for protein detection. Multiple antibodies are immobilized on a planar support, and the captured proteins are probed using biotinylated detection antibodies, followed by fluorescence detection using phycoerythrin-labeled streptavidin. **b** Glycan detection on antibody arrays. This format is similar to above, but the detection reagents target the glycans on the capture proteins rather than the core proteins. The glycans on the immobilized antibodies are chemically derivatized to prevent lectin binding to those glycans. **c** Imprinting hydrophobic boundaries for practical, high-throughput processing of antibody microarrays. Wax is melted by the hotplate under the bath, and a slide is inserted upside-down into the holder. Bringing the lever forward raises a stamp out of the wax bath to touch the slide, imprinting the design onto the slide to form borders around multiple arrays. Two stamps are shown in front of the machine. **d** Loading samples onto a slide containing 48 arrays. The arrays are spaced at 4.5 mm, which is compatible with the 9 mm spacing of standard multichannel pipettes. **e** Example antibody array results for core protein detection (*left*) and glycan measurement (*right*). SA-PE, streptavidin-phycoerythrin

13.4.3 Convenient Detection of Both Core Protein and Glycan Levels

In order to properly interpret the amount of glycan on a protein, one must also know the underlying protein concentration. For example, if a change in glycan level is observed, it is valuable to know whether the underlying core protein concentration changed, or the amount of glycosylation per protein. Both the protein and glycosylation levels may be conveniently obtained using antibody microarrays (Fig. 13.3a and b). A sample may be incubated multiple times on replicate microarrays, each time probed with a different lectin, to characterize glycan levels (Fig. 13.3b), or with antibodies, to characterize core protein levels (Fig. 13.3a). A previous study (Chen et al. 2007) showed the value of using both formats to detect glycosylation differences between samples, in which it was observed that core protein levels did not change between samples, but the amount of glycosylation did.

13.4.4 Low-Volume, High-Throughput Sample Processing

Several studies have examined glycosylation changes in biological samples using conventional glycobiology methods, but due to the requirements for large amounts of material and the time involved to analyze each sample, these studies generally used either cell culture material or a small number of patient samples. Therefore, while many cancer-associated glycans have been identified, it is not known how often they appear, how closely they are associated with particular disease states, or the distribution of protein carriers on which they appear. The ability to conveniently process multiple samples, combined with low consumption of precious samples, would enable such studies.

A practical method for the high-throughput processing of low-volume microarrays was demonstrated earlier (Forrester et al. 2007). Multiple, replicate microarrays are printed onto a microscope slide, and the arrays are segregated from one another by hydrophobic wax borders that are precisely imprinted onto the slide. The borders are imprinted using a device (The Gel Company, San Francisco, CA) that elevates a stamp out of a wax bath, which sits atop a hotplate to melt the wax, to contact a microscope slide suspended above the wax bath (Fig. 13.3c). The wax borders prevent liquid from spilling from one array to another, and they remain on the slide throughout the processing steps and the fluorescence scanning (Fig. 13.3d). Any size or pattern of arrays could be accommodated by using the appropriate stamp. A high-throughput strategy using this format is to incubate sets of samples in a randomized order on a microscope slide and then probe the captured proteins on the slide with a lectin (Fig. 13.3e). Multiple, different detection reagents can be used as glycan-binding probes, so that a variety of glycan structures can be probed on each protein in each sample. Such a strategy has been used for high-throughput antibody array processing in multiple studies (Gao et al. 2005; Orchekowski et al. 2005; Chen et al. 2007). The ability to capture much information by processing many samples, with many measurements per sample, should be useful in many glycobiology applications.

13.5 ALSA in Pancreatic Cancer Research

13.5.1 Types of Experiments Using ALSA

Above we described some of the features of the ALSA platform that make it a valuable complement to the existing glycobiology tools. Here we describe some of the ways in which the platform could be applied to pancreatic cancer research, and we follow with a section on specific examples of applications.

13.5.1.1 Characterizing Glycosylation Variation in Populations

In most studies examining disease-associated glycosylation, constraints in the technologies limited the examinations of glycan alterations to just a few samples. Therefore, the population variation of particular alterations, or the prevalence with which they appear, is not known. Two aspects of ALSA make it possible to obtain pictures of population variation of glycans: high-precision measurements and the ability to run samples in high-throughput. These features allow one to run many samples and to make precise determinations about the relative levels of protein and glycosylation levels. Such a capability could be used to determine whether a particular glycan alteration could be used as a biomarker, whether it associated with certain disease states, or whether it is correlated with any other conditions or clinical factors.

13.5.1.2 Identifying Glycan Changes on Specific Proteins in Model Systems

Glycans are dynamically regulated as a function of a cell's activation or differentiation state, although in most cases, the details of those changes are not known. Model systems have been developed in which cell differentiation or activation states may be precisely controlled, for example in the activation of T cells by IL-2 or the conversion of monocytes to macrophages by phorbol 12-myristate 13-acetate (PMA). These model systems provide a good opportunity to study dynamic glycan regulation using ALSA, which could be used to conveniently look at both the abundance and glycosylation of multiple proteins at multiple time points in particular conversion processes. Samples may be collected from media, to examine changes to the secretory compartment, and compared to changes in the membrane and cytosolic fractions.

Such systems may also provide ideal settings for studying the factors that control glycosylation. By monitoring glycan changes using ALSA, one may perturb the culture system, perhaps through RNAi strategies or chemical strategies, to examine the resulting effects on glycosylation. Since samples can be run in high-throughput, many conditions could be explored in parallel. This type of study may have implications for disease biology or drug discovery since the glycosylation states of certain cytokine receptors, which may control critical functions of cells, greatly affect the ability of those receptors to receive and transmit molecular signals.

13.5.1.3 Characterizing Protein Carriers of Specific Glycans

In some cases it is known that a particular glycan is associated with cancer, but the range of proteins on which the glycan is found is not known. This question may be particularly important in cancer if the protein carriers of a particular glycan are different in disease compared to healthy states, indicating a disease-specific shift in function of those proteins. The antibody microarray provides a useful tool for characterizing the proteins on which a glycan is found. Arrays of antibodies targeting multiple, candidate proteins could be incubated with patient samples, and the level of the glycan on each of the proteins could be determined by probing the captured proteins with a lectin or antibody targeting that glycan.

13.5.2 Example Applications in Pancreatic Cancer Research

13.5.2.1 Serum Biomarkers for Pancreatic Cancer

Most pancreatic cancers—about 85%—are inoperable at the time of diagnosis because the disease has progressed too far without detection. The late-stage detection combined with few treatment options leads to five-year survival rates of less than 5% (Yeo et al. 1995). Earlier detection of pancreatic cancer would very likely lead to higher survival rates. A benefit of improved diagnostics would be to increase the percentage of patients who might benefit from surgical intervention. For patients who are operable (about 15% of patients), survival rates can be improved to 20–30% (Yeo et al. 1995). In addition, cancer cells in higher states of differentiation, more closely resembling the cell type of origin, may also respond better to the current chemotherapeutics used for pancreatic cancer. For these reasons earlier detection of pancreatic cancer is an important research goal.

Serum or plasma markers would be particularly useful for the early detection of pancreatic cancer. Blood tests are simple and minimally invasive, can be performed regularly and routinely, and are inexpensive (Anderson and Anderson 2002). As noted above, the measurement of glycan levels on specific proteins may yield improved performance over measuring just the protein levels, as both proteins and glycans can be altered in disease. ALSA provides an ideal way to screen for markers that have both protein abundance and glycosylation alterations. The quantitative nature of the method enables precise views of the changes between samples; the multiplexing allows many candidate markers to be screened; the low-volume assays means that minimal amounts of precious sample will be consumed; and the ability to run the assays in a high-throughput mode enables studies on large populations, as required for biomarker studies. These capabilities will be employed in various areas in which serum biomarkers could be useful, including early detection, discriminating benign from malignant disease, or in drug trials as markers of tumor burden.

13.5.2.2 Cyst Fluid Biomarkers

An increasingly important diagnostic challenge facing clinicians is how to handle pancreatic cysts (Singh and Maitra 2007). With the improving resolution and broader use of abdominal scans in clinical practice, pancreatic cysts—fluid-filled sacks with epithelial lining—are being found with greater regularity. Some cysts are known cancer precursors, and as such should be surgically removed to prevent the development of cancer. However, other cysts are completely benign without any danger of advancing to cancer, and those should not be removed, in order to avoid unnecessary surgery. While evaluations of the images of the cysts and the clinical data often correctly predicts the status of the cyst, much diagnostic improvement is needed in order to effectively impact patient care.

The fluid inside the cysts may be accessed by biopsy, using endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), which provides an ideal sample source in which to search for biomarkers of cancer precursors. Since the cyst fluid is confined and surrounded by the epithelia, molecules secreted by cancer precursor cells will be much less diluted than in blood, and much easier to detect. Otherwise, the same advantages cited above in using ALSA for serum biomarker studies also apply to cyst fluid studies. The benefit of low-volume sample consumption is more important in this application, because sample collection can be difficult and minimal.

13.5.2.3 Glycosylation in Cancer Cell Subpopulations

The cancer cells within tumors are known to be in varying states of differentiation. For example, some cancer cells within a tumor may have higher proliferation rates than others, or some cells may become highly motile while others remain stationary. This variability may result from divergent genetic alterations and divergent signals impinging from the environment. A cancer cell's phenotype or differentiation state is often described by morphology or the presence of various cell-surface markers. The glycan structures produced by the cell may also be an accurate indicator of the state of the cell. In immunobiology and developmental biology, extensive remodeling of cell-surface glycans has been observed in response to changes in differentiation or activation. Therefore, in the development of the transformed and invasive states of a cancer cell, it is reasonable to presume that similar glycan remodeling takes place.

The use of ALSA to investigate changes to protein glycosylation could reveal much information about the involvement of glycan alterations as cancer cells progress. Considering the importance of glycans in a wide variety of functions, the glycan alterations associated with cancer progression are likely to be functionally important to the tumor. Characterizing the alterations will enable the development of strategies to interfere with the functions. Accurate models systems will be important for such studies. Cell culture systems can reproduce the transition from stationary to migratory cells, and mouse models have accurately recapitulated the entire sequence of the development of invasive cancer from cancer precursors. ALSA applied to the study of samples from those systems could be highly valuable.

13.5.2.4 Defining Targets for Immunotherapy

A promising strategy for targeting cancers is to stimulate immune responses against cancer-specific antigens. Since certain glycans can be abnormally present or elevated on particular proteins, the use of tumor-associated glycans for immunotheraphy has generated much interest. Dozens of new carbohydrate-related compounds are under development, aimed at treating conditions ranging from inflammation, tissue rejection, hepatitis to cancer (Alper 2001). Glycans can be conjugated to keyhole limpet hemocyanin (KLH), a strongly immunogenic protein isolated from a marine mollusc, prior to delivery with another immune booster for augmenting the immunogenicity (Gilewski et al. 2001, 2007; Ragupathi et al. 2005). Some of the carbohydrates tested include well-known cancer-associated structures, such as sialyl-Tn and some glycolipids, individually or together in polyvalent cancer vaccines (Ragupathi et al. 2005). Advances in the enzymatic production of carbohydrates are available to facilitate the production of such compounds.

While immunotherapy trials are testing the most commonly observed carbohydrate antigens, the real prevalence with which certain antigens appear is not known. ALSA would be useful to complement such work by providing information on which antigens were most prevalent and how they correlated with clinical parameters that might be likely to influence the success or choice of the treatment. Having a convenient means to characterize the glycans on particular molecules may open the possibility of tailored immunotherapy, in which patients could be first tested to determine their glycan profiles, and then assigned to vaccine regimens based on those profiles. Such a strategy might be a highly effective use of diagnostics to guide therapy.

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Chapter 14
The Development of Pharmacodynamic Endpoint Models for Evaluation of Therapeutics in Pancreatic Cancer

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Abstract The lack of development of new, more effective, therapies for pancreatic cancer has been disappointing. Among the factors limiting the development of targeted therapy approaches has been the inherent molecular heterogeneity of this disease combined with the challenge of obtaining fresh tumor tissue for the identification of pharmacodynamic biomarkers to segment patients based on target expression and resistance factors. Although many pre-clinical studies include pharmacodynamic studies, few large clinical studies have included similar biological correlative endpoints. Gemcitabine, although only modestly effective, still remains the standard of care. Emerging studies have identified potential molecular and genetic markers for cellular transporters and metabolism of gemcitabine which may be useful in predicting both clinical benefit and toxicity from this agent. The continued development of new therapeutics combined with identification of pharmacodynamic biomarkers to predict and monitor response to anti-cancer agents represents an exciting opportunity to individualize therapy and improve outcomes in this challenging disease.

14.1 Introduction

Drug development is increasingly utilizing pre-clinical information about the pharmacology of new agents to help design clinical trials and select patient populations who may derive the greatest benefit from treatment. The selection of the subset of patients within a given tumor type who will benefit from each treatment approach remains challenging. Both pre-clinical and clinical pharmacodynamic studies have the potential to help identify both positive and negative predictive determinants, and biomarkers for response. The term biomarker has been defined by the National

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Institutes of Health Director's Initiate on Biomarkers and Surrogate Endpoints as: a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention. In this chapter, we use the term pharmacodynamic (PD) to refer to the study of the biochemical and physiological effects of drugs on the body, the mechanisms of drug action, and the relationship between drug concentration and effect. In essence, a PD biomarker confirms that the drug has reached its intended target. Alternatively, functional response biomarkers can be used to measure if a drug has affected a target pathway. Both PD and functional biomarkers can be useful in identifying a biologically active dose of drug, as opposed to the maximally tolerated dose. The combination of assessment of target expression prior to treatment, termed pharmacogenomic (PG) biomarkers, modulation of target in response to therapy (PD biomarkers), and clinical benefit related to target modulation will help validate new drug targets. Due to the heterogeneous genetic profiles associated with pancreatic cancer, the development of both PG and PD biomarkers for pancreatic cancer is extremely important (Kang and Saif 2008).

14.2 Assay Validation

Another challenge in developing PD endpoints for clinical testing is assay validation. In order to evaluate an assay's predictive accuracy the precision, sensitivity, and specificity of an assay should be established. Unfortunately, it is both expensive and time-consuming to standardize laboratory methodologies and institute the quality control measures necessary to validate an assay. Ultimately, the level of assay validation must be considered within the context in which they will be utilized.

14.3 In Vitro Models

The correlation between drug target modulation and anti-tumor activity is typically established first in pre-clinical in vitro studies. Many human pancreatic cancer cell lines are commercially available and have been described in Chap. 1. Pre-clinical models can be useful for initial mechanism of action studies and to identify the molecular context necessary for optimal biological response. Genetic abnormalities including gene mutations (K-Ras, p53, breast cancer type 2 susceptibility protein (BRCA2)), gene amplification (c-MYC, epidermal growth factor receptor (EGFR)), and gene deletions (Smad4/Dpc4 and p16) are common in pancreatic cancers (and are reviewed recently by Friess et al. 1999; Koliopanos et al. 2008), reflecting the necessity to use a variety of cell line models when testing new agents. Assessing the efficacy of new agents is limited in two-dimensional culture conditions because the dynamic interactions between the tumor and the microenvironment cannot be

simulated in this setting. Co-cultures with other cell types and three-dimensional culture systems offer alternative approaches. For angiogenesis-targeting compounds, modulation of endothelial cell growth viability, growth factor and cytokine secretion are commonly measured when new agents are being evaluated. In addition, in vitro cultures can be grown in hypoxic conditions and low pH to simulate features of the tumor microenvironment. Ultimately, PD activity is best evaluated using in vivo models where pharmacokinetics (PK) parameters can also be assessed.

14.4 Xenograft Models

The most common in vivo models include human pancreatic cell lines grown as flank (subcutaneous) or orthotopic xenografts which are initiated through the injection of tumor cells from culture, or through transplantation of a small tumor mass. Tsuji et al. (2006) described a novel method for establishing orthotopic ductal pancreatic tumors via injection of cells into the common bile duct. Although subcutaneous pancreatic xenograft tumors are easy to measure, they seldom metastasize in vivo. In contrast, orthotopic pancreatic xenografts often metastasize, but are difficult to serially measure. Utilization of luminescent, green fluorescent protein, and/or red fluorescent protein expressing pancreatic cell lines allow for serial monitoring of the formation and growth of metastasis (Bouvet et al. 2002; Amoh et al. 2006; Medicherla et al. 2007; Harada et al. 2007). The capability of monitoring in real-time both macro-and micro-metastasis is ideal for investigating the correlation of anti-tumor effect with drug target modulation. In parallel with anti-tumor activity assessment, target modulation, and PK should be monitored. Ideally, the efficacy of targeted agents should correlate with target modulation within the tumor and be dose-dependent. One general, obvious flaw when using high-passage cell lines is that they may actually bear very little resemblance to human tumors. It is not surprising that the activity of cytotoxic agents tested in these xenografted models does not predict their clinical efficacy in many instances (Danhof et al. 2005).

14.5 Hetero-Transplanted Human Xenografts

A new and intuitive way of overcoming the deficiency of high-passage xenograft cell line models is to establish low-passage xenografts directly from patients with pancreatic cancer (Rubio-Viqueira et al. 2006). Presumably, the molecular characteristics of these low-passage tumors passaged in mice more accurately reflect the heterogeneity normally present in human tumor tissue that is lost with serial passaging of cancer cell lines in culture. This is a new and promising platform for testing both efficacy and PD endpoints for therapeutics for pancreatic cancer. Another potential advantage of this concept is that it creates an opportunity to validate “in

vivo" findings by testing the activity of a specific agent in an individual patient (Rubio-Viqueira and Hidalgo 2008).

14.6 Genetically Engineered Mouse Models

Genetically engineered mouse (GEM) models also offer a promising alternative for the pre-clinical testing of novel cancer therapeutics and some of the most commonly utilized pancreatic models are reviewed by Olive and Tuveson (2006); Liao et al. (2007) and further described in Chap. 4. The increasing sophistication of pancreatic GEM models is impressive and will hopefully accelerate pre-clinical drug screening and serve as models for identification and development of PD biomarkers.

14.7 Clinical Assessment of PD

Although pre-clinical modeling can provide some predictive information, as mentioned earlier, neither cell-based nor xenograft models have been very successful in predicting pancreatic cancer drug-sensitivity in humans. The outcome for both patients with locally advanced and metastatic pancreatic cancer is dismal with median survivals of only nine and six months, respectively (with therapy). Despite the obvious need to develop and test new agents, only two new anticancer agents have been approved for pancreatic cancer in the past 15 years, gemcitabine and erlotinib (Tarceva™). This underscores the limited predictive value of currently used pre-clinical models. For example, many cell line models and xenograft tumors are sensitive to the anti-tumor activity of gemcitabine, but clinically it has limited activity with only a small percentage of patients achieving objective response (ranging from 5 to 10%). Similarly, Phase II studies in humans have had limited success in predicting drug responses in Phase III studies, perhaps reflecting the heterogeneous nature of the disease. Few Phase I or Phase II studies in pancreatic cancer have included PD studies. In contrast to this, many elegant pre-clinical PD studies in pancreatic cancer have been reported, but translating these studies into the clinical setting remains challenging and expensive. This lack of inclusion of selective PG biomarkers or PD biomarkers into early-phase clinical trials or incorporation of these biomarkers into the larger Phase III studies to segment patients may be one reason why so many Phase III trials in pancreatic cancer patients have shown such limited activity. Perhaps, the reason for this discrepancy lies in the fact that pancreatic cancers harbor multiple genetic alterations that affect some of the key cancer cell pathways involved in metabolic adaptation, neo-angiogenesis, cell cycle regulation, cytoskeletal and surface interactions, and DNA repair mechanisms as reported by Jones et al. (2008). The authors of this study suggest that because of this redundancy and also because of inherent heterogeneity of pancreatic adenocarcinomas the best hope for advancing the treatment may be achieved with agents that are less selective and target downstream

mediators involved in key pathways. A challenge to personalizing therapy based on the tumor molecular profile is the difficulty of obtaining tumor biopsies in patients with pancreatic cancer. Pancreatic tumors are not very well visualized using standard imaging methods, which presents a challenge for obtaining image-guided diagnostic biopsies. Even then, the cancer cells are usually very scarce, as they are usually embedded in fibrotic, stromal tissue. In addition, only about 20% of all pancreatic cancer patients are candidates for radical resection (pancreatectomy). Thus, gaining serial biopsies for PD drug target modulation studies and identification of optimal "biological" dose is even more problematic.

14.8 Imaging Biomarkers

Molecular and functional imaging approaches are also being evaluated as PD biomarkers in many tumor types (Stephen and Gillies 2007). Examples include diffusion contrast-enhanced magnetic resonance imaging (DCE-MRI), diffusion-weighted MRI, and fluoro-18-deoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) (Fleming and Brekken 2003; Delbeke and Pinson 2004). O'Connor et al. (2007) have reviewed the utilization of DCE-MRI as a biomarker for several anti-angiogenic agents being investigated in cancer therapy, which has demonstrated mixed success. To date, DCE-MRI has not been investigated as a biomarker in any large trials containing anti-angiogenic agents in pancreatic cancer. Although FDG-PET has shown great promise as a biomarker in other malignancies, FDG-PET sensitivity/specificity for visualizing and monitoring pancreatic cancer in humans is not as good as for some other malignancies (Lemke et al. 2004). This specifically applies for the imaging of lymph node and distant metastases from pancreatic cancer. Thus, FDG-PET cannot be considered a universal molecular imaging modality for all pancreatic cancers.

14.9 PD Models for Gemcitabine

Gemcitabine (Gemzar™) was approved by the FDA in 1998 for patients with locally advanced, or metastatic (stage IV) adenocarcinoma of the pancreas based on a reported improvement in quality of life. Gemcitabine is a deoxycytidine analog that requires cellular uptake and intracellular phosphorylation to its di- and triphosphate forms for activity (Fukunaga et al. 2004). The initial phosphorylation step requires deoxycytidine kinase, which is the rate-limiting enzyme in gemcitabine activation. The diphosphate form is a potent inhibitor of ribonucleotide reductase, resulting in depletion of cellular dNTP pools. The triphosphate-nucleoside leads to inhibition of DNA synthesis. Several biomarkers have been explored in relationship to gemcitabine activity, resistance, and toxicity.

14.9.1 Equilibrative Nucleoside Transporter 1

An in vitro study using human pancreatic cancer cell lines identified equilibrative nucleoside transporter 1 (ENT1) as one of the primary nucleoside transporters responsible for gemcitabine uptake (Garcia-Manteiga et al. 2003). In a study performed on 21 formalin-fixed, paraffin-embedded pancreatic tumor sections, immunohistochemical analysis for ENT1 demonstrated two patterns of staining, including a uniformly positive staining of adenocarcinoma cells or heterogeneous regions lacking hENT1 in adenocarcinoma cells ranging from 10 to 100%. Patients with detectable ENT1 expression had significantly longer survival (median 13 months) than those patients with heterogeneous areas lacking ENT1 expression (median 4.0 months) (Spratlin et al. 2004). Interestingly, ENT1 expression has been shown to be down-regulated in response to hypoxia via hypoxia-induced transcription factor 1 alpha (HIF-1α) (Eltzschig et al. 2005), which is often elevated in pancreatic adenocarcinomas and is associated with poor prognosis (Sun et al. 2007; Miyake et al. 2008). In a larger study of 102 patients with pancreatic cancer, the RNA expression of hENT1 was measured and correlated with clinical outcome (Giovannetti et al. 2006). High expression of ENT1 was significantly associated with longer overall survival (median 25.7 months) compared to patients with medium (median 15.74 months) or low expression (median 8.48 months) of ENT1. Multivariate analysis in this same study found ENT1 gene expression to be an independent prognostic marker. Although these results are promising, the utility of hENT1 as a predictive biomarker for gemcitabine sensitivity will require further investigation. Clinical trials are now being conducted with gemcitabine in combination with experimental therapies in pancreatic cancer patients in which ENT1 expression is being measured and correlated with response (Gemcitabine and S-1 for Locally Advanced Unresectable or Metastatic Pancreatic Cancer, NCT00429858).

14.9.2 Cytidine Deaminase Activity and Deoxycytidylate Deaminase

Metabolic de-activation of gemcitabine occurs by cytidine deaminase activity (CDA), or, after phosphorylation, by deoxycytidylate deaminase (DCTD). Variation in therapeutic response and toxicity to gemictabine has been hypothesized to correlate with the expression in these enzymes, with decreased expression or activity being related to increased toxicity or with increased enzyme activity resulting in under-treatment. In a small study evaluating dose rate infusion of gemcitabine with autologous hemopoietic support in advanced pancreatic adenocarcinoma, 16 patient samples were evaluable for CDA activity. Low (<10 U) CDA showed improved clinical benefit compared to patients with high (>10 U) CDA activity (Bengala et al. 2005). Polymorphisms have been reported for CDA which may contribute to variation in therapeutic response and toxicity to gemcitabine (Fitzgerald et al.

2006; Sugiyama et al. 2007; Giovannetti et al. 2008). The Ala208thr polymorphic variant occurs with a 3.7% frequency in the Japanese population and is associated with decreased gemcitabine clearance values (Sugiyama et al. 2007). Recently, Giovannetti et al. (2008) reported that the Lys27Lys polymorphism results in significantly lower CDA enzymatic activity than in patients with CDA Lys27Gln or Gln27Gln. A recent case report described a patient who received standard dose gemcitabine/carboplatin and died from drug toxicity related to down-regulation of CDA due to a 79 A > C (Lys27Gln) single-point mutation (Mercier et al. 2007).

14.9.3 Ribonucleotide Reductase

In pancreatic cell lines in vitro, the reduced expression of regulatory subunits of ribonucleotide reductase 1 and 2 (RRM1) and (RRM2) has been shown to correlate with increased gemcitabine sensitivity (Ohhashi et al. 2008), and increased expression of RRM1 and RRM2 correlates with acquired resistance to gemcitabine (Nakano et al. 2007). In a small study of 18 patients with pancreatic cancer, a significant correlation was observed between gemcitabine response and RRM1 expression, with patients with high RRM1 expression demonstrating poorer survival than patients with low levels of RRM1 (Nakahira et al. 2007). However, in a larger study by Giovannetti et al. (2006) consisting of 102 patients treated with gemcitabine, high RRM1 expression was not correlated with longer overall survival; however, it was significantly associated with longer time to progression (5.85 months vs 12.30 months) when compared to patients with low RRM1 message expression. In this same study, no statistical differences were observed for time to progression or overall survival for RRM2. In vitro studies have correlated the RRM1 2464G > A polymorphism with gemcitabine sensitivity (Kwon et al. 2006) but no large clinical studies have yet been reported linking this polymorphism with gemcitabine response in pancreatic cancer.

14.9.4 Survivin: An Anti-Apoptotic Gemcitabine Resistance Marker

Alterations in apoptotic signaling pathways have also been reported to mediate resistance to gemcitabine (reviewed in El et al. 2008). The expression of inhibitors of apoptosis (IAP) family members has been reported to be upregulated in pancreatic cancer, including survivin. Survivin blocks apoptosis induced by various stimuli including radiation and chemotherapeutic drugs through binding to caspase-3 and caspase-7 preventing their activation, or by direct interaction with Smac/DIABLO (reviewed in Yamamoto et al. 2008). Survivin expression has been reported in pancreatic cancer in several studies, with expression ranging from 77 to 94% of tumors positive by immunohistochemical analysis (Satoh et al. 2001; Sarela et al. 2002;

Lee et al. 2005; Sun et al. 2007). In addition to these studies, Bhanot et al. (2006) demonstrated increasing expression of survivin RNA expression levels from low-grade lesions to high-grade lesions and further to pancreatic adenocarcinoma. In a study in breast cancer, survivin expression was reported to be regulated by EGFR activation of the PI3Kinase pathway resulting in activation of HIF-1α, which binds to the hypoxia response element in the promoter of survivin resulting in increased gene expression (Peng et al. 2006). A correlation between HIF-1α message and survivin expression has been observed in pancreatic cancer (Wei et al. 2006). Interestingly, vaccination with survivin peptides has been reported to have resulted in a complete remission of a pancreatic tumor hepatic metastasis (Wobser et al. 2006). Knockdown of survivin in vitro pancreatic cancer cell line models significantly sensitizes cells to gemcitabine (Liu et al. 2008). Therefore, survivin represents a promising new therapeutic target for pancreatic cancer both alone and in combination with gemcitabine.

14.9.5 CA 19-9 as a Prognostic Factor for Response to Gemcitabine

Change in the serum levels of circulating tumor marker carbohydrate antigen CA 19-9 is perhaps the most widely used marker utilized in pancreatic cancer patients. Some early trials suggested that there is a strong correlation of CA 19-9 with radiologically-assessed tumor area, and time to progression (Rocha Lima et al. 2002). A review by Boeck et al. (2006) suggested that a 20% reduction of CA 19-9 baseline levels within the first eight weeks of chemotherapy appeared to be sufficient to define a prognostic relevant subgroup of patients as "CA 19-9 responders." However, subsequent studies to confirm the value of CA 19-9 as a surrogate endpoint for tumor response and survival have reported conflicting results. Hess et al. (2008) reported on 175 patients with pancreatic cancer who had baseline and post-chemotherapy values (gemcitabine or gemcitabine combined with capecitabine) response to treatment CA 19-9 levels. Although baseline CA 19-9 levels predicted for overall survival, a 50% decline in CA 19-9 after two cycles of chemotherapy did not correlate with overall survival. A study by Wong et al. (2008) examined the change in CA 19-9 in 73 patients treated with gemcitabine in relation to objective response rate and found a decline in CA 19-9 to be a strong predictor for time to progression and overall response rate. An additional study in 72 patients with advanced pancreatic cancer by Nakai et al. (2008) found that a CA 19-9 change (decrease) following the first cycle of gemcitabine treatment was significantly correlated with overall survival and time to progression. Berger et al. (2008) investigated CA 19-9 as a predictive marker in pancreatic cancer patients post-resection treated with chemoradiation in a prospective, Phase III trial consisting of 385 accessible patients. In this study, patients with lower CA 19-9 levels (<180, $p = 0.001$) showed a significantly longer overall survival rate. Overall, there is sufficient evidence to incorporate CA 19-9 measurements as one general biomarker for predicting early response to

gemcitabine therapy, but the results should be cautiously interpreted and evaluated in combination with traditional imaging response criteria.

14.10 PD Models for Erlotinib

Erlotinib (Tarceva™) was approved by the FDA in 2005 for first-line treatment of patients with locally advanced or metastatic pancreatic cancer in combination with gemcitabine based on a modest survival advantage. Erlotinib is an orally available quinazoline-based small molecule TKI with highly selective activity against the EGFR (in-vitro IC_{50}: 0.02 μmol/L; intact cells IC_{50}: 0.2 μmol/L) (Moyer et al. 1997; Arteaga 2001). EGFR immunohistochemical expression does not correlate with sensitivity to erlotinib. In a study using direct pancreatic xenografts (Jimeno et al. 2008), global EGFR pathway activation appeared to be the best predictor for benefit compared to any one genetic alteration. As in other tumor types, skin rash may be a biomarker for clinical benefit. In the NCIC Phase III study, a sub-group analysis suggested an association with skin rash and survival; median survival for patients with grade 0 skin rash ($n = 79$) was 5.29 months, with grade 1 skin rash ($n = 108$) was 5.75 months and with grade 3 skin rash ($n = 103$) was 10.51 months (Moore et al. 2007). K-Ras mutations have been shown to predict for lack of response to antibodies directed toward EGFR in other diseases, but K-Ras mutations have not been identified as a biomarker for lack of benefit from erlotinib in pancreatic cancer.

14.11 PD Models for Investigational Angiogenesis-Targeting Agents in Pancreatic Cancer

14.11.1 Introduction to Angiogenesis in Cancer

Angiogenesis is the formation of new blood vessels from the proliferation and migration of pre-existing, completely differentiated endothelial cells. This process is one of the primary mechanisms by which tumor cells create their own oxygen and nutrient supply, resulting in increased growth and facilitation of metastasis (Folkman 1971). There are many regulators of angiogenesis. One of the best characterized and broadly studied is vascular endothelial growth factor (VEGF). Similar to other cancers, angiogenesis represents an important mechanism driving tumor growth and metastasis in pancreatic cancer (Ikeda et al. 1999). Tumor and circulating VEGF-A, circulating VEGF-C, circulating VEGF-A:sVEGFR-1 ratio, tumor HIF-1α, and platelet-derived growth factor (PDGF) have all been reported to be prognostic indicators for poor survival in pancreatic cancer patients (Fujimoto et al. 1998; Couvelard et al. 2005; Fjallskog et al. 2007).

14.11.2 Angiogenesis as a Therapeutic Target

Angiogenesis is a validated drug target for malignancies such as renal carcinoma (Motzer and Basch 2007), metastatic colon cancer (Marshall 2005), non-small cell lung carcinoma (Sandler 2007) and breast cancer (Sirohi and Smith 2008). Bevacizumab (Avastin®), an antibody directed against VEGF-A, and sunitinib (Sutent®) and sorafanib (Nexavar®), small-molecule VEGF-R inhibitors, are currently FDA-approved anti-angiogenic agents (Kesisis et al. 2007). While initial studies provided some glimpse of hope, the relevance of angiogenesis as a target for pancreatic cancer has been challenged with the recent reports from two large Phase III trials combining gemcitabine with bevacizumab (Kindler et al. 2005; Ko et al. 2008; Saif 2008; Kindler et al. 2007; Vervenne et al. 2008). Multiple hypotheses can be generated in order to explain this apparent lack of clinical activity of bevacizumab such as lack of vascularization in pancreatic tumors, redundant angiogenesis pathways, and resistance to hypoxia; however, it is possible that only a smaller subset of patients/tumors will respond to bevacizumab. Obviously, when a relatively small subset of potential responders is "diluted" with a large number of resistant patients the clinical outcome is likely to be non-significant, as we have seen with some studies utilizing EGFR inhibitors in patients with lung cancer. It is clear that we need better markers for angiogenesis pathway activation for pancreatic tumors in order to identify tumors that may be more sensitive to this class of anti-cancer agents.

14.11.3 Biomarkers of Angiogenesis in Cancer

There are a number of potential biomarkers that have been explored in the solid tumor setting to predict and monitor the response to anti-angiogenic therapies. As yet, these approaches have had limited application in the pancreatic setting, but represent potential new areas of study as newer anti-angiogenic agents are developed and tested in pancreatic cancer. Some of these approaches will be discussed below, including measurement of soluble angiogenic proteins and circulating endothelial cells.

14.11.3.1 Soluble Angiogenic Proteins

A large number of angiogenic factors are released into the circulation and can be detected in plasma or serum at increased concentrations in patients with cancer compared to healthy individuals. Some angiogenic proteins can also be measured in urine, including VEGF-A. Many commercial assays are available to measure soluble proteins including enzyme-linked immunosorbent assays (ELISA) and multiplexed bead-based immunoassays (xMAP). Peripheral blood and urine both offer the advantage of being minimally invasive to collect, allow serial measurements,

can be stored easily for future analysis, and are relatively inexpensive to analyze. Accurate quantification of VEGF in peripheral blood has been challenging due to release of VEGF-A from platelets into the plasma after collection. Variability in processing and shipping of plasma samples may explain the lack of correlation between circulating VEGF levels and response to anti-VEGF therapies (Hormbrey et al. 2002). Urine VEGF although less susceptible to processing and storage conditions than plasma, should still be handled according to guidelines outlined by Hayward et al. (2008). Other circulating angiogenic factors including soluble basic fibroblast growth factor (bFGF) may be more stable and should be investigated as potential PD biomarkers for VEGF-targeted therapies.

14.11.3.2 Cellular PD Markers: Circulating Endothelial Cells

Mature circulating endothelial cells (CEC) and endothelial precursor cells (CEP) are measurable in the blood at an increased frequency in patients with cancer and are being investigated as surrogate markers for tumor angiogenesis. In healthy individuals CEC cells are rare, compromising on average 1 in 10^6 blood cells (Robb et al. 2008). In one study, cancer patients had an average 3.3-fold increase in CEC cells compared to normal controls, and patients with progressive disease had higher CEC levels than patients with stable disease (Beerepoot et al. 2004).

In mouse models, maximally tolerable dose chemotherapy was found to provoke an increase in CEP levels and metronomic chemotherapy reduced the number of CEPs (Shaked et al. 2008). Epigallocatechin-3-gallate (EGCG), a polyphenolic compound from green tea that has anti-angiogenic activity also decreases circulating endothelial cell numbers in mice with xenograft pancreatic tumors (Shankar et al. 2008). Measurement of CEC or CEP cells in large clinical trials in pancreatic cancer patients is still needed to assess their value as PD biomarkers to predict or monitor response to therapy. In a recent Phase II trial evaluating bevacizumab with fixed-dose gemcitabine and low-dose cisplatin circulating endothelial cells collected at baseline and after two months of treatment did not correlate with overall survival (Ko et al. 2008); however, the clinical benefit of this drug regimen was only modestly effective.

14.12 PD Models Utilized in Assessing Mechanism and Activity of COX-2 Expression and Inhibition in Pancreatic Cancer

14.12.1 COX-2 is Highly Expressed in Pancreatic Cancers

The cyclooxygenase-2 (COX-2) enzyme catalyzes the rate-limiting step in the conversion of arachidonic acid to prostaglandins and other eicosanoids. COX-2 is

over-expressed in a number of malignancies, including pancreatic cancer where its expression is associated with poor prognosis (Juuti et al. 2006; Matsubayashi et al. 2007). The level of expression of COX-2 in pancreatic adenocarcinomas has been studied by a number of investigators, with reported over-expression ranging from 36 to 72% (Yip-Schneider et al. 2000; Merati et al. 2001; Kong et al. 2002; Juuti et al. 2006). COX-2 expression is associated with increased cell proliferation, decreased apoptosis, invasion, metastasis, and angiogenesis (reviewed in Juuti et al. 2006). Colby et al. (2008) recently reported the finding that forced over-expression of COX-2 in a mouse transgenic model results in a chronic pancreatitis-like state with alterations in biomarkers associated with inflammation and neoplasia. This phenotype was not observed when the mice were treated with celecoxib, a selective COX-2 inhibitor. In human intraepithelial neoplasia (PanIN), COX-2 expression has been reported to be elevated and is postulated be represent a link with chronic pancreatitis and pancreatic cancer (Albazaz et al. 2005).

14.12.2 Selective COX-2 Inhibitors

Selective COX-2 inhibitors were developed as a strategy to avoid the gastrointestinal side-effects that are associated with traditional NSAIDs. Unfortunately, selective COX-2 inhibitors have demonstrated an increased incidence of cardiovascular risks due to their ability to lower the levels of the major urinary prostacyclines (reviewed by Vardeny and Solomon 2008). The mechanism by which COX-2 inhibitors confer their anti-tumor activity has been controversial. For example, in addition to COX-2 inhibition, celecoxib can also act through COX-2-independent mechanisms including modulation of Ca^{2+} ATPase, protein-dependent kinase 1 (PDK1), cyclin-dependent kinases, in complex with cyclins, and carbonic anhydrase (El-Rayes et al. 2004; Grosch et al. 2006; Schonthal 2007). Notably, the concentration of celecoxib required to inhibit cell proliferation is higher than that required to inhibit COX-2 activity (Grosch et al. 2001).

14.12.3 PD Models for COX-2 Inhibition in Pancreatic Cancer

Unfortunately, the addition of celecoxib to gemcitabine-containing regimens has not resulted in significant improvements in survival rates in patients with pancreatic cancer, even in patients with COX-2 over-expressing tumors (El-Rayes et al. 2005; Dragovich et al. 2008). This is perhaps explained by a recent investigation of celecoxib pharmacodynamics in pancreatic cancer in which a small number of patients scheduled for resection of an infiltrating adenocarcinoma of the pancreas were randomized to receive celecoxib at a dose of 400 mg twice a day ($n = 3$) or placebo ($n = 3$) (Jimeno et al. 2006). In addition, a direct pancreatic xenograft model was utilized ($n = 9$). No statistical differences in prostaglandin E_2 (PGE_2)

levels were observed between the patients tumors from the placebo vs. treatment groups ($p = 0.10$). However, in the xenografts six of the nine tumors demonstrated a significant change in PGE_2 levels following celecoxib administration. In spite of the observed inhibition of PGE_2 levels only two tumors showed a significant tumor growth inhibition, and there were no differences between the baseline proliferation measured by Ki67 and microvascular density (CD31) levels. One interpretation of these observations is that the dose of celecoxib used clinically may be sufficient to lower COX-2 activity, but sub-optimal for anti-tumor response mediated by the non-COX-2 targets of celecoxib such as PDK1. This study is an exciting example of how the direct xenograft model may be a more meaningful predictor of anti-tumor effects than traditional xenografts using high-passage pancreatic cell lines.

14.13 Targeting Stroma: Secreted Protein Acid and Rich in Cysteine

Secreted protein acidic and rich in cysteine (SPARC)/osteonectin/BM 40 is a matricellular glycoprotein involved in tissue repair, wound healing, cell migration and neo-angiogenesis (Clark and Sage 2008). SPARC is often hypermethylated and the expression is suppressed in pancreatic cancer cells (Hong et al. 2008). Interestingly, SPARC is over-expressed in pancreatic cancer stromal cells (Sato et al. 2003). This deregulation of SPARC in pancreatic tumors raised an interest in its role as a prognostic marker and/or potentially a therapeutic target. Indeed, patients with pancreatic cancer whose stromal fibroblasts over-express SPARC tend to have significantly worse prognosis than patients whose tumor stroma does not express SPARC (15 months vs. 30 months, $n = 299$) (Infante et al. 2007). Serendipitously, a new nano-particle taxane drug (nab-paclitaxel, Abraxane®) has been found to accumulate in SPARC-expressing tissues (Desai et al. 2008). A recent preliminary report from a Phase I trial of gemcitabine plus nab-paclitaxel suggests very encouraging activity of this combination, and possibly higher response rate for patients with tumors expressing SPARC (Von Hoff, Proc AACR 2008). Further investigations are needed to establish if stromal and/or intatumoral SPARC expression can predict an increased sensitivity to nab-paclitaxel.

14.14 Other Potential PD Endpoints: Circulating Tumor Cells

Circulating tumor cells (CTCs) can be detected in the peripheral blood of cancer patients. It is postulated that CTCs are the origin of metastatic disease. In breast cancer, the number of CTCs are a prognostic indicator. CTCs can be distinguished from hematological cells by epithelial markers including cytokeratins and EpCAM (Deng et al. 2008). Detection methods for CTC enumeration are becoming increasingly sophisticated, with automated immunomagnetic separation, microfluidics,

and microchips (Jacob et al. 2007). The detection of CTCs in pancreatic cancer patients using microchip technology has been described (Nagrath et al. 2007). In the small group of patients studied who were receiving treatment for their disease, CTC number correlated with radiographical measurement of disease progression. These results are encouraging and suggest that CTC enumeration may eventually be a useful PD endpoint. Even more exciting would be the ability to perform molecular profiling on pancreatic CTCs to segment patients for specific treatment regimens.

14.15 Concluding Remarks

Recent studies utilizing microarray and proteomic technologies have greatly improved our understanding of the underlying molecular characteristics of pancreatic cancer, resulting in the identification of potentially more sensitive diagnostic and prognostic biomarkers (Liang et al. 2009) and rational targets for anti-cancer therapies (Middleton et al. 2008). However, due to the complexity of this disease, the development of PD biomarkers to select patients for individualized therapy may be essential for optimal clinical benefit. The development of assays to identify target expression and modulation in combination with the presence of resistance markers will be critical for selecting optimal therapeutic regimens. The difficulty in obtaining tumor samples, especially pre-and post-drug treatment, necessitates the investigation of surrogate tissue, imaging biomarkers, and circulating cells, and soluble proteins as potential PD biomarkers.

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