Review

Tumor-stroma interactions in pancreatic ductal adenocarcinoma

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Abstract

The host stromal response to an invasive epithelial carcinoma is frequently called a desmoplastic reaction (DR) and is a universal feature of pancreatic ductal adenocarcinoma (PDA). This DR is characterized by a complex interplay between the normal host epithelial cells, invading tumor cells, stromal fibroblasts, inflammatory cells, proliferating endothelial cells, an altered extracellular matrix, and growth factors activating oncogenic signaling pathways by autocrine and paracrine mechanisms. Hence, the tumor microenvironment is a dynamic process promoting tumor growth and invasion through mechanisms likely to include anoikis resistance, genomic instability, and drug resistance. Cell coculture models, murine models (xenograft and genetic), and gene expression profiling studies on human PDA biopsies have identified several key molecules, such as collagen type I, fibronectin, laminin, matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of MMP), growth factors (transforming growth factor β, platelet-derived growth factor, connective tissue growth factor, and hepatocyte growth factor), chemokines, and integrins as constituents of the DR. Despite these findings, it is unclear which molecular-cellular events initiate and drive desmoplasia in PDA. Accumulating evidence indicates that pancreatic stellate cells when activated switch to a myofibroblast phenotype that produces components of the extracellular matrix, MMPs, and tissue inhibitors of MMPs by activating the mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) pathway. Based on current evidence, several therapeutic strategies are been evaluated on identified potential therapeutic targets. This review summarizes our current understanding of the mechanisms that potentially drive the DR in PDA and future possibilities for therapeutic targeting of this critical process. [Mol Cancer Ther 2007;6(4):1186–97]

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Introduction

Tumors are complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda (1). In this prevailing model, a three-dimensional structure supports epithelial carcinoma cells through an altered extracellular matrix (ECM), maintained by diffusible paracrine growth factors and cytokines, tumor-associated vasculature, inflammatory cells, and stromal fibroblasts. However, the emerging model indicates a more important role for stromal fibroblasts in carcinogenesis than appreciated previously. New evidence indicates that mutations arising in stromal fibroblasts and consequent manifestation of paracrine factors promote growth and proliferation of carcinoma cells (2). Therefore, the paradigm that stromal fibroblasts are mere bystanders in the oncogenic process is changing and a more active role is warranted (Fig. 1).

A hallmark in pancreatic ductal adenocarcinoma (PDA) is the presence of 'desmoplasia,' which is defined as proliferation of fibrotic tissue with an altered ECM conducive to tumor growth and metastasis. Histopathologic analyses of human PDA compared with normal pancreas depict dense collagen (types I and III) bundles associated with fibroblasts with loss of basement membrane integrity and invasion of malignant cells into the interstitial matrix with exposure to collagens (Fig. 2). This desmoplastic reaction (DR) is associated with an abnormal vasculature with numerous circuitous small leaky blood vessels and capillaries (3). The abundant connective tissue is due to manifestation of growth factor production, such as transforming growth factor β (TGFβ), by the tumor microenvironment (TME). This in turn activates autocrine and paracrine oncogenic signaling pathways leading to a growth advantage to proliferating tumor tissue. In addition, several recent studies have added another layer of complexity by showing that the TME can drive oncogenesis via matrix metalloproteinase (MMP)-3 (activates Rac1b) or matriptase (activates phosphatidylinositol 3-kinase/Akt; refs. 4, 5) or integrin (activates Rho and extracellular signal-regulated kinase) through a rigid ECM (6). Thus, a paradigm shift is in progress where aberrant signals from the extracellular compartment can promote the initiation of oncogenesis in the context of normal epithelial physiology (Fig. 1).

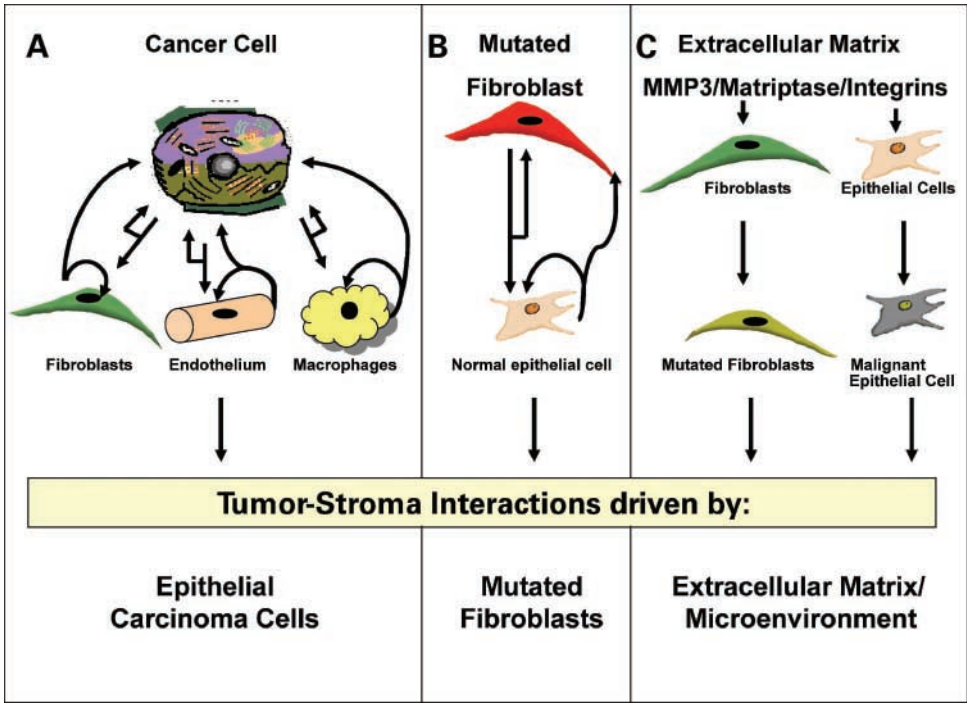

Figure 1. Models of tumor-stroma interactions driven by the (**A**) mutated epithelial cells (prevailing model), (**B**) mutated fibroblasts (emerging model), and (**C**) altered ECM/TME (new model). Matriptase, a type II transmembrane trypsin-like serine protease, is expressed by epithelial cells and is overexpressed in a variety of human cancers, including PDA. This protease has been implicated in facilitating cellular invasiveness and activating oncogenic (phosphatidylinositol 3-kinase/Akt) pathways. The prosurvival effects of the ECM proteins laminin and fibronectin are mediated through their integrin receptors, which are expressed by the tumor cells. *Arrows,* factors secreted by the respective cells that act in both an autocrine and a paracrine manner.

Cell Culture Models of Tumor-Stroma Interactions

Tissue coculture experiments have shown that factors derived from tumor fibroblasts (7, 8) or senescent fibroblasts (9) contribute to the transformation of immortalized epithelia. In contrast, virus-transformed epithelial cells (e.g., submandibular glands transformed by polyoma virus) only grow in culture in the presence of normal embryonic mesenchyme (10), indicating that both the transformed epithelia and the tumor fibroblasts are essential for growth, proliferation, angiogenesis, and invasion of carcinomas. However, their relative contribution varies and is tumor type specific. A pancreas cancer–specific study using Panc-1 cells grown in coculture with normal skin fibroblasts in Transwell plates showed induction of desmoplasia (collagens I and III and fibronectin) with an associated increased expression of TGFβ1 and fibroblast growth factor (FGF)-2. Panc-1 cells transfected with TGFβ1 (Panc-1/TGFβ1 cell) grown in culture induced the production of collagen I and platelet-derived growth factor (PDGF)-AA whereas grown in coculture with normal skin fibroblasts led to the proliferation of both cell types and the production of collagen I and connective tissue growth factor (CTGF) by skin fibroblasts (Northern analysis). Moreover, the level of tyrosine phosphorylation was severalfold higher in fibroblasts cocultured in the presence of Panc-1/TGFβ1 cells, which seems to correlate with an associated increased phosphorylation of extracellular signal-regulated kinase 1/2 and 3 (11). The TGFβ signaling pathway is complex in that it is tumor suppressive to normal epithelial cells but can promote invasion and metastasis during later stages of many cancers, including PDA progression. This is due to a loss of growth-inhibitory response to TGFβ as a result of mutations (e.g., loss of SMAD4) in the components of its signaling pathway and/or overexpression of TGFβ. The increased TGFβ in the TME seems to drive autocrine-paracrine signaling, epithelial-stromal interactions, inflammation, neoangiogenesis, and immune evasion leading to an insidious progression of epithelial carcinomas (12).

Several lines of evidence show that sublethal damage to fibroblasts with radiation treatment and subsequent

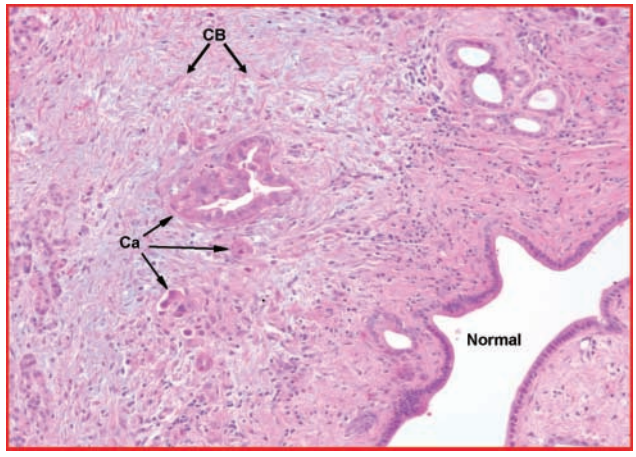

Figure 2. Histopathologic analyses of the tumor-stroma interactions in human PDA depict dense collagen bundles (*CB*) associated with fibroblasts with loss of basement membrane integrity and invasion of malignant cells (*Ca*) into the interstitial matrix with exposure to collagens. This DR is associated with an abnormal vasculature. Normal pancreatic ductal epithelium (*Normal*).

growth in the presence of nontransformed (mammary epithelial cells; ref. 13) or tumor (pancreatic cancer cells) cells (14) leads to breast cancer development or to a more aggressive, invasive pancreatic cancer, respectively. Therefore, radiation-induced deleterious mutations within stromal fibroblasts seem to release factors that promote enhanced tumor growth. Pancreatic cancer cells (Suit-2 or Capan-1) cocultured in the presence of irradiated normal human lung fibroblasts (MRC5) promoted the invasiveness of nonirradiated pancreatic cancer cells. In addition, when pancreatic cancer cells were also irradiated and cocultured with irradiated fibroblasts, there was further enhancement of invasiveness. Several growth factors [hepatocyte growth factor (HGF), basic FGF, TGFβ1, vascular endothelial growth factor (VEGF), and epidermal growth factor] and matrix modifying proteins (MMP-2, MMP-9, and urokinase-type plasminogen activator) were assayed in the culture medium of postirradiated fibroblasts. Only basic FGF was modestly elevated compared with nonradiated fibroblast. When Suit-2 cells were exposed to supernatant from irradiated fibroblasts, the mitogen-activated protein kinase pathway was activated in a biphasic pattern, which seemed to correlate with c-Met expression and activation via an autocrine mechanism, which could explain the observed invasive potential, although this is not conclusive. NK4 (a 447-amino acid protein), which is a specific antagonist of HGF, inhibits invasiveness at concentrations of 5 to 10 μg/mL with decreased Met phosphorylation, implying the HGF-c-Met axis is promoting a DR.

More recently, pancreatic stellate cells (PSC), which are of mesenchymal origin, have been identified in normal pancreas (15, 16), chronic pancreatitis (16), and PDA (17). PSCs express intermediate filament protein desmin and glial fibrillary acidic protein and, together with the presence of intracellular fat droplets, serve to discriminate PSCs from normal fibroblasts (15, 16). PSCs have the ability to transdifferentiate from a 'quiescent' retinoid/lipid storing phenotype in the normal pancreas to an 'activated' α-smooth muscle actin producing myofibroblastic phenotype. In culture, primary PSCs continually change from a quiescent to an activated phenotype and, during this change, they pass through a series of temporal states of transformation (18). Activators of PSCs *in vivo* include cytokines [interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α], growth factors (PDGF and TGFβ), and reactive oxygen species released by damaged inflammatory cells recruited in response to injury to the pancreas. Activated PSCs, in turn, can produce autocrine factors, such as PDGF, TGFβ, cytokines (IL-1, IL-6, and tumor necrosis factor–related apoptosis-inducing ligand), and proinflammatory molecules [cyclooxygenase-2 (COX-2)], which may potentiate an activated phenotype. Further, activin-A, a member of the TGFβ family, also functions in an autocrine manner to increase collagen I secretion and MMPs tissue inhibitors of MMPs and augment TGFβ expression and secretion (19, 20), which regulate ECM turnover. Human PDA tumor biopsy samples have an abundance of PSCs intermingled in fibrotic collagen bands and are in close proximity to the malignant epithelial cells compared with normal pancreas tissue (21). However, chronic activation of PSCs leading to the fibrosis associated with the DR in PDA is poorly understood. However, a better understanding of the biology of PSCs offers potential therapeutic targets for the treatment of the DR in PDA. Several studies have identified major signaling pathways involved in the regulation of PSC function, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, Rho kinase, Janus-activated kinase/signal transducer and activator of transcription, activator protein-1, nuclear factor-κB, and TGFβ/SMAD. In addition, studies using peroxisome proliferator-activated receptor γ ligands implicate this pathway in down-regulation of PSC activation (20). In summary, these signaling pathways are potential therapeutic targets for the modulation of PSC function in PDA.

A tissue culture model showed a >5-fold increase in PSC proliferation ([^{3}H]thymidine incorporation) when exposed to conditioned medium from pancreatic cancer cell lines (Panc-1, Mia PaCa-2, and AsPC-1). However, there was only a modest increase in collagen secretion but a large increase in MMP-2 and tissue inhibitors of MMP by PSCs in the presence of conditioned medium. Moreover, the addition of TGFβ1 enhanced collagen synthesis by PSCs and not in pancreatic cancer cell lines (21). These results indicate that other autocrine and paracrine factors play an important role in generating a DR. An *in vitro* DR, in the form of clonogenic assays, which used growing pancreatic cancer cells on type I collagen, had a higher proliferative capacity due to a rapid transition through S phase and/or G_2/M with an associated morphologic change that seems to provide a protective effect to 5-fluorouracil chemotherapy by overexpressing antiapoptotic protein Mcl-1 (21). However, this observation is not universal to all pancreatic cancer cell lines (except AsPC-1 cells) evaluated and caution should be exercised when interpreting and extrapolating these observations to PDA. The combined use of cultured primary PSCs and human pancreatic cancer cells, coupled with the use of coculture systems, are likely to provide additional mechanistic insights into the biology of the DR in PDA.

Because some members of the intermediate filaments (intracellular matrix), the ECM (Table 1) and growth factors/chemokines (Table 2), are key players in the DR, matrix proteins, such as collagen and fibronectin, are likely to interact with cell surface integrin receptors to provide survival signals to PSCs and PDA cells. Support for this concept comes from studies on Mia PaCa-2 cells cultured on fibronectin that result in a dose-dependent increase in IL-8 (CXC cytokine family member) secretion, a chemokine thought to be important in the progression of PDA (22). This dose-dependent increase was RGD dependent, implying integrin binding and is accompanied by cell spreading and proliferation. Disruption of the cytoskeleton with cytochalasin D resulted in a large increase in IL-8 secretion, which was reduced by fibronectin. However, this effect could not be accounted for by anti-integrin antibodies inhibiting integrins $\alpha_5\beta_1$ or $\alpha_v\beta_5$ alone but when inhibited

Table 1. Intracellular matrix and extracellular secreted and matrix components of prognostic significance in the DR of PDA

ECM protein	Functional role	Reference
Collagens I, III, IV	Angiogenesis, invasion, and metastasis	(11, 41, 69)
Decorin	Matrix assembly	(41)
Versican	Matrix remodeling	(41)
TIMP-1	Matrix degradation and cell proliferation	(41)
Fascin*	Actin bundling motility protein	(44, 45)
Fibronectin	Angiogenesis, invasion, and metastasis	(68)
Osteonectin/SPARC	Regulate cell growth and shape	(44, 46)
MMP-2, 11	Binds integrin $\alpha_V\beta_5$ and ECM degradation	(44)
Apolipoprotein C-1, D	Unknown in ECM biology	(44)
α2-macroglobulin	Protease inhibitor and cytokine transporter	(44)
α-Smooth muscle actin*	Cell motility	(69)
Desmin*	Intermediate filament	(69)
Laminin	Binds integrins and angiogenesis	(69)
Biglycan	Binds collagen and transfers TGFβ1	(70)

Abbreviation: TIMP, tissue inhibitors of MMP.
*Intracellular matrix.

in combination completely abolished the response to fibronectin. These results suggest a latent stimulatory effect of α_v or $\alpha_5\beta_5$ integrin on IL-8 secretion and provide evidence that integrin cross-talk may limit the induction of IL-8 secretion by fibronectin (23). However, the magnitude of IL-8 secretion cannot be explained solely by a fibronectin-integrin–driven process, and other signaling pathways, such as TGFβ and IL-10, are implicated (24).

Global gene expression profiling (GEP) using DNA microarrays on pancreatic cancer cells (CFPAC1) and primary human pancreatic stromal fibroblasts induced by coculture (48 h) identified multiple genes to be differentially expressed in pancreatic cancer cells and in fibroblasts as a consequence of their mutual interactions. Analysis of the GEP patterns in CFPAC1 (monoculture and coculture) and fibroblasts (monoculture and coculture) identified 718 transcripts that had the greatest variation among the four samples. Hierarchical cluster analysis with these 718 transcripts identified two major clusters that discriminated between the different cellular origins (epithelial versus stroma). Scatter plot and fold change analyses between monoculture and coculture conditions revealed only a small fraction of transcripts that displayed differential expression and these findings imply that genes regulated through tumor-stroma interactions represent only a small percentage of the total transcripts. This small subset of genes could be sufficient for the phenotypic changes observed. Of the 18,462 transcripts analyzed, only 55 (0.30%) were expressed at levels >3-fold in coculture than in monoculture; conversely, only 88 (0.48%) transcripts were expressed at levels >3-fold down-regulated in coculture. A subset of five overexpressed genes validated by reverse transcription-PCR were *COX-2, hyaluronan synthase 2* (*HAS2*), *MMP-1*, and *trefoil factor 1* (*TFF1*) and down-regulation of gravin. These genes together may explain increased propensity for tumor invasion by promoting cell motility (*TFF1, HAS2*, and *MMP-1*) in cell culture systems but need validation in mouse models and in human PDA. Genes differentially regulated in fibroblasts by coculture compared with monoculture showed 43 (0.23%) transcripts to be >3-fold overexpressed in coculture, whereas 31 (0.17%) were >3-fold down-regulated.

Table 2. Growth factors elaborated by fibroblasts at the tumor-stroma interface

Growth factor	Expressing cells	Responding cells	Functional role
TGFβ-1,2,3	Fibroblasts	Epithelia, fibroblasts	+Proliferation +Morphogenic
FGF-2	Fibroblasts	Epithelia	+Proliferation +Transformation
FGF-7/KGF	Fibroblasts	Epithelia	+Proliferation +Morphogenic
FGF-10	Fibroblasts	Epithelia	+Proliferation
HGF	Fibroblasts	Epithelia	+Proliferation +Transformation +Morphogenic
IGF-1,2	Fibroblasts	Epithelia	+Proliferation
CXCL12	Fibroblasts	Epithelia	+Proliferation +Transformation
Wnt-1,3	Fibroblasts	Epithelia	+Proliferation +Transformation
IL-6, LIF, oncostatin M	Fibroblasts	Epithelia	+Proliferation +Transformation
NGF	Fibroblasts	Epithelia	+Transformation

Abbreviations: IGF, insulin-like growth factor; KGF, keratinocyte growth factor; NGF, nerve growth factor; LIF, leukemia inhibitory factor.

Reverse transcription-PCR validated five genes identified as up-regulated by coculture, which include *annexin 14* (*ANX14/ANXA10*), *monocyte chemoattractant protein-1* (*MCP-1*), *IL-8, growth-regulated oncogene 1* (*GRO1*), and *COX-2*. Of interest are the overexpressed genes of members of the CXC/CC chemokine subfamilies, including *MCP-1* (CCL2), *IL-8* (CXCL8), *GRO1* (CXCL1), and *GRO2* (CXCL2), which may play pertinent roles in generating a DR with a phenotype associated with tumor invasion, metastasis, and angiogenesis (25). This study is tantalizing and provocative but firm conclusions cannot be drawn until further extensive studies are conducted. For example, this study does not mention some of the more pertinent features of tumor-stroma biology, such as overexpression of collagen types, fibronectin, or TGFβ1.

Mouse Models of Desmoplasia

Several growth factor and chemokine families are implicated as autocrine and paracrine mediators of tumor-stroma interactions inherent in the carcinogenic process (Table 2). Mouse xenograft models of PDA provide an excellent system to evaluate the relative importance of these growth factors and chemokines. Most of these factors are activators of the carcinogenic process; however, the TGFβ family is different in that TGFβ initially acts as a growth inhibitor and therefore a tumor suppressor. However, loss or attenuation of this pathway enhances carcinogenesis by increased expression of TGFβ by an autocrine mechanism (26).

Xenograft Models. The role of TGFβ in the DR is supported by a study that showed that TGFβ1-transfected Panc-1 cells induced a rich stroma after orthotopic transplantation into a nude mouse pancreas. The transfer of a single growth factor, TGFβ1, conveys the ability to induce a fibroblast response similar to that seen in DR in human PDA. This effect cannot only be attributed to TGFβ1 but also results from the up-regulation of several other factors, including collagen type I, fibronectin, CTGF, and PDGF-AA, evident both on the tumor margin toward the normal mouse pancreas as well as within the tumor (9, 12). However, this study did not assess the aggressiveness and/or invasiveness of the TGFβ1-transfected Panc-1 compared with the mock-transfected cell line in relation to overall survival.

Apart from growth factors, ECM modifying proteins have been evaluated in promoting a DR. SERPINE2, also known as protease nexin I is overexpressed in >80% of human PDA biopsy samples and enhances the local invasiveness of the s.c. Suit-2 (S2-007) pancreatic cancer xenograft tumor, accompanied by a large increase in ECM production in the invasive tumors. ECM deposits were positive for type I collagen, fibronectin, and laminin, resembling the DR commonly observed in PDA. Moreover, pancreatic cancer cells in invasive SERPINE2-expressing tumors tended to adapt a spindle-shaped morphology and strongly expressed the mesenchymal intermediate filament marker vimentin. However, vascularization estimated by immunostaining with CD31 was not altered by overexpression of SERPINE2 (27). Hence, given the complexity of the DR, the overexpression of SERPINE2 by itself is insufficient to increase the overall metastatic phenotype because it does not promote anoikis resistance. Accumulating evidence suggests that the interplay of extracellular proteases and their inhibitors in invasion and metastasis is complex and extends beyond their roles as protease inhibitors. For example, the closely related SERPINE1 (plasminogen activator inhibitor type 1), an inhibitor of urokinase-type plasminogen activator and tissue plasminogen activator, when elevated is a poor prognostic factor in several solid tumors, including PDA, indicating that it has other roles in cancer progression that as a mere matrix protease inhibitor (28). In Table 1, some of the ECM components that may be of clinical prognostic significance in PDA are listed and requires further validation.

One of the adjuvant treatment modalities used for PDA is radiation or chemoradiation and despite these therapies overall survival is still poor. This is most likely due to the DR that provides a milieu for resistance. The treatment modality of adjuvant radiation or chemoradiation is inferior to chemotherapy in a randomized clinical trial for the treatment of PDA (29), implying that added radiation may facilitate mutations in stromal fibroblasts (PSCs), which in turn create a milieu for resistance. In fact, patients treated with radiation-based therapy did worse than those who only received chemotherapy. This observation correlates in a mouse model of pancreatic cancer where irradiated fibroblasts and nonirradiated pancreatic cancer cells mixed well, implanted into the pancreas of nude mice, grown for 7 days, and showed a high invasive potential compared with nonirradiated fibroblasts. This invasive pancreatic cancer phenotype enhanced the expression of phosphorylated c-Met (14), suggesting that radiation therapy may enhance the DR. Mouse xenograft models provide useful systems to evaluate the DR in human PDA but are still in its infancy and require more extensive evaluations and corroboration using human biopsy samples.

Genetic Models. A genetic progression model of PDA akin to that of colorectal cancer has emerged, which is predicted to recapitulate the full spectrum human PDA (30). Activating mutations in the KRAS proto-oncogene present in over >90% in human invasive PDA is hypothesized to represent an initiating event. Endogenous mutant KRAS (G12D) expression in the progenitor cells of the pancreas induces the entire progression of PDA from preinvasive (PanIN-1 to PanIN-3) to invasive and metastatic disease. In mice that lived longer, the pancreas had extensive ductal lesions and the acinar parenchyma was replaced by an intense DR composed of collagen, fibroblasts, and inflammatory cells reminiscent of human PDA (31). However, a cellular and molecular analysis of the DR in the invasive and metastatic pancreas cancer was not described. Instead, a molecular analysis of PanINs showed that normally quiescent oncogenic signaling pathways are activated, which include the *Notch* signaling pathway (manifested by *Hes1* transcription factor expression) that determines cell fate in embryogenesis, *COX-2*, a component of the prostaglandin pathway involved in inflammation,

and *MMP-7* that is involved in ECM modeling, each of which has been known to be inappropriately overexpressed in human PDA specimens (31). How these pathways alone or more likely in combination may promote or initiate a DR is yet to be gleaned. However, clinical trials thus far targeting RAS (via inhibition of farnesyl transferase) or MMPs or COX-2 have been negative (32), indicating that invasive and metastatic PDA has evolved with the acquisition of further molecular and cellular aberrations manifesting in a chemotherapy resistant DR.

A second mouse model used mutations in mice engineered to sustain pancreas-specific Cre-mediated activation of mutant KRAS (G12D) and deletion of a conditional CDKN2/Ink-4a/Arf tumor suppressor allele. This led to the early appearance of PanIN lesions and rapid progression to highly invasive and metastatic PDA. The tumors appear to bear striking resemblance to human PDA with a proliferative stromal component and ductal lesions with a propensity to advance to a poorly differentiated state. However, lung and liver metastases were rarely observed in these mice (33), indicating that directed activation of an oncogene and loss of a tumor suppressor in a genetic mouse model of PDA may not be a true reflection of human PDA, which may be due to species-specific gene expression and differential hard wiring of signaling pathways.

Several other tumor suppressors are also functionally lost in human PDA, which include TP53 (75%), SMAD4 (DPC4 or MADH4; 55%), and BRCA2 (<10%). The two genetic models described above do not manifest mutations in any of the other tumor suppressor gene pathways and therefore raises questions as to whether the other tumor suppressor pathways alter PDA progression and/or each pathway constitutes a distinct genetic route to PDA with a unique phenotype. Hence, a third mouse model was developed to evaluate tumor progression where mutant Trp53 (R172H, Li-Fraumeni human orthologue) endogenously expressed in the context of concomitant KRAS (G12D) expression. These mice developed invasive and widely metastatic carcinoma with a high degree of genomic instability manifested by nonreciprocal translocations without telomere erosion leading to chromosomal instability. No mutations were found in other tumor suppressors normally found in human PDA. A molecular analysis focused on ErbB1 (HER1), ErbB2 (HER2), E-cadherin, and Sonic hedgehog was done to glean the phenotypes detected. ErbB1 showed considerable heterogeneity in expression in invasive and metastatic lesions compared with PanIN lesions, ErbB2 was robustly overexpressed in PanIN lesions, and both epidermal growth factor receptors were absent in metastatic lesions. However, the expression of Sonic hedgehog and E-cadherin was present in both preinvasive and invasive lesions that were moderately well differentiated but not in poorly differentiated lesions (34). This study did not analyze tumor-stroma interactions and how mutant Trp53-driven genomic instability in the context of mutant RAS correlates with aberrant expression of cell cycle mitotic protein kinases, such as Aurora (35), Polo-like kinase 1 (36), and Nek2 (37). In human PDA, Aurora A and Aurora B are overexpressed and have been identified as markers of genomic instability. Aurora A regulates centrosome maturation and spindle assembly, whereas Aurora B (and C), a chromosome passenger protein, regulates chromosome orientation on the spindle and cytokinesis. All three Auroras when overexpressed are associated with distinct polyploid phenotypes containing multiple centrosomes and in an appropriate genetic background function as oncogenes by overriding cell cycle checkpoints leading to errors in mitosis (aneuploidy) with subsequent chromosomal instability (38).

To explore early pancreatic cancer initiation through the TGFβ pathway, Smad7, a specific inhibitor of TGFβ signaling, was expressed under the elastase I promoter in a pancreas-specific expression in a transgenic mouse model. At age 6 months, most of the transgenic mice developed PanIN, which were accompanied by increased proliferation of the ductal cells and acinar cells and an increased fibrosis around the ductal lesions (39). This study shows that *in vivo* inactivation of TGFβ signaling pathway leads to the development of premalignant pancreatic lesions and provides a promising animal model for molecular dissection of this pathway and a model for early therapeutic intervention.

Human GEP to Dissect Tumor Desmoplasia

Although mouse models (xenograft and genetic) can provide insights into the tumor-stroma interactions in the DR, it is important that these interactions be sought in human PDA patient biopsy samples. To tackle the DR therapeutically by either supporting or suppressing its development, it is essential to study the etiology and to attribute this feature either to the tumor cells or to the host or both (40). Large-scale GEP studies have become a useful method for characterizing pathologic processes when compared with normal physiology. Human PDA is an excellent but problematic model system to evaluate GEP due to the dense DR with tumor cells often representing a minor population. Fine-needle aspiration of six patients with PDA was evaluated on a cDNA array of 588 cancer-related genes (Human Cancer Atlas cDNA Expression Array Membranes) enriched for tumor cells or bulk tumor tissue consisting of stromal elements. The bulk carcinomas contained 30% to 40% of tumor cells with differentially expressed genes common to the DR, such as collagens I and III, decorin, and versican. These molecules are implicated in the remodeling and maintenance of the ECM during inflammation, fibrosis, and proliferation. Two other genes differentially overexpressed in the bulk tumor were *Rac-1* and *CD9* (41). Rac-1 is overexpressed in ~70% tumor samples and is implicated in regulating cell morphology, motility, and cytokinesis by reorganizing the actin cytoskeleton and promoting cross-talk with cadherin-dependent intercellular adhesion (42). Rac-1 acts downstream of Ras and because mutated activated Ras is an initiating event in PDA, it may be critical to Ras-induced transformation and may be a likely promoter of the DR. It has been shown that the TME drives oncogenesis via MMP-3 by activating Rac1b (6) and this evidence together supports a signaling

network that seems to provide a mechanistic basis for the DR in PDA. CD9, a tetraspanin, forms complexes with integrins, other tetraspanins, HLA antigens, and associates with small GTP-binding proteins (43), such as Rac-1, and may modulate cell-cell and cell-ECM interactions in PDA.

To better characterize the GEP of invasive PDA and their associated DR, *in situ* hybridization was done on six patient biopsy samples to characterize the expression of 12 genes identified by serial analysis of gene expression as highly expressed in invasive pancreatic cancer tissues but not in pancreatic cancer cell lines. *In situ* hybridization showed that eight genes were expressed within the stromal and/or angioendothelial cells of the DR compared with the invasive tumor and four of these genes were specifically expressed by the stromal cells (apolipoprotein C-1 and D, MMP-2, and α2-macroglobulin) immediately adjacent to the invasive neoplastic epithelium, suggesting regional differences in gene expression within the host DR. In contrast, four genes were specifically expressed by the invasive neoplastic epithelium (*CTGF, β-catenin, ICAM-1*, and *MMP-14*), indicating important differences between *in vivo* and *in vitro* gene expression of human epithelial neoplasms (44). Because α2-macroglobulin receptor is expressed on the neoplastic epithelium, whereas α2-macroglobulin is expressed in the juxtatumoral stroma, it is conceivable that the latter acts as a growth factor. Moreover, the α2-macroglobulin receptor is also the receptor for CTGF, which could also stimulate the neoplastic epithelium in an autocrine manner. MMP-2 present in the stroma is a substrate of the membrane-bound MMP-14 metalloproteinase and may cleave pro-MMP-2 at sites within the stroma to enhance the invasive process. A more extensive analysis was done by the same research group, which included 17 (45) and 26 (46) resected PDA patient samples. It was shown that 79 genes were significantly overexpressed in pancreas cancer compared with normal pancreas (45). Several of these genes are associated with several cellular functions, including cell-cell, cell-ECM, cytoskeletal remodeling, proteolytic activity, and calcium homeostasis. A cluster of genes seemed to be related to the DR, which included the well-characterized collagen type I, MMPs, tissue inhibitors of MMPs, apolipoprotein C-I and C-II, and the less well-characterized markers, such as hevin, osteonectin, and biglycan. Further, 217 genes were shown to be overexpressed of which 75 have been reported previously, whereas 142 genes are reported as novel (46). Among the most differentially expressed genes are *mesothelin, Muc* (4, 5A/C), *kallikrein 10, transglutaminase 2, fascin, TMPRSS3*, and *strattifin*. However, when an analysis of genes identified by both serial analysis of gene expression and Affymetrix (Santa Clara, CA) U133 arrays were done, only two genes were identified as significant: *CEACAM6* and tumor-associated calcium signal transducer 2. Both these molecules are implicated in adhesion, invasion, and metastasis and are under active investigation. However, despite these extensive GEP studies, the molecular mechanisms leading to PDA-associated DR profile are yet to be elucidated.

Therapeutic Strategies

Gemcitabine and more recently gemcitabine plus Tarceva (an epidermal growth factor receptor tyrosine kinase inhibitor) are the only approved therapies for unresectable and metastatic PDA. However, none of therapies are curative and at best provide only 3 to 4 months of survival advantage over supportive care (47). The altered stromal ECM proteins with their cognate integrin receptors are implied in mechanisms of acquired resistance to chemotherapy. An *in vitro* model of pancreatic cancer cell lines with different grades (Mia PaCa-2, grade 3; Panc-1, grade 2; and Capan-1, grade 1) of differentiation cultured in the presence of collagen (I or IV) or fibronectin or laminin showed that fibronectin promoted Mia PaCa-2 cells to proliferate, whereas collagens I and IV and laminin were suppressive. In contrast, Panc-1 and Capan-1 cells were proliferative in the presence of collagens I and IV and fibronectin but not in laminin. When these cells were grown on any of the above ECM proteins in the absence or presence of chemotherapy agents (cisplatin, doxorubicin, gemcitabine, and 5-fluorouracil) for up to 72 h, Mia PaCa-2 cells showed increased chemoresistance to all of the chemotherapy agents except gemcitabine. Capan-1 cells were also chemosensitive to gemcitabine but this effect was not observed with Panc-1 cells. These results suggest that grade of differentiation of the tumor may determine matrix-driven sensitivity to chemotherapy (47) and inhibiting ECM-integrin function in combination with chemotherapy may be a potential therapeutic intervention that could specifically target the DR. One such therapeutic is the monoclonal antibody targeting $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrin (Vitaxin). However, this study lacks direct evidence of how PSCs or matrix fibroblasts influence chemotherapy effects on tumor cells and thus requires further evaluation of this phenomenon.

A Transwell coculture model using the chemosensitive human pancreas cancer cell lines T3M4 and PT45-P1 cultured in the presence of murine pancreatic fibroblasts showed that both tumor cell lines became resistant to etoposide compared with cells grown under standard conditions. In this model system, it was shown that the murine fibroblasts released nitric oxide that induced the secretion of IL-1β by T3M4 and PT45-P1 cells. This effect could be inhibited by IL-1β receptor blockade, abolishing etoposide resistance developed during cocultivation. Incubation of tumor cells with the nitric oxide donor *S*-nitroso-*N*-acetyl-D, L-penicillamine up-regulated IL-1β secretion and conferred resistance to etoposide-induced apoptosis. This effect was abolished when an inhibitor specific to the inducible nitric oxide synthase, aminoguanidium, was added during coculture. Immunohistochemical studies (IHC) done on ~20 to 22 human PDA biopsy samples confirmed ~60% expression of IL-1β in tumor cells and >70% expression of inducible nitric oxide synthase in stromal cells (48). The main problems with this study are that the fibroblasts are of murine origin, etoposide is not an approved therapy for PDA, IHC were not done for the presence of IL-1β receptor, and commonly used pancreatic

cancer cell lines, such as Mia PaCa-2 and Panc-1, were not fully evaluated. Despite the IHC on patient samples, firm conclusions cannot be drawn as to the causality of the effects observed.

In PDA, the TGFβ/SMAD signaling pathway is implicated in invasive tumor progression and associated poor prognosis. The MADH4 tumor suppressor on chromosome 18 undergoes loss of heterozygosity in >90% PDA patients (49) and, in >50% cases, it is biallelically inactivated by homozygous deletion or missense or nonsense mutations of the second allele (50). TGFβ is generally activated in response to tissue injury, which induces an epithelial-to-mesenchymal transdifferentiation and produces ECM components leading to a fibrotic scar (51) that is dependent on $\alpha_v\beta_6$ integrin-dependent mechanism (52). This TGFβ-driven injury response in human cancers and particularly in PDA may contribute to the invasive and metastatic potential of tumors. Therefore, targeting of the TGFβ signaling pathway would be a rational therapeutic approach in PDA (53, 54). The treatment of several pancreatic cancer cell lines with intact SMAD4 (Mia PaCa-2 and Panc-1) or absent SMAD4 (BxPC-3, CFPAC-1, CaPan-2, AsPC-1, and Hs766T) with a specific TGFβ receptor (TGFβR) I serine/threonine kinase inhibitor SD-093 had no effect on cell growth or apoptosis but inhibited BxPC-3 cell motility and invasiveness by 50% but without any effect on Panc-1 cell motility. It seems that BxPC-3 cell motility and invasiveness is in part mediated by the TGFβRI and is independent of SMAD4 but dependent on SMAD2 and SMAD3. Coculture studies of BxPC-3 with TMLC cells (mink lung epithelial cells transfected with a luciferase promoter driven by the plasminogen activator inhibitor type 1 promoter) showed that TGFβ secreted by BxPC-3 cells resulted in a dose-dependent increase of luciferase activity in TMLC cells and treatment with SD-093 inhibited this activity. Because $\alpha_v\beta_6$ integrin is involved in TGFβ-driven signaling, inhibition with a neutralizing antibody to $\alpha_v\beta_6$ blocks activation in TMLC cells (55). However, this study did not evaluate the effect BxPC-3 cells (or other pancreas cancer cell lines, such as, Mia PaCa-2 or Panc-1) on stromal fibroblasts and vice versa in coculture for TGFβ-driven DR or the efficacy of SD-093 in a relevant coculture system or in a mouse xenograft model of PDA. Therefore, it is not possible to make firm conclusions based on the above studies. The authors believe that inhibition of the TGFβRI in an appropriate murine model of PDA would lead to slowing of tumor growth by interfering with tumor-stroma interactions and will likely add or synergize with gemcitabine therapy.

Overexpression and activation of c-Met is a common event in human epithelial carcinomas. Moreover, the irradiation of stromal fibroblasts seems to activate c-Met on pancreas cancer cells (14). These observations predict that inhibiting the activation of c-Met may be a rational therapeutic approach. A soluble c-Met receptor (decoy Met) does interfere with HGF binding and inhibits c-Met activation by homodimerization. Lentiviral vector-based delivery of local or systemic decoy c-Met in mice inhibited tumor cell proliferation and survival, impaired tumor angiogenesis by preventing host vessel arborization, inhibited the formation of spontaneous metastases, and synergized with radiotherapy in inducing tumor regression without affecting housekeeping functions (56). Therefore, the targeting HGF-c-Met axis in pancreatic cancer and its microenvironment may be an effective therapy. A humanized monoclonal antibody to HGF is currently in clinical trials and small molecular tyrosine kinase inhibitor(s) of the c-Met kinase domain is undergoing preclinical validation. Preclinical and clinical studies are awaited with interest.

Tumor invasion and metastasis occur in the context of the ECM, and secreted protein a nd rich in cysteine (SPARC; osteonectin), a matricellular glycoprotein, mediates cellular interactions with the ECM, the levels and deposition of which are controlled in part by SPARC. Tumor-derived SPARC and its homologue hevin have de-adhesive effects on cultured cells and are reported to stimulate or retard tumor growth depending on the tumor type (57). Both proteins are produced at high levels in many types of cancers, especially by cells associated with tumor stroma and vasculature. These matricellular proteins do critical functions in the DR of tumors that result in their dissemination and eventual colonization of other sites. GEP identified *SPARC* as one of the genes induced by treatment with a DNA methylation inhibitor in pancreatic cancer cells (58). The loss of SPARC expression was associated with aberrant hypermethylation of its CpG islands and IHC staining revealed that SPARC protein was overexpressed in the stromal fibroblasts immediately adjacent to the tumor epithelium in PDA but rarely expressed in the cancers themselves. Primary fibroblasts derived from pancreatic cancer strongly expressed SPARC mRNA and secreted SPARC protein into the conditioned medium, and treatment of pancreatic cancer cells with exogenous SPARC resulted in growth suppression. These findings suggest that SPARC is a frequent target for aberrant methylation in pancreatic cancer and that SPARC expression in fibroblasts adjacent to pancreatic cancer cells is regulated through tumor-stromal interactions (58). The growth of pancreatic tumors in SPARC-null [SP(−/−)] mice and their wild-type [SP(+/+)] counterparts injected s.c. grew significantly faster in SP(−/−) mice than cells injected into SP(+/+) animals. Lack of endogenous SPARC resulted in decreased collagen deposition and fiber formation, alterations in the distribution of tumor-infiltrating macrophages, and decreased tumor cell apoptosis. Tumors grown in SP(−/−) had a lower percentage of blood vessels that expressed smooth muscle α-actin (pericyte marker; ref. 59). These data reflect the importance of ECM deposition in regulating tumor growth and show that host-derived SPARC is a critical factor in the response of host tissue to tumorigenesis. Recently, albumin-bound paclitaxel (ABI-007; Abraxane) was shown to target SPARC in advanced nonhematologic malignancies (60) and hence should be considered as a therapeutic that interferes with the DR in PDA.

A substantial body of evidence shows that inflammatory cells at tumor sites contribute to proliferation and invasion of human tumors, including PDA. IHC of 134 PDA patient biopsies showed significantly more mast cells and macrophages than in normal pancreas. The number of mast cells directly correlated with the presence of lymph node metastases. IHC also showed that the mast cells, macrophages, and tumor cells overexpress VEGF-A, VEGF-C, and basic FGF and were highly correlative with intratumor microvessel density assessed using CD34 (61). Hence, mononuclear inflammatory cells of the nonspecific immune response are recruited to PDA and may influence its metastatic capacity, adversely, thus contributing to the development of tumors with high angiogenic activity. Hence, antiangiogenic therapeutic strategies are likely to be effective in PDA. Another study did IHC on 38 PDA patient biopsies for thymidine Pi-deoxyribosyltransferase, which was overexpressed in tumor cells, endothelium, and infiltrating macrophages. The Pi-deoxyribosyltransferase–positive tumor cells and endothelial cells had significantly higher intratumor microvessel density compared with adjacent normal tissue. Because PDA is sensitive to 5-fluorouracil, Pi-deoxyribosyltransferase–activated oral capecitabine in tumor, endothelial cells, and infiltrating macrophages could increase the concentration of 5-fluorouracil at tumor site and result in an enhanced antitumor activity (62).

Phase I/II clinical trials are currently evaluating or proposing to evaluate several tyrosine kinase inhibitors in PDA. Targeting the vasculature in human cancers has been shown to be therapeutically effective. One such vasculature targeting agent is PTK787 (Novartis, East Hanover, NJ), which inhibits the tyrosine kinase domains of VEGF receptor/PDGF receptor/c-kit (63). Clinical trials with Avastin, a monoclonal antibody to VEGF in combination with gemcitabine, has shown modest activity (64). Hence, the clinical efficacy with PTK787 alone in PDA would be of importance for future combination studies with vascular targeting agents. The Src family comprises a family of nonreceptor tyrosine kinases that are overexpressed in a variety of human tumors (colon, breast, and pancreas) and are an integral part of tumor cell signaling pathways associated with migration, proliferation, adhesion, and angiogenesis. The blockade of Src kinase by daily oral administration of a novel Src tyrosine kinase inhibitor AZM475271 (AstraZeneca, Wilmington, DE), alone or in combination with i.p. gemcitabine,

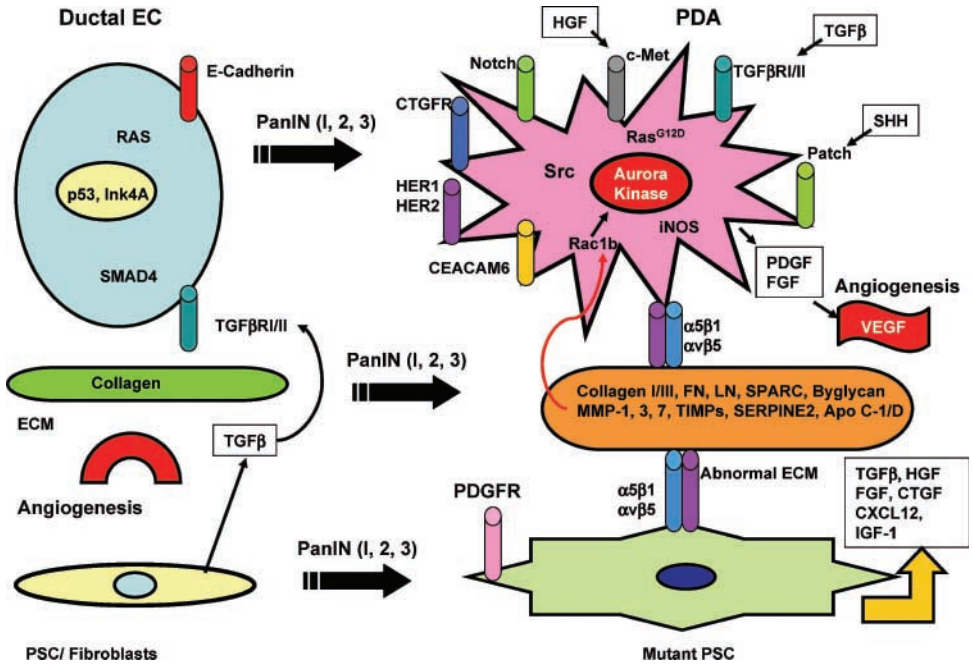

Figure 3. A predictive model of the tumor-stroma interactions and potential targets for therapeutic intervention in PDA. The normal exocrine pancreas is composed of ductal epithelial cells (*EC*), an intact basement membrane, ECM, stromal fibroblasts/PSCs, and vasculature. Invasive PDA evolves through three preinvasive lesions (PanIN) of increasing aggressiveness. Normal ductal epithelial cells have an intact Ras signaling pathway with normal p53 and Ink4A/Arf. TGFβ activation of the TGFβRI/II complex signaling through SMAD4 and E-cadherin provide a growth-suppressive effect. When mutations arise in Ras, Ink4A, p53, and SMAD4 in a sequential manner, an aggressive invasive PDA phenotype develops leading fibroblast proliferation and an associated increase in paracrine and autocrine growth factors with modification of the ECM, which in turn activates several oncogenic signaling pathways that lead to genomic instability [Src, Rac1b, and inducible nitric oxide synthase (*iNOS*)]. Several key cell surface receptors [Notch, Patch, c-Met, TGFβRI/II, PDGF receptor (*PDGFR*), HER1/2, CTGF receptor (*CTGFR*), CEACAM6, and $\alpha_5\beta_1/\alpha_v\beta_5$] are overexpressed and activated. In addition, an altered ECM [collagen I/III, fibronectin (*FN*), laminin (*LN*), SPARC, byglycan, MMPs, and tissue inhibitors of MMPs (*TIMPs*)] coupled to elaboration of several growth factors [TGFβ, HGF, PDGF, insulin-like growth factor-1 (*IGF-1*), CXCL12, and Wnt] and an abnormal vasculature (VEGF) is a basis of the DR in PDA.

inhibited the growth and metastasis of orthotopically implanted human pancreatic carcinoma cells in nude mice. Treatment with AZM475271 alone reduced the primary pancreatic tumor volume by ~40%. Gemcitabine plus AZM475271 reduced tumor volume by 90%, reduced metastasis, and was associated with reduced tumor cell proliferation, decreased tumor microvessel density, and increased apoptosis *in vivo* (65). However, this study did not analyze the efficacy of the Src tyrosine kinase inhibitor in resolving the DR. The opportunity to biopsy or harvest mouse xenograft tumors at the end of treatment and/or at the end of the study, in the absence or presence of the Src tyrosine kinase inhibitor, and to analyze by IHC the DR (e.g., collagen I) and correlate this with target inhibition (e.g., Src tyrosine phosphorylation) might have provided insights into the effectiveness of untangling the DR. The efficacy of the novel Src tyrosine kinase inhibitor dasatinib (Bristol-Myers Squibb, Princeton, NJ) untangling the tumor-stroma interactions in a mouse model should provide relevant information that can be incorporate into future clinical trial in PDA.

Finally, a novel way to target PDA is to inhibit broad spectrum of proteases of the TME. Legumain, an extracellular asparaginyl endopeptidase, is highly expressed by tumor, stroma, and endothelial cells. A novel legumain-activated, cell-impermeable doxorubicin prodrug LEG-3 designed to be activated exclusively in the TME showed a profound increase of the end product doxorubicin in nuclei of cells in tumors but little in other tissues. This TME-activated prodrug completely arrested growth of a variety of tumor types, including multidrug-resistant tumor *in vivo*, and significantly extended survival without evidence of myelosuppression or cardiac toxicity in mice models (66). This approach of targeting the TME is likely to be feasible in PDA. The direct targeting of the DR is in general not well established except to mention that clinical trials targeting MMPs in PDA did not show meaningful activity (32). This is most likely due to redundancy present within the MMP system and a more complex matrix biology that is not well understood.

Conclusion

One of the hallmarks of PDA is the marked stromal fibroblast proliferation and deposition of ECM components, a phenomenon known as 'desmoplasia' that seems to promote tumor growth and invasion (67). Paracrine and autocrine growth factor-induced fibrotic events seem to be targeted to the myofibroblast-like PSC cell, which has the capability to increase expression of ECM proteins (68), including collagens, fibronectin, laminin, and matricellular proteins, such as SPARC. The understanding of the molecular and cellular interactions between the genetically altered malignant epithelial cells, stromal fibroblasts (PSC), and altered ECM/TME will be critical to deciphering the pathogenesis of PDA. Figure 3 describes a predictive model of the tumor-stroma interactions that cause a DR and potential targets for therapeutic intervention in PDA. The most attractive therapeutic targets in our opinion would be cell surface receptors and/or their ligands amenable to therapeutic monoclonal antibodies (PDGF receptor, c-Met, VEGF receptor, Sonic hedgehog, and CTGF receptor), small molecular inhibitors to receptor and nonreceptor protein kinases (TGFβRI, Src, Aurora kinase, and Polo-like kinase), and signaling proteins (Ras and Rac1b). Other novel attractive targets are located to the TME/ECM (CEACAM-6, integrins, and SPARC). Future studies would focus on tumor-stroma markers identified in human PDA, validating these in a relevant pancreas cancer cell line(s) through mouse models, biological characterization, and potentially targeting these proteins as therapies for pancreatic cancer.

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