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Stromal Biology of Pancreatic Cancer

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Abstract The genetic paradigm of cancer, focused largely on sequential molecular aberrations and associated biological impact in the neoplastic cell compartment of malignant tumors, has dominated our view of cancer pathogenesis. For the most part, this conceptualization has overlooked the dynamic and complex contributions of the surrounding microenvironment comprised of non-tumor cells (stroma) that may resist, react to, and/or foster tumor development. Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease in which a prominent tumor stroma compartment is a defining characteristic. Indeed, the bulk of PDAC tumor volume consists of non-neoplastic fibroblastic, vascular, and inflammatory cells surrounded by immense quantities of extracellular matrix, far exceeding that found in most other tumor types. Remarkably, little is known about the composition and physiology of the PDAC tumor microenvironment, in particular, the role of stroma in tumor initiation and progression. This review attempts to define key challenges, opportunities and state-of-knowledge relating to the PDAC microenvironment research with an emphasis on how inflammatory processes and key cancer pathways may shape the ontogeny of the tumor stroma. Such knowledge may be used to understand the evolution and biology of this lethal cancer and may convert these insights into new points of therapeutic intervention. J. Cell. Biochem. 101: 887–907, 2007.

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Extensive progress has been made in elucidating the molecular genetic determinants of cancer, including the identification and characterization of oncogenes and tumor suppressor genes. Many of these genetic aberrations impact the biologic potential of the cancer cell in processes such as replication, apoptosis, and invasion. From the first observations through the light microscope, we have come to recognize that a "tumor" consists far more than a collection of homogenous cancer cells, but also includes *stroma*—the extracellular and cellular tissue framework that surrounds and interacts with cancer cells. The nature of these stromal cells associated matrix and how it may contribute to the neoplastic phenotype in pancreatic cancer is the subject of this review.

The composition of tumor stroma can vary significantly from tumor type to tumor type, and from location to location, suggesting that stroma formation depends on a complex set of interactions between cancer cells, non-malignant cells and extracellular matrix (ECM) in a particular tissue. Despite this structural heterogeneity, tumor stroma can be broken down into constituent parts. *Mesenchymal cells* represent a heterogeneous population of spindled-shaped cells including *fibroblasts*, which normally reside in connective tissue. Upon stimulation, fibroblasts may proliferate, secrete copious amounts of ECM rich in collagen, as well as induce intracellular markers of activation proteins such as α-smooth muscle actin (α-SMA) [Kalluri and Zeissberg, 2006]. These activated fibroblasts, known as myofibroblasts, based on their smooth muscle cell-like contractile properties, typically constitute the descriptive category of "carcinoma-associated fibroblasts"

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(CAFs) due to their abundance in many tumor types. Other mesenchymal cells, such as the stellate cells found surrounding pancreatic exocrine glandular units (see below), may normally have specialized functions but when activated show myofibroblast-like properties. *Vascular cells* consist of endothelial cell precursors and intimately associated pericytes which form the basis for blood vessels [Armulik et al., 2005]. *Inflammatory/immune cells* consist of cells of adaptive and innate immunity including lymphocytes, dendritic cells, neutrophils, macrophages, mast cells, and eosinophils. Finally, any tissue resident cell type in proximity to a growing tumor can be incorporated into the stroma, such as organ-specific cells, adipose and nervous tissue, and also influence tumor biological processes. One may note these constituents of tumor stroma are very similar to components that arise in response to tissue injury, and indeed, the description of a tumor as a "wound that never heals" [Dvorak, 1986] holds merit to a first approximation.

Strong evidence now exists that perturbations in the normal host compartment may drive tumorigenesis [Tlsty and Coussens, 2006]. Analogous to cell-autonomous pathways, non-malignant stromal cells can exert "tumor suppressor" activity via immunosurveillance, for example. Reciprocally, stromal cells can play pro-carcinogenic roles particularly in the setting of inflammation where fibroblasts and immunocytes show a variety of gain-of-function properties, [Coussens and Werb, 2002; Bhowmick and Moses, 2005]. Moreover, growing evidence suggests that, after the establishment of an incipient tumor, there are elaborate heterotypic interactions in which genetic alterations in cancer cells can elicit facilitatory responses in the surrounding host microenvironment to enable further tumor growth. These responses include the formation of new blood vessels, the promoting of proliferation and activation of ECM-producing fibroblasts that synthesize growth factors such as stromal cell-derived factor 1 (SDF-1), and the provocation of an active inflammatory response. Reciprocal effects of the stroma on the overt cancer cells further promote tumor behavior, such as effects of SDF-1 on tumor proliferation and stimulation of angiogenesis [Orimo et al., 2005; Orimo and Weinberg, 2006]. These cellular responses may be a combination of generalized, reactive responses intrinsic to tissue injury, as well as instructive responses from tumor cells. In pancreatic ductal adenocarcinoma (PDAC), cancer cells are enveloped in a rich tumor stroma more exuberant than in most tumor types. On this burgeoning foundation of tumor microenvironment research and knowledge, we summarize the state of knowledge of the PDAC microenvironment and possible roles in processes of cancer initiation and progression. Before covering this topic, the next subsection provides a brief general review of PDAC emphasizing the histopathologic and genetic evolution of the cancer cell compartment [see Hezel et al., 2006 for a more extensive review of PDAC].

PANCREAS CANCER 101

PDAC is the fourth leading cause of cancer death in the United States and exhibits a median survival of less than 6 months and a 5-year survival rate of 3–5% [Warshaw and Fernandez-del Castillo, 1992; Li et al., 2004]. These dismal statistics relate to the lack of early detection tests resulting in advanced disease presentation and to the inherent resistance of PDAC tumors to virtually all-therapeutic modalities including conventional and targeted agents and radiation therapy. Recent advances in our understanding of PDAC include a histopathological roadmap of evolving neoplasms and associated signature genetic events, a catalog of recurrent oncogenomic events, and the development of faithful mouse models driven by classical mutations. Despite this progress, many unanswered questions remain, particularly those relating to the role of the tumor microenvironment in disease evolution and maintenance.

Histopathologic Evolution of Pancreatic Neoplasms

Extensive histopathologic analysis of pancreatic neoplasms has identified a series of lesions with the potential to progress to the highly malignant and invasive PDAC. These pathologic entities are termed pancreatic intraepithelial neoplasm (PanIN), mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN) [Brugge et al., 2004; Maitra et al., 2005]. PanINs are the most extensively studied and appear to be the most

common type of precursor lesion. PanINs are typically found in the smaller caliber pancreatic ducts and demonstrate a spectrum of divergent morphological alterations relative to normal ducts that seem to represent graded stages of increasingly dysplastic growth [Hruban et al., 2001; Maitra et al., 2005]. PanINs are graded from stages I–III with the earliest stage characterized by the appearance of a columnar, mucinous epithelium and with increasing architectural disorganization and nuclear atypia through stages II and III. The high-grade PanINs can ultimately transform into frank invasive PDAC with invasion through the ductal basement membrane. A number of molecular profiling studies have subsequently reinforced the PanIN-to-PDAC progression model through documentation of an increasing number of gene alterations in higher grade PanINs [Heinmoller et al., 2000; Hruban et al., 2000, 2001; Wilentz et al., 2000; Yamano et al., 2000; Luttges and Kloppel, 2001; Maitra et al., 2003]. The less common MCN and IPMN lesions also exhibit a characteristic genetic progression towards PDAC, sharing a number of key genetic features with the PanIN-PDAC progression (for more complete review see Hezel et al. [2006]).

Signature Genetic Events

KRAS: Numerous genetic events are associated with PDAC pathogenesis and several appear to correlate with key early or late histopathologic stages. The oncogenic KRAS mutation, which activates this small GTPase, appears to be almost universal in PDAC and is seen in early neoplastic lesions. KRAS acts a central regulator of growth factor signaling and leads to the activation of multiple downstream signaling cascades involved in cellular proliferation, survival, and differentiation (reviewed in Campbell et al. [1998]) and may also regulate angiogenesis [Arbiser et al., 1997; Fleming et al., 2005]. Consistent with a role for KRAS in driving initiation of PDAC, mice engineered with an activated KRAS mutant allele rapidly develop premalignant PanINs with low potential for progression unless combined with tumor suppressor gene mutations [Aguirre et al., 2003; Hingorani et al., 2003].

INK4A/ARF and p53 Tumor Suppressors: INK4A and ARF are critical tumor suppressors involved in multiple cancer types and are encoded by the same gene via distinct first exons and alternative reading frames in shared downstream exons. Given the juxtaposition of these coding sequences, many pancreatic cancers sustain loss of both gene products with a single genetic event. In PDAC, the INK4A/ARF locus is inactivated by homozygous deletion, somatic mutation, or promoter hypermethylation in 80–95% of sporadic PDAC [Rozenblum et al., 1997; Hustinx et al., 2005]. Emerging evidence supports the view that each gene product functions to constrain PDAC development. Along these lines, there are INK4A-specific mutations in human PDAC which spare ARF coding sequences. Conversely, recent genetic evidence in mouse models has also established the importance of ARF in blocking PDAC progression in the context of activating KRAS mutations [Aguirre et al., 2003; Bardeesy et al., 2006].

The p53 tumor suppressor is mutated in up to 50% of cases of PDAC [Rozenblum et al., 1997]. This event generally occurs in later stage PanINs that have acquired significant features of dysplasia, suggesting a role in disease progression [Boschman et al., 1994; Maitra et al., 2003]. Loss of p53 may contribute to the well known rampant genomic instability seen in PDAC. This observation is substantiated by genetically engineered mouse models where mutant p53 cooperates with activated KRAS to produce PDAC with increased genomic instability and metastatic potential [Hingorani et al., 2005; Bardeesy et al., 2006a].

SMAD4: Another prominent mutation associated with PDAC progression is loss of the SMAD4 (DPC4) transcriptional regulator [Hahn et al., 1996], which serves as a key component in the paracrine transforming growth factor-β (TGF-β) signaling cascade [Massague et al., 2000]. The SMAD4 gene is targeted for deletion or intragenic point mutations in nearly 60% of PDAC cases [Hahn et al., 1996]. SMAD4 has been designated a progression allele for PDAC on the basis of its loss in later stage PanINs [Wilentz et al., 2000; Luttges et al., 2001; Maitra et al., 2003]. The mechanism by which SMAD4 loss contributes to tumorigenesis likely involves its role in TGF-β-mediated growth inhibition. The tumor biological impact of TGF-β signaling is extremely complex and depends on cell type and tumor stage, for instance TGF-β may act as an apoptotic agent for epithelial cells but may serve as a growth factor for fibroblasts (see below).

PRE-TUMOR MICROENVIRONMENT, PDAC INITIATION, AND THE POTENTIAL ROLE OF PANCREATITIS AND INFLAMMATION

The pancreas is composed of both exocrine and endocrine tissues which operate independently to regulate the digestion in the GI tract and to control glucose homeostasis through the secretion of insulin and glucagon. The endocrine cellular compartments are organized into discrete units called islets of Langerhans. The exocrine pancreas contains clusters of acinar cells forming functional units termed acini. Individual acinar cells comprising these acini contain numerous vesicles containing zymogen, or inactive pancreatic digestive enzymes, including trypsin, chymotrypsin, carboxypeptidase, amylase, and lipase. Zymogen is secreted through a network of ducts starting at the centroacinar cells which mark the beginning of the intercalated duct. Intercalated ducts merge to form intralobular ducts which in turn coalesce to form interlobular ducts, ultimately merging to form the main pancreatic duct which drains into the duodenum. Zymogen activation typically occurs only after reaching the duodenal lumen.

The study of host–tumor interactions must consider the cellular constituents surrounding incipient and evolving cancerous lesions. In the case of PDAC, this "pretumor microenvironment" is often dominated by active inflammatory processes. The pathogenetic relevance of inflammation is readily evident in a medical condition known as chronic pancreatitis. In this condition, inappropriate activation of zymogen occurs in the acinus, resulting in tissue autodigestion, cytokine release, and inflammation which results in acute pancreatitis. Repetitive acute injury or a self-perpetuating inflammatory process can lead to chronic pancreatitis, characterized by additional tissue damage leading to fibrosis and ultimately exocrine insufficiency.

As with other well-established links between inflammation and cancer, such as gastritis and stomach cancer or hepatitis and hepatocellular carcinoma, there is strong evidence that pancreatitis is a major risk factor for PDAC [Coussens and Werb, 2002; Whitcomb, 2004]. Notably, prospective analysis has demonstrated a striking 27-fold increase in PDAC incidence in patients with chronic pancreatitis relative to disease-free individuals in the general population [Malka et al., 2002]. Chronic pancreatitis may thus cause an altered environment resulting in a landscape that fosters tumorigenesis. Chronic pancreatitis may be provoked by genetic and/or environmental factors. Alcoholic pancreatitis accounts for 70% of cases of chronic pancreatitis, an additional 10% of cases are linked to obstructive (e.g., gallstone) pancreatitis, lymphoplasmacytic sclerosing pancreatitis, hereditary and genetically-linked pancreatitis, and the remaining 20% of cases have no readily identifiable cause and are termed "idiopathic pancreatitis" [Whitcomb, 2004].

In patients with hereditary pancreatitis caused by germline gain-of-function mutations in the cationic trypsinogen gene PRSS1, there is a 53-fold increased incidence of PDAC [Lowenfels et al., 1997]. Mutations in the pancreatic secretory trypsin inhibitor (SPINK1) gene are associated with "tropical pancreatitis," an idiopathic chronic pancreatitis seen in tropical Asia and Africa. In patients with tropical pancreatitis, there is a 100-fold risk of PDAC with onset approximately 14 years earlier than in sporadic cases [Whitcomb and Pogue-Geile, 2002; Whitcomb, 2004]. Finally, a link between chronic pancreatitis and PDAC is seen in patients with cystic fibrosis (CF). Mutation in the CFTR gene impairs proper secretion of pancreatic digestive enzymes resulting in a ductal obstruction with pancreatitis and associated fibrosis [Noone et al., 2001; Whitcomb, 2004]. Carriers of one CF allele are at increased risk for pancreatitis and for PDAC [McWilliams et al., 2005].

Regardless of the etiology, histological and molecular evidence show a strong association between PDAC genesis and chronic pancreatitis. The common precursor lesion, PanIN, has been detected in 63% of specimens resected for chronic pancreatitis but only 28% in incidental pancreatectomies [Andea et al., 2003]. The intimate link between pancreas neoplasia and chronic pancreatitis is reinforced by the molecular observation of activating K-RAS mutations in up to a third of chronic pancreatitis cases, raising speculation that the molecular origins of PDAC may be fueled by processes related to chronic pancreatitis [Lohr et al., 2000]. Similarly, the developmentally regulated Hedgehog family of secreted proteins plays a significant role in both chronic pancreatitis and PDAC. During embryonic development, Sonic

hedgehog (Shh) and Indian hedgehog (Ihh) are expressed in the developing gut and negatively regulate pancreas formation (reviewed in Lau et al. [2006]). Berman et al. [2003] demonstrated a widespread requirement of Hedgehog signaling in gastrointestinal tumors, including PDAC. Shh, typically absent in normal adult pancreas, is expressed in PanINs and 70% of PDACs in humans, and when overexpressed in transgenic mice can induce PanIN lesions [Thayer et al., 2003]. Hedgehog and its receptors Patched and Smoothened are found to be upregulated in ducts in chronic pancreatitis and likely contribute to its pathogenesis [Kayed et al., 2003]. Thus, dysregulation of Hedgehog signaling may reflect a pathophysiological reacquisition of an early developmental state and thereby contribute to tumorigenesis.

Activation of the NFκB pathway also occurs in response to stimulation by proinflammatory cytokines and growth factors, and is known to regulate numerous cancer-relevant processes including immune modulation, angiogenesis, and apoptosis (reviewed in Hayden and Ghosh [2004]). Interestingly, both primary pancreatic cancers and chronic pancreatitis but not normal pancreas show constitutive NFκB activity [Gukovsky et al., 1998; Wang et al., 1999; Chandler et al., 2004]. Activation of the NFκB pathway occurs in response to a variety of cell stresses through stimulation of factors including tumor necrosis factor-alpha (TNF-α) [Hayden and Ghosh, 2004]. TNF-α is synthesized by multiple cell types, including macrophages as well as inflamed pancreatic acinar cells, and can have paracrine effects in both chronic pancreatitis as well as PDAC cells. NFκB regulates the transcription of a host of genes governing these biological processes including vascular endothelial growth factor (VEGF), urokinase, and other pro-invasive or angiogenic factors which thus further shapes the tumor microenvironment [Fujioka et al., 2003; Xiong et al., 2004]. These autocrine and paracrine effectors and targets of NFκB signaling are complex and highlight the need to further explore the actions of this pathway in PDAC pathogenesis.

Another important downstream mediator of inflammation is cyclooxygenase-2 (COX-2), a key enzyme responsible for synthesis of prostaglandins and eicasinoids that is upregulated in a wide range of epithelial cancer and their precursor lesions [Dubois et al., 1998]. COX-2 is induced by a wide range of oncogenic, inflammatory, and growth factor stimuli [Brown and DuBois, 2005]. This range of inducing agents and pathways is reflected by a large number of transcription factor binding sites in the COX-2 promoter which can be engaged downstream of paracrine and autocrine signals. COX-2 is upregulated in inflamed acinar and ductal components of chronic pancreatitis, in PanINs correlating with the histologic grade of the PanIN lesions, and is overexpressed in the vast majority of PDAC [Tucker et al., 1999; Schlosser et al., 2002; Albazaz et al., 2005]. Moreover, transgene-directed COX-2 expression in pancreatic ductal cells results in predominantly cystic lesions with focal areas of mucinous epithelial differentiation [Muller-Decker et al., 2006]. The roles of these signaling molecules and associated pathways fit well with observed clinical associations between inflammation, cell turnover, and cancer. To what extent activation of NFκB and COX-2 pathways by chronic pancreatitis and PDAC represent convergent activation of distinct initiating stimuli, and to what extent they represent mechanistically linked process with a common pathological etiology, remain important areas of active investigation.

Similar to inflammation seen in the setting of other tumors, pancreatic inflammatory microenvironment leading to tissue damage and fibrosis might also promote tumorigenesis in part by promoting the local release of growth factors, cytokines, and reactive oxygen species (ROS), thereby inducing cell proliferation, disrupting cell differentiation states, and selecting for oncogenic mutations [Farrow and Evers, 2002]. RAS transformed cells have increased ROS levels, which may be necessary for full cellular transformation [Irani et al., 1997] and these cells also upregulate multiple antioxidant proteins that allow them to survive in the setting of high levels of ROS [Young et al., 2004]. Increased intracellular ROS production may also cause DNA damage and may also contribute to telomere shortening which is widely observed in PanINs [van Heek et al., 2002]. DNA damage, telomere shortening, and mutation of proto-oncogenes might affect a pluripotent reserve, resulting in accumulation of genetic aberrations in this cell population which promote oncogenic transformation [Beachy et al., 2004].

TRACING THE ORIGINS OF PDAC STROMA

Tumor stroma is a complex, dynamic entity whose formation depends on instructive signals from the cancer cell, reactive nature of resident cells, recruitment of non-resident cell types, and generation of ECM. In PDAC, the major components of the tumor microenvironment/tumor stroma are a complex population of fibroblasts forming the bulk of the stroma, vasculature, and inflammatory/immune cells (leukocytes). *Desmoplasia* can be defined as the exuberant proliferation of stromal cells elicited by an invasive tumor. Abundant ECM synthesis with extensive collagen production characterizes desmoplastic stroma. Like all stroma across different tumor types, desmoplastic stroma likely depends on a combination of instructive signals from the tumor as well as site-dependent differences in resident stromal precursor cells.

In the following subsections, the origins of PDAC stroma will be traced temporally, first by briefly describing the stroma surrounding PanIN lesions, followed by a description of PDAC stromal components. Next, particular signaling pathways which mediate tumor-stroma cross talk, and how these signals contribute to tumor initiation and progression will be highlighted.

Formation of PanIN Stroma/The PanIN Microenvironment

PanINs, likely representing a PDAC precursor lesion, are thought to arise from small ducts, ductules, and possibly the centroacinar cell at the start of the pancreatic ductular axis [Hruban et al., 2001; Orimo et al., 2005; Stanger et al., 2005]. As an in situ/intraductal neoplasm, PanINs are confined by a basement membrane [Hruban et al., 2001]. Depending on the duct caliber, there is a variable amount of periductular connective tissue consisting primarily of resident fibroblasts. Similar to stroma surrounding ductal carcinoma-in situ of the breast [Ronnov-Jessen et al., 1996], the stroma surrounding PanINs may undergo proliferation and show specialization [Detlefsen et al., 2005]. In mouse models in which oncogenic Kras is expressed in the pancreas, PanIN lesions are formed in which a variable stromal response is present depending on location and severity of lesion [Aguirre et al., 2003; Hingorani et al., 2003]. Intriguingly, a mouse model which activates oncogenic Kras and eliminates Smad4 receptor in the pancreas show enhanced stromal proliferation and collagen I synthesis around PanIN lesions [Bardeesy et al., 2006b].

In addition to fibroblastic proliferation, the PanIN-associated stroma is characterized by the birth of new blood vessels, and by the infiltration of inflammatory cells often seen at the stroma periphery (Fig. 1). Similar changes have been better described in the preinvasive stage of neoplastic lesions of other tissues [Lee et al., 1997; Mueller and Fusenig, 2004], but their significance with respect to tumorigenesis is unclear. As in a mouse model of squamous epithelial carcinogenesis [Bergers et al., 1998; Coussens et al., 1999, 2000; Hoffman et al., 2003], one might speculate that the stroma around the in situ PanINs may promote tumor growth, angiogenesis, ECM remodeling, and assist in tumor invasion, although a neutral or inhibitory role for this stroma is equally plausible.

Formation of PDAC Stroma (I): Fibroblasts and Other Mesenchymal Cells

Pancreatic stellate cell/fibroblast (PSC). The pancreatic stellate cell (PSC) has received close scrutiny in the past decade as a major contributor to fibroblastic proliferation and fibrosis in both chronic pancreatitis and PDAC (reviewed in Jaster [2004]). PSCs were recently identified in the pancreas based on morphologic and functional similarity to vitamin A-storing stellate cells of the liver (Ito cells), which contribute to fibrosis in cirrhosis and some liver cancers [Saotome et al., 1997; Apte et al., 1998; Bachem et al., 1998]. By electron microscopy, these flat, fairly inconspicuous mesenchymal cells show cytoplasmic lipid droplets and "gift-wrap" acini. In cell culture, PSCs adopt an activated phenotype characterized by acquisition of a spindled-shape, expression of α-SMA and desmin, and production of large amounts of ECM proteins such as type I and III collagen, fibronectin, and laminin [Bachem et al., 1998]. Based on their appearance and function, activated PSCs have been variably termed "fibroblast" or "myofibroblast." Activation and proliferation of PSCs and induction of these proteins can be observed in human chronic pancreatitis as well as chemical models of pancreatitis in the rodent [Haber et al., 1999; Sparmann et al., 1997].

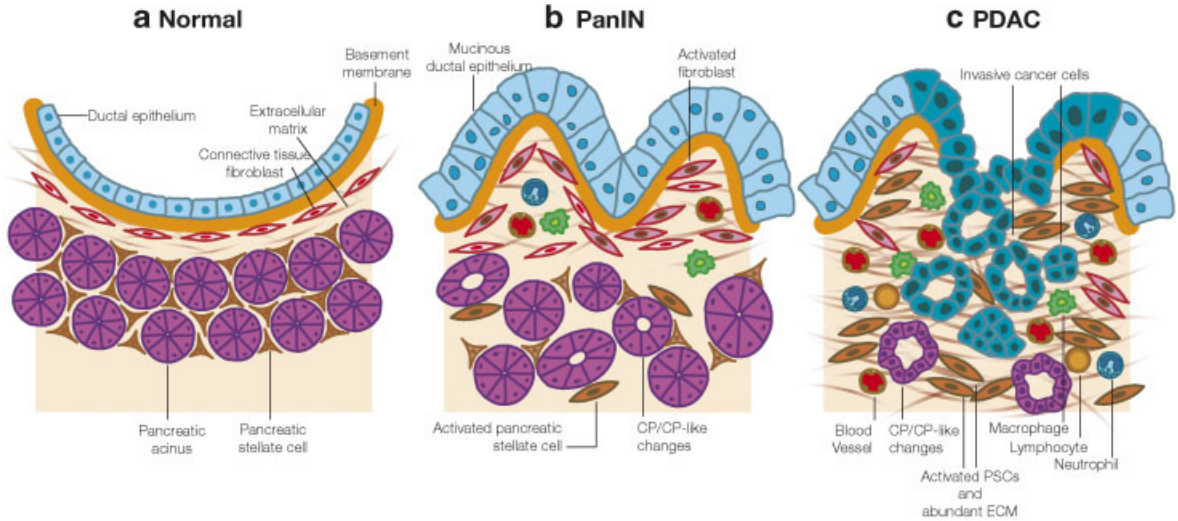

Fig. 1. Proposed tumor-stroma interactions during pancreatic ductal adenocarcinoma (PDAC) tumorigenesis. **a**: In the normal exocrine pancreas, the pancreatic ductal epithelium is separated from connective tissue fibroblasts and extracellular matrix (ECM) by a basement membrane. Located adjacent to ducts are pancreatic acini, units of cells secreting zymogen granules containing digestive enzymes. These acini are ensheathed by the pancreatic stellate cell (PSC), a specialized mesenchymal cell located in close apposition to acinar cells. **b**: In intermediate grades of pancreatic intraepithelial neoplasia (PanIN 2), the ductal epithelium is replaced by an epithelium consisting of columnar mucin-containing cells exhibiting nuclear atypia. The basement membrane around these lesions remains intact. Stromal alterations are seen, including fibroblastic and early vascular proliferation. The exocrine pancreas often shows concomitant chronic pancreatitis-like changes including ductal metaplasia and activation of PSCs. **c**: In pancreatic ductal adenocarcinoma, the basement membrane is breached and tumor cells invade in to the surrounding pancreatic parenchyma. Invasive tumor cells may form duct-like structures. An extensive stromal reaction takes place, characterized by activation of pancreatic stellate cells exhibiting a myofibroblastic phenotype, vascular proliferation, infiltration of inflammatory cells, and exuberant ECM formation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Evidence for activated PSCs in PDAC stroma comes from similarities to stromal cells in chronic pancreatitis. PDAC stroma stains intensely for α-SMA, smooth muscle myosin heavy chain, GFAP, and collagen I, and hence shows a molecular profile highly suggestive of activated PSCs [Yen et al., 2002; Apte et al., 2004]. Supernatants of human PDAC cell lines added to purified rodent PSC cultures stimulated PSC proliferation and synthesis of ECM proteins. Subcutaneous injection into nude mice of PDAC cells (from established lines) mixed with primary rodent PSCs resulted in fibrotic tumors showing enhanced tumor growth as well as more intense staining for collagen I and III, and fibronectin, suggesting that a large portion of PDAC stroma could be accounted for by PSC activation [Bachem et al., 2005]. A large number of signaling pathways potentially involved in mediating tumor-stroma interactions have been described for the activated PSC. PSCs have been shown to respond to extrinsic signals including platelet derived growth factor (PDGF), TGF-β1, fibroblast growth factor 2, TNF-α, IL-1 and IL-6 [Jaster, 2004]. These factors have all been described in the PDAC microenvironment and could potentially play a role in PSC activation and fibrosis. In addition, PSCs also secrete matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) which are important for ECM remodeling. One should note, however, that many of these molecules are also induced in chronic pancreatitis and thus may represent general signals of pancreatic tissue injury [Apte and Wilson, 2003]. In this regard, establishing cause or consequence relationship is a key priority for the field.

Thus, as PanIN progresses to invasive PDAC, the ductular basement membrane is eroded and the tumor, no longer bounded by the basement membrane, is free to invade into the surrounding pancreatic parenchyma. At the periphery of a growing tumor, quiescent PSCs are activated, likely due to a combination of specific signals directed by the tumor, as well as through activation through generalized wound healing mechanisms. PSCs proliferate, synthesize abundant ECM, interact with inflammatory and angiogenic cells (see below)

and feedback to modulate subsequent tumor behavior.

Connective tissue-type fibroblasts and other mesenchymal cells. Based on the morphological appearance and location, it appears that multiple cell types with discrete origins comprise the PDAC fibroblast component. One cell type described above is the activated PSC. Other cell types from which PDAC stroma may arise include "traditional" fibroblasts. Such fibroblasts, forming an integral part of resident connective tissue support, are found around ducts, as well as blood vessels, nerves, and around pancreatic lobules. These interlobular fibroblasts, normally modestly apparent, show proliferation in many forms of pancreatic injury such as interlobular pancreatitis [Kloppel et al., 2004] and are markedly accentuated in the reactive areas surrounding human PDAC as well as in PDAC of mouse models. Thus, interlobular stroma from traditional fibroblasts may also contribute to the tumor stroma and microenvironment. Other mesenchymal cells present in the pancreas include pericytes, located adjacent to endothelial cells (see below), and adipocytes. In a mouse model for pancreatic islet cell tumorigenesis, stromal cells derived from bone marrow were found to be capable of contributing up to 25% of the fibroblasts in the mild stromal reaction seen around these tumors, showing markers of activation such as α-SMA and collagen I [Direkze et al., 2004; Song et al., 2005; Direkze and Alison, 2006; Lamagna and Bergers, 2006]. Pericytes have similarly been shown to derive in part from bone marrow [Ryu et al., 2001; Iacobuzio-Donahue et al., 2002; Song et al., 2005; Lamagna and Bergers, 2006]. Thus, an extra-pancreatic source for mesenchymal cells in mouse and human PDAC, although not established experimentally, is a possibility that deserves future attention.

In line with the multiple cell types that comprise the PDAC stroma, recent molecular analysis has begun to show that there are molecular patterns of stromal heterogeneity in these tumors. Using serial analysis of gene expression (SAGE), genes associated with pancreatic tumor invasion were identified. Through in situ hybridization on resected human PDAC, several of these invasion-associated genes were found to show patterns of stromal expression with histologic correlates, including genes expressed throughout the stroma and others expressed in the stroma immediately adjacent to the tumor glands, that is, juxtatumoral expression [Ryu et al., 2001; Iacobuzio-Donahue et al., 2002]. Genes showing marked juxtatumoral expression include ApoC1, ApoD, and the matrix metalloproteinase MMP11-gene expression patterns suggesting the presence of discrete interactions between tumor and stroma which may mediate the biological behavior of PDAC [Ricci et al., 2005].

The aforementioned studies reinforce the idea that the CAF is not a homogenous entity, but rather a complex collection of cellular profiles reflecting different cellular ontogeny and perhaps influenced by the presence of disease processes such as chronic pancreatitis (CP) and the occurrence of distinct genetic events in the PDAC cancer cell compartment. The molecular heterogeneity can be assessed both by fibroblast identity and functionally in terms of the synthesis of particular constellations of growth factors, chemokines and ECM ultimately leading to paracrine effects on the tumor and on angiogenesis [Kalluri and Zeisberg, 2006]. (In PDAC biology, activated PSCs exhibit many molecular and biological features akin to CAFs, but due to the role of activated PSCs in the non-neoplastic entity of CP, this term should be avoided.) Like in advances in breast carcinoma stromal biology, functional markers of tumor stromal heterogeneity need to be investigated in order to understand tumor biology and for developing targets in cancer therapies [Sugimoto et al., 2006]. For example, S100A4 (FSP1), a marker of a subset of fibroblasts, is expressed in some PDAC stromal cells. There are likely multiple other markers of particular subsets of fibroblasts that, once identified, will allow for crucial studies to understand these complex cellular interactions. Given the prominence of stroma in PDAC, these distinctions should be clearly defined to truly understand the disease.

Formation of PDAC Stroma (II): Inflammation, Angiogenesis, and Dynamic Interactions

Inflammation and chronic pancreatitis-like stromal responses. Similarities between granulation tissue (inflammation, angiogenesis, and fibroblastic reaction in the acute phase of wound healing) and desmoplasia (inflammation, angiogenesis, and fibroblastic reaction seen in particular invasive tumors) have been described above and noted elsewhere [Coussens

and Werb, 2002; Tlsty and Coussens, 2006]. The role of inflammation in the form of CP as the pancreatic pre-tumor microenvironment has been described above. But conversely, the stromal changes seen in PDAC bear significant resemblance to that of CP [Jaster, 2004], highlighting the role of inflammation in tumor stroma. Some of the similarities or common pathogenesis are the focus of this section.

Histologically, the stroma of CP and PDAC are related, consisting largely of cell-poor tissue with abundant collagen-rich ECM, vessels, and inflammatory cells. Indeed, the advancing PDAC front causes parenchymal changes adjacent the tumor that is histologically similar to CP. PanINs and PDAC may also cause ductal obstruction or tissue damage, engendering a CP-like condition. Standard markers which stain CP and PDAC stroma include the intracellular marker α-SMA and the ECM components such as collagen I, collagen III, decorin, tenascin, and fibronectin. A series of expression profiling studies of PDAC have collectively identified a lengthy list of genes upregulated not only in PDAC stroma but also in CP stroma as well [Crnogorac-Jurcevic et al., 2001; Binkley et al., 2004; Sato et al., 2004; Shen et al., 2004; Fukushima et al., 2005; Esposito et al., 2006; Koninger et al., 2006]. Indeed, serum biomarker studies for tumor routinely use CP for controls [Koopmann et al., 2006]. Finally, as mentioned above, inflammation is thought to activate PSCs, leading to their proliferation and synthesis of ECM proteins in both PDAC and CP.

In support of the concept that inflammatory immunocytes modulate tumor stroma [Aoyagi et al., 2004] described an invasive front of granulocytes, predominantly neutrophils, at the edge of the PDAC tumor periphery, the site of the greatest amount of tissue damage. These neutrophils showed high levels of TGF-β secretion which correlated with high levels of collagen production, suggesting that TGF-β, in combination with the unique acinar pancreatic environment, may play a role in stimulating stroma synthesis by fibroblasts/PSCs. Stroma with increased numbers of T cells and macrophages has also been described [Emmrich et al., 1998]. Detailed studies need to be performed to demonstrate which other type of immune cells, including lymphocytes, macrophages, dendritic cells, and mast cells play a functional role on cancer cell growth either directly or indirectly by altering the tumor microenvironment.

Tumor vasculature. Angiogenesis plays a fundamental role in tumor growth and metastasis. In PDAC as in other tumor types, blood vessels likely form as a result of dynamic neovascularization and vascular remodeling, in which the density and architecture of neoplastic blood vessels depends on angiogenic factors from the cancer cell, stromal cells, and the ECM, as well as temporal and geographic considerations. Formation of new vessels also reflects a dynamic balance between pro-angiogenic factors and endogenous angiogenesis inhibitors of both cellular and extracellular origins. As descriptive and experimental data on PDAC angiogenesis is less advanced than mechanisms elucidated from tumors of its geographic neighbor, the endocrine pancreas, the lessons learned from the study of angiogenic stages of a mouse model of pancreatic islet carcinogenesis will be briefly discussed. In the prototypical RIP-Tag2 mouse model, Hanahan and colleagues have described a multi-step process in which angiogenesis is initiated in dysplastic nodules (angiogenic islets), prior to tumor formation in what has been dubbed an "angiogenic switch" [Hanahan and Folkman, 1996]. The VEGF-A angiogenic growth factor plays a central role, as revealed by gene knockout and pharmacological inhibition [Bergers et al., 2000, 2003; Inoue et al., 2002]. Notably, VEGF-A activity is modulated in this tissue, not by upregulation of gene expression, but rather by release from ECM by a variety of matrix-degrading enzymes, including MMP-9 [Bergers et al., 2000], cysteine cathepsins [Joyce et al., 2004; Gocheva et al., 2006], and heparanase [Joyce et al., 2005].

In addition to endothelial cells, other cell-types play a role in the angiogenic process. Pericytes are mesenchymal cells defined by their close association with endothelial cells that comprise the inner lining of a vessel wall. Recently, pericytes have become recognized as vital regulators of angiogenesis including vascular development, stabilization, and remodeling. In addition, pericytes may show functional plasticity, including the ability to differentiate into vascular smooth muscle cells and may contribute to collagen-synthesizing stromal cells in wound healing and in tumors [Armulik et al., 2005; Lamagna and Bergers, 2006]. An emerging concept in the field is that pathologic tumor vasculature exhibits structural and molecular differences compared to mature

vasculature which may in part be due to altered gene expression [Carmeliet and Jain, 2000]. In the RIP-Tag2 model of pancreatic islet carcinogenesis, the persistence and functionality of the VEGF-driven tumor vasculature has been revealed to be maintained in significant part by PDGF signaling from endothelial cells to PDGF receptors on pericytes [Bergers et al., 2003; Pietras and Hanahan, 2005; Song et al., 2005]. In PDAC, both PDGF ligands and receptors are variously expressed in tumor, fibroblastic, and endothelial cells, but experimental data is lacking to determine the precise role of these molecules in tumor angiogenesis.

These parameters of the angiogenic phenotype described in pancreatic islet cell tumorigenesis, including angiogenic growth factors, matrix-degrading enzymes, and accessory cells (pericytes and innate immune cells) stand as reasonable candidates for rigorous evaluation in PDAC angiogenesis. Indeed, many of these parameters have been described (albeit incompletely validated) in PDAC. For instance, VEGF-A is present in PDAC cancer cells, and moreover several but not all studies have shown that high expression of VEGF predicts early recurrence and poor prognosis after curative resection for PDAC [Niedergethmann et al., 2002], reviewed in Korc [2003]. MMPs such as MMP-9 are also expressed in PDAC epithelia and may help to mobilize VEGF-A. However, there are significant differences between invasive PDAC and islet cell tumors that suggest that additional angiogenic mechanisms should be sought. For instance, while desmoplastic stroma is abundant in PDAC, islet cell tumors

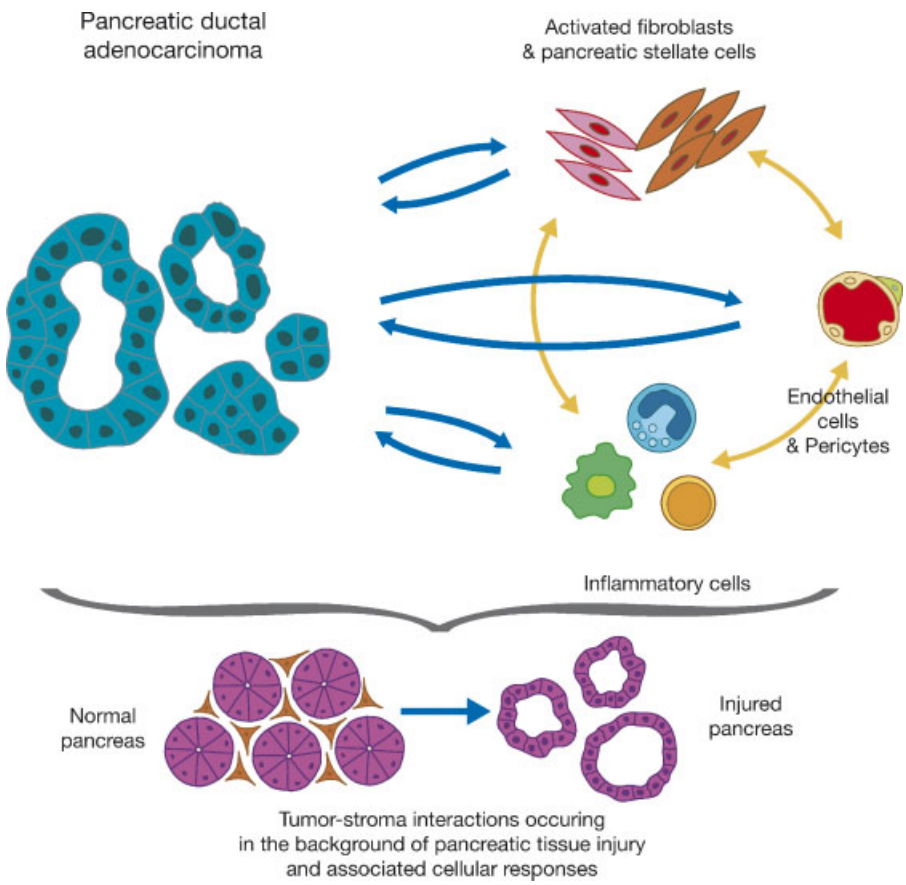

Fig. 2. Crosstalk between PDAC cancer cells and PDAC stromal constituents. Cancer cells secrete numerous growth factors including such factors as TGF-β, HGF, and VEGF as well as ECM-modifying matrix metalloproteinases, which all serve to stimulate angiogenesis, fibroblastic proliferation, ECM proliferation, and recruitment of inflammatory cells. In a paracrine feedback loop, these components can stimulate cancer cell proliferation and promote further invasive behavior. Additional paracrine interactions among stromal constituents such as those which occur during tissue injury and wound healing may serve to amplify signals that further promotes tumor growth. Moreover, these processes occur in a pancreas-specific setting, including the presence of pancreatic stellate cells as well as injury due to zymogen release and activation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

have minimal fibroblastic stroma. Signals arising from the fibroblast/activated PSC-rich stroma may instruct new vessel growth (Fig. 2).

In addition to signaling via fibroblastic cells, another mechanism by which PDAC cancer cells may indirectly shape its vascular microenvironment is via inflammation. Tissue damage resulting from chronic pancreatitis and PDAC exhibits profoundly greater levels of inflammation compared to the modest levels of inflammation seen in islet cell tumors. Infiltrating immune cells, principally neutrophils and macrophages have been implicated as pro-angiogenic [Coussens et al., 2000; Imhof and Aurrand-Lions, 2006; Nozawa et al., 2006]. Pancreatic tissue injury may possibly elicit a distinct wound-healing response including neovascularization in a mechanism common to both chronic pancreatitis and PDAC [Kuehn et al., 1999]. Proangiogenic factors such as VEGF-A, VEGF-C, and basic fibroblast growth factors secreted by infiltrating monocytes and mast cells have been implicated in contribute to angiogenic activity in PDAC.

FUNCTIONAL TUMOR-STROMA INTERACTIONS IN PDAC

TGF-β/SMAD Signaling in the PDAC Microenvironment

The TGF-β signaling pathway plays a vital role in many processes, including embryonic development, inflammation, angiogenesis, and neoplastic ontogeny such as tumorigenesis, tumor invasion, migration, and epithelial-mesenchymal transition [Massague et al., 2000, 2005; Bierie and Moses, 2006]. As mentioned, the SMAD4 tumor suppressor gene is deleted more frequently in human PDAC than in any other type of tumor [Hahn et al., 1996]. SMAD4 encodes a signaling molecule central to the signaling pathways initiated by a superfamily of TGF-β ligands and cognate receptors. The type II TGF-β receptor gene (Tgfbr2) encodes a receptor serine/threonine kinase which upon activation results in nuclear translocation of SMAD4 complexed with additional receptor Smad molecules (SMAD2, 3) and transcription factors. Tgfbr2 is mutated in a small subset of PDAC [Goggins et al., 1998]. The secreted ligands which bind to the TGF-β II receptor, TGF-β -1, -2, and -3, are significantly upregulated in PDAC and enhanced expression of these correlates with decreased survival [Friess et al., 1993a,b]. Moreover, enhanced expression of TGF-β type II receptor is associated with decreased survival in PDAC [Wagner et al., 1999]. Many of the same TGF-β signaling constituents also play a role in chronic pancreatitis and associated fibrosis [Detlefsen et al., 2005].

The effects of TGF-β/SMAD4 signaling intrinsic to tumor epithelial cells, are complex and often seemingly paradoxical [Bierie and Moses, 2006]. Within these cells, TGF-β signaling may either be growth-inhibitory or growth-promoting, depending on cellular context. Blockade of TGF-β signaling by expression of soluble type II TGF-β receptor attenuates tumor growth in vitro as well as in xenografts [Rowland-Goldsmith et al., 2001, 2002] conversely, exogenous addition of TGF-β enhances in vitro measures of aggressive tumor behavior in some cell lines [Ellenrieder et al., 2001a,b]. The cell-autonomous role of SMAD4 is under scrutiny. In human PDAC cell lines, SMAD4 status does not seem to predict TGF-β-induced cytotoxicity. To investigate the role of SMAD4 in a tumor xenograft model, reintroduction of SMAD4 in SMAD4-deficient human PDAC cells lines show only modest impact on cell growth (see mouse studies below).

In PDAC, elucidation of the role of SMAD4-dependent signaling in cancer cells and on stromal cells is still in its infancy. SMAD4 restoration experiments in PDAC cell lines suggest an inhibition of PDAC xenografts mediated through downregulation of angiogenesis by decreasing expression of VEGF and increasing expression of thrombospondin-1, as well as by reducing invasion and possibly ECM remodeling through downregulation of MMP-2 and -9 [Schwarte-Waldhoff et al., 2000; Duda et al., 2003]. A PDAC cell line engineered to over-express TGF-β1 resulted in an ability of conditioned media to promote fibroblast proliferation [Lohr et al., 2001].

Similarly, the role of TGF-β signaling within stromal cells from paracrine sources is poorly understood. PSCs treated with TGF-β as well as pancreatic stroma exposed to TGF-β1-overexpressing pancreatic epithelium show dose-dependent induction of activation markers such as SMA as well as production of ECM proteins [Apte et al., 1999; Vogelmann et al., 2001]. Interestingly, stromal cells show TβRII expression at significantly higher levels in PDAC than in CP stroma. As recent literature shows that

modulation of TβRII-dependent signaling in a defined subset of fibroblasts in mice can result in the initiation or promotion of epithelial carcinomas, [Bhowmick et al., 2004; Cheng et al., 2005], closer examination of TGF-β signaling in the PDAC stroma is warranted. TGF-β may have a direct impact on angiogenesis or an indirect effect by stimulation on VEGF pathways. Neutrophil-derived TGF-β may stimulate stromal collagen synthesis in PDAC, thus linking inflammation and desmoplasia [Aoyagi et al., 2004]. TGF-β signaling thus epitomizes a pathway in which tumor interacts with stromal components, and stromal components may act synergistically, ultimately affecting tumor biology (Fig. 2).

Recent advances have utilized genetically engineered mouse models to study more precisely the effects of TGF-β signaling in PDAC. In order to model genetic mutations observed in human PDAC, Bardeesy et al. [2006b] engineered Smad4 deficiency in the context of an activated Kras mutation. Tumors resembling IPMNs developed, surrounded by proliferative α-SMA-positive cells enveloped by collagenous ECM, suggestive of activated PSCs. These IPMNs were lethal in most cases due to pancreatic insufficiency, although a subset (~10%) of these mice developed invasive PDAC that appeared to arise from malignant progression of the IPMN lesions. In combination with heterozygous mutations of the Ink4a/Arf tumor suppressor locus, these mice developed IPMN and showed rapid progression to PDAC. These results suggest that while Smad4 inactivation promotes Kras-directed pancreatic tumorigenesis, additional loss of Ink4a/Arf function is required for full malignant progression. In a related study, Ijichi et al. [2006] examined the effect of Tgfbr2 deletion in concert with Kras activation. These mice developed invasive PDAC with high penetrance and rapid lethality. Hence, in this context, Tgfbr2 appears to be a more potent PDAC tumor suppressor than Smad4, pointing to receptor activities that extend beyond its links to SMAD4-dependent signaling.

These studies of mouse models harboring activated Kras in conjunction with different combinations of tumor suppressor gene alterations provided the opportunity to relate tumor genotypes to specific tumor biological phenotypes. Notably, while PDAC from mice with Ink4a/Arf mutations frequently showed an undifferentiated phenotype—reminiscent of epithelial-to-mesenchymal transition (EMT)—those harboring combined Ink4a/Arf and Smad4 inactivation and those with Tgfbr2 mutation retained a well-differentiated epithelial phenotype. Collectively, these results verify that TGF-β-Smad4 signaling plays a tumor suppressive function in the pancreas while indicating that tumors that arise bearing lesions in this pathway are impaired in their ability to undergo EMT. A provocative implication of these results may be that the status of TGF-β pathway may dictate the clinical presentation of PDAC in humans, and therefore that different therapeutic approaches may be required for subtypes of PDAC so differing in their TGF-β status. Ongoing studies will be required to identify whether these subsets of tumor have fundamental differences in oncogenic circuitry or in their acquisition of cooperating genetic alterations. A more detailed analysis of stromal composition in these various models will also be of critical importance given the presumed roles of TGF-β pathway in regulating tumor desmoplasia, angiogenesis, and inflammatory cell recruitment.

HGF/Met in the PDAC Microenvironment

Molecular tumor-stroma interactions are also well illustrated by interactions between the receptor tyrosine kinase Met and its ligand, hepatocyte growth factor (HGF). In a variety of tumors, the Met-HGF axis exerts multiple tumor-promoting properties including increasing cell motility, invasion and proliferation through autocrine and paracrine effects (reviewed in Corso et al. [2005], Matsumoto and Nakamura [2006]). Expression of Met is markedly upregulated in PanIN lesions and PDACs [Di Renzo et al., 1995; Furukawa et al., 1995]. The met ligand, HGF, is expressed both in PanIN lesions, and in PDAC stroma but minimally in PDAC epithelium [Paciucci et al., 1998].

In vitro HGF promotes PDAC cancer cell motility which is accompanied by downregulation of E-cadherin. Inhibition of this pathway through blocking antibodies or via expression of a dominant negative HGF fragment, called NK4, results in inhibition invasive growth and angiogenesis of xenografts. Moreover, the increased motility by HGF is accompanied by an increase of the serine protease urokinase plas-

minogen activator (u-PA), an effector of the plasmin proteolytic system which may suggest an in vivo mechanism for enhanced tumor invasiveness [Paciucci et al., 1998; Maehara et al., 2001].

The receptor tyrosine kinase RON is a family member of Met and is also implicated in tumor progression and metastasis. RON modulates multiple signaling pathways known to regulate tumorigenic properties such as adhesion, migration, invasion, and apoptosis and may interact with c-Met [Camp et al., 2005]. RON is expressed in the fetal pancreas and is present in pancreatic cell lines [Okino et al., 2001; Bardella et al., 2004]. The ligand for RON known as macrophage-specific protein (MSP) shows homology to HGF. Interestingly, ablation of the TGF-β receptor TβIIR in mammary fibroblasts results in upregulation of MSP- and HGF-mediated pathways in mammary carcinoma, linking these molecules together in tumor-stroma interactions [Cheng et al., 2005].

Matrix Metalloproteinases

The ECM-degrading family of MMPs has pleiotropic effects which serve to maintain the extracellular environment. Many MMPs can promote remodeling of ECM structure, and thus may help enhance neoplastic progression. Recently, additional roles have been documented, including regulating the bioavailability of growth factors and cytokines and thus processes such as tumor cell proliferation, angiogenesis, and metastasis (reviewed in Deryugina and Quigley [2006]; Overall and Kleifeld [2006b]). MMPs are expressed by PDAC cancer cells as well as fibroblasts, activated PSCs, and immunocytes. MMP-1, -2, -3, -7, -9, -11, -13, MT1-MMP, and tissue inhibitors TIMP-1 and -2 have been described in at least one PDAC cellular compartment [Yamamoto et al., 2001; Shek et al., 2002; Phillips et al., 2003; Kordes et al., 2005]. Additional MMPs have been reported yet require more extensive validation. Of note, MMP-2 and -9 (gelatinases A and B) are robustly expressed by PDAC tumors cells but are also expressed by activated PSCs and macrophages. MMP-9 has been shown to mobilize VEGF and thus manifest proangiogenic activity in other tumor types [Coussens et al., 1999; Bergers et al., 2000]; however, anti-angiogenesis factors such as a proteolytic fragment of type IV collagen, called tumstatin, is also cleaved by MMP-9 [Hamano et al., 2003]; thus the biological role of this enzyme is evidently more complex that just stimulation of angiogenesis. MMP-7 (matrilysin) is present in most PDAC, in particular in epithelial cells in the invasive tumor front. MMP-7 positivity was significantly correlated with advanced pathologic stages, and patients with MMP-7-positive carcinoma had a shorter survival time than those negative for MMP-7 [Yamamoto et al., 2001]. MMP11 (stromelysin-3) is expressed in PDAC stromal cells in a "juxta-tumoral" distribution and may indicate direct communication between tumor and stromal cells [Iacobuzio-Donahue et al., 2002; Ricci et al., 2005].

THERAPEUTIC IMPLICATIONS OF THE PDAC TUMOR MICROENVIRONMENT

The clinical success of the anti-VEGF monoclonal antibody, bevacizumab, across a range of malignancies has validated the concept of targeting a tumor's supporting microenvironment, and more specifically the supporting vasculature. Despite success in these other tumor types, PDAC has thus far remained refractory to such efforts. Large randomized trials evaluating Bevacizumab in addition to standard gemcitabine-based chemotherapy have failed to demonstrate benefit in PDAC. To what extent this reflects the lack of dependence of PDAC on a supporting vasculature as well as VEGF as opposed to a lack of drug efficacy in shutting down angiogenesis in PDAC remains to be evaluated.

Beyond angiogenic targets additional novel strategies targeting the tumor microenvironment are underway, though generally at earlier stages of investigation. One exception that has already undergone extensive clinical testing is the MMP inhibitors (MMPIs, e.g., Marimastat and BAY 12-9566) which have also failed in Phase 3 trials. Again the reason for failure is unclear, but could be related to target redundancy as there are many closely related MMPs as discussed above, of which only a subset are inhibited by any one of the above-mentioned agents, or because activated MMPs may have complex pro-homeostatic as well as pro-tumorigenic roles that have both been altered [Overall and Kleifeld, 2006a].

Other approaches aim to disable PDAC-associated fibroblasts including a vaccine against fibroblast activation protein that seems

to improve the efficacy of chemotherapy by increasing intratumoral drug uptake [Loeffler et al., 2006]. The TGF-β signaling cascade, which acts in a context-specific manner, is being evaluated by a number of pharmaceutical companies [Arteaga, 2006]. Among investigational agents are receptor and ligand blocking antibodies, small molecules that inhibit TGF-β receptor catalytic activity, and an antisense oligodeoxynucleotide (AP 12009) that is designed to inhibit TGF-β2. Complicating the use of TGF-β pathway modulators is the unclear role of the pathway in fully established tumors. Though GEM studies have demonstrated TGF-β signaling constrains tumor progression at early stages how this pathway functions, and to what degree it is important to the maintenance of the tumor cell versus surrounding stroma is unclear. Furthermore, it is possible that the resulting inflammation and stromal reaction to PDAC could serve to restrain proliferation and growth, thus inhibiting these constituents through TGF-β blockade could enhance tumor growth.

Neutralization of inflammatory cells is being tested clinically with the COX-2 inhibitor (Celecoxib). COX-2 is expressed both by tumor cells and inflammatory cells, stimulating inflammatory pathways and angiogenesis. Sunitinib (Sutent, Pfizer) is a multitargeted RTK inhibitor which exhibits in vitro and in vivo activity against multiple targets including c-kit, VEGFR1-3, PDGFR α, PDGFR β, Flt-3, CSF-1R, ret, and potentially others. Sunitinib is presently being tested in combination with gemcitabine. Given the possible role of the PDGF pathway in PSC proliferation, success with this agent should further motivate a more careful evaluation of this compartment in the Sunitinib trials.

The failure of anti-angiogenic therapies, multiple MMPIs, and other agents in PDAC may speak to differences in the efficacy of therapies targeting the microenvironment in the primary site of disease versus metastatic lesions. PDAC is rarely surgically removed and is often fatal due to local spread and growth of the cancer around the pancreas and through the abdomen. Most other common malignancies are fatal due to metastatic disease—often involving the liver and lungs. It is possible that different local tumor environments—primary site in the pancreas versus liver versus lungs—each have a unique molecular dependence on certain growth and angiogenic factors. Thus, the success of any given therapy targeting a specific microenvironment molecule may be somewhat dependant on where the tumor resides. In PDAC, where local spread contributes significantly to the disease's mortality, understanding the cellular heterogeneity of the cancer stroma and key governing pathway seems crucial for meaningful clinical progress.

CHALLENGES AND OPPORTUNITIES IN PDAC STROMAL BIOLOGY

Recent years have witnessed numerous critical scientific advances in PDAC including a better definition of the histopathological and molecular evolution of the human disease, high-resolution oncogenomic analyses pointing to the existence of many novel oncogenes and tumor suppressor genes, and the development of genetically engineered mouse models linking signature mutations with specific aspects of disease pathogenesis. Remarkably, while progress in this area has been brisk, PDAC's most prominent feature—its extensive stroma—remains enigmatic and essential uncharted territory. This limited knowledge, coupled with the likelihood that stroma plays fundamental roles in tumor progression and therapeutic responsiveness, demands concerted and integrated efforts to dissect the molecular and biological evolution and characteristics of PDAC stroma and its functional relationship to cancer cell compartment. There have been many scientific advances in the understanding of PDAC that have occurred over the past decade. These include high-resolution oncogenomic analysis, pointing to the existence of many potentially novel oncogenes and tumor suppressor genes, global expression analysis, and the utilization of mouse genetics to define the individual roles of signature mutations in disease initiation and progression. The field appears poised to exploit many of the genomic technologies and model systems used to advance our understanding of the PDAC cancer cell in the area of stromal biology. This is a daunting challenge in light of stroma's many components and myriad heterotypic interactions among tumor fibroblasts/mesenchymal cells, blood vessels, innate and adaptive immunocytes, and the ECM.

An important initial task will be to precisely define the molecular, cellular, and extracellular

constituents of the PDAC microenvironment, and determine the origins and evolution of these components. Many basic questions remain, for instance, in the characterization of all the cellular and extracellular components. Even at the most basic level, the origin of mesenchymal cell populations, PSC-derived or otherwise, are as of yet undefined. How to approach these questions in an attempt to gain answers is complex and will likely require advances on multiple fronts involving 3D model systems, integrative and quantitative biological approaches, genome-wide analyses with emphasis on the epigenetic changes, among others.

Borrowing a page from cancer cell analysis, one can envision that utilizing expression profiling of morphologically distinct stromal constituents, such as differing populations of fibroblasts, could be an excellent starting point, although an important caveat is the assumption that we have the means of accurately identifying these constituents. Nevertheless, results from these studies could allow for the identification of signature expression patterns that may reveal useful identity markers as well as point to their cellular origins as cells tend to retain signatures reflecting their ontogeny. As a first step, differential expression analysis between PSCs and "traditional" pancreatic fibroblasts would be most productive in assessing the cellular relationship and allow for the identification of differing cell surface proteins which could ultimately result in reagents for immunohistochemistry as well as antibodies to prospectively isolate these different populations. Lastly, the identification of promoters that are transactivated in specific cell subtypes would generate useful tools for the directed expression of oncogenes, Cre recombinase or various toxins for selective ablation in mouse models. Such diphtheria toxin ablative models have lead to the understanding of the roles of multiple cell types in different organ systems [Buch et al., 2005] and could certainly prove useful in this setting to both understand the role of individual stromal cell types in both normal pancreatic development as well in neoplasia.

Another aspect that should be investigated is whether functional differences exist that are dependent on geographic regionalization, for example, activated fibroblastic cells at the invasive front versus, compared to cells surrounded by mature ECM in the tumor center. These are important questions that can only be answered once appropriate tools, such as cell-type specific markers, are identified.

As defining cell populations serves merely as an entry point to heterotypic interactions in PDAC, the ability to interrogate cancer cell interactions with individual stroma components, and vice versa, is of utmost importance (Fig. 2). Once these classes of cells can be isolated prospectively by markers, a variety of in vitro systems can then be utilized to the study the interactions between them and the cancer cells themselves. Co-culture experiments in 3-D matrix systems can allow for the study of this crosstalk. They will also allow for a detailed biochemical analysis of both the stroma and cancer cells to identify what particular signaling pathways are necessary for the various biological effects seen both in vivo and in vitro. Intricacies in these systems will need to be developed to study the role of direct contact between cancer cells and stroma as well as those effects mediated by soluble factors. Additionally, assays could be undertaken to assess whether alteration of epithelial cancer cell behavior is due to transient interactions with tumor stroma or relates to sustained interaction. The results from these studies and others will have direct impact on the utility and design types of therapies that involve targeting the stromal compartment. In addition to the above in vitro assays, the interactions of these various cell types/subtypes will ultimately require validation in genetically defined in vivo model systems. Subcutaneous xenograft transplants in the mouse using a mixture of cancer cells and fibroblasts have been shown to have good utility in studying tumor growth [Bachem et al., 2005]. Intrapancreatic (orthotopic) transplants should be used whenever feasible, for endogenous stromal constituents such as PSCs are undoubtedly necessary to model stromagenesis. Furthermore, efforts to perform such studies in mice with intact immune systems are imperative, as use of nude or SCID mice neglects this key dimension of host–tumor interactions. The availability of genetically engineered mouse models of PDAC makes the use of syngeneic tumor cell lines a more reasoned approach.

This review highlights the numerous similarities between PDAC stroma and CP stroma. Many of the cellular and extracellular components of microenvironment are similar: tissue damage, cytokine release, inflammation, angiogenesis, and activation of PSCs and connective

tissue fibroblasts. While these may reflect reactive processes of wound healing, it will be crucial to identify ways in which CP and PDAC stroma share similarities and differences in cellular composition and in biological functions. Expression profiling stromal elements and functional tumor-stroma recombination experiments are likely to be highly productive avenues of pursuit. Far from being an academic question, identification of a PDAC-specific stromal signature may one day lead to the development of diagnostic tests that would allow for screening of the disease at a much earlier stage.

Finally, comparative array-CGH analysis of human PDAC has allowed us to identify a wealth of candidate genes involved in PDAC formation [Aguirre et al., 2003]. In evaluating candidate oncogenes and tumor suppressor genes, particular emphasis should be placed on identifying those genes that may interact with the tumor microenvironment. Attention must not be placed solely on assays which emphasize cell autonomous biological properties, such as colony-forming assays, but should be placed with greater emphasize on cell non-autonomous assays. As these assays currently rely principally on in vivo techniques, such as tumor xenograft growth, greater emphasis must be placed on developing in vitro assays which will allow the assessment of paracrine cell interaction. Advances above will allow us to design more intelligent therapies that attack PDAC at the tumor-stroma interface.

In summary, pancreatic cancer remains a daunting scientific challenging and utmost crisis for the patient and health care provider. Advances in treatment of this disease have not come as rapidly as one would hope and early detection strategies as well as targeted therapy approaches have yet to yield much in the way of tangible results. Pancreatic cancer, because of its robust and tumor defining stromal reaction, is the ideal system to study the role of the tumor microenvironment in epithelial malignancies and whether such knowledge can be translated into novel effective approaches for early diagnosis and therapy.

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