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Fibroblasts in Pancreatic Ductal Adenocarcinoma: biological mechanisms and therapeutic targets

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Abstract

The desmoplastic reaction of pancreas cancer may begin as a wound healing response to the nascent neoplasm, but it soon creates an insidious shelter that can sustain the growing tumor and rebuff therapy. Among the many cell types subverted by transformed epithelial cells, fibroblasts are recruited and activated to lay a foundation of extracellular matrix proteins and glycosaminoglycans that alter tumor biophysics and signaling. Their near-universal presence in pancreas cancer and ostensible support of disease progression make fibroblasts attractive therapeutic targets. More recently, however, it has also become apparent that diverse subpopulations of fibroblasts with distinct phenotypes and secretomes inhabit the stroma, and that targeted depletion of particular fibroblast subsets could either provide substantial therapeutic benefit or accelerate disease progression. An improved characterization of these fibroblast subtypes, along with their potential relationships to tumor subtypes and mutational repertoires, is needed in order to make anti-fibroblast therapies clinically viable.

Keywords

PDA; fibroblast; desmoplasia; GEMM

Introduction

Pancreatic ductal adenocarcinomas (PDA) consist of a minority of malignant tumor cells embedded in a complex environment of extracellular matrix (ECM), suppressive immune cells and fibroblasts[1]. Although recent improvements to chemotherapeutic regimens against tumor epithelia have increased patient survival[2, 3], much of the current research and development of novel treatments for PDA have increasingly focused on targeting the various

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stromal constituents because of their important roles in facilitating disease progression and resistance. One example involves targeted degradation of the ECM component hyaluronan, or hyaluronic acid (HA), which has been shown to fundamentally alter the biophysical properties of the tumor by increasing interstitial gel-fluid pressures, contributing to the collapse of blood vessels and preventing drugs from efficiently entering the tumor bed[4–6]. A pegylated form of hyaluronidase, PEGPH20, may enhance the efficacy of standard-of-care chemotherapy by increasing tumor penetration and drug exposure[4, 5, 7]. In addition, agents aimed at relieving immunosuppression and adoptive immune cell strategies are also being rigorously pursued with some encouraging results[8–12].

Fibroblasts have long been known to be a fundamental and near-universal component of PDA[13], but their role in tumor progression and maintenance has proven to be unexpectedly nuanced, with conflicting reports suggesting that they can provide both pro-tumorigenic support and anti-tumorigenic restraint[14–17]. Recent advances in genetically engineered mouse models (GEMM) of PDA along with improved characterization of fibroblast subpopulations are providing a clearer understanding of their diverse roles and suggest new therapeutic approaches that may be effective alone or in combination with other therapies.

Fibroblasts accumulate in the pancreas during acute and chronic pancreatitis[18], as well as in early pre-invasive lesions such as pancreatic intraepithelial neoplasms (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN)[19–25], but increased numbers are seen in PDA. The fibrotic responses likely originate from the activation and subsequent expansion of local, so-called quiescent pancreatic stellate cells (PSC), but infiltration of fibroblasts derived from mesenchymal precursors in the circulation may also occur[26]. What begins as essentially a wound response to microscopic lesions evolves into a comprehensive transformation of the nascent microenvironment, involving complex paracrine signaling to and from fibroblasts, activated deposition of diverse ECM components and, ultimately, establishment of an immune- and drug-privileged sanctuary for the tumor epithelia. While tumor epithelial cells orchestrate these changes, fibroblasts are in many ways complicit in the remodeling of the tumor microenvironment (TME) as direct mediators of paracrine signaling and matrix deposition. However, lumping cancer-associated fibroblasts (CAF) into a single group is insufficient to describe the many diverse, and sometimes conflicting, roles they play. Fibroblasts can be categorized by expression of specific marker proteins into subpopulations with distinct phenotypes, gene expression patterns and functions, not unlike the hierarchical stratification of immune cell populations[27, 28]. Understanding the diversity of fibroblast subtypes, including their engagement in various paracrine signaling pathways, distinct programs of TME remodeling and differences in relative abundance within tumor subtypes, will be critical to the design of safe and effective therapies targeting this facet of the PDA neo-organ.

Delineation of PDA-associated fibroblast subtypes or activation states

At the most fundamental level, fibroblasts can be considered to be either quiescent or activated. Quiescent fibroblasts are primarily thought of as “normal”, tissue-resident fibroblasts or PSC that inhabit the pancreas prior to the development of preinvasive or invasive disease. Protein markers characteristic of this population include desmin and glial

fibrillary acidic protein (GFAP), among others, and they are also usually non-proliferative (i.e. Ki67-negative)[29–31]. Quiescent PSC also exhibit cytoplasmic lipid droplets that are reservoirs for vitamin A[30, 32], but the primary role of quiescent PSC is to monitor the integrity of the surrounding pancreatic tissues and, due to the precarious nature of an organ filled with digestive enzymes, respond quickly and robustly to injury to contain and heal tissue damage. Indeed, PSC can become activated by mechanical injury, chemical insult (e.g. alcohol), toxins (e.g spider venom) or inflammatory injury (e.g. autoimmune pancreatitis) to produce a fibrotic wound-healing response, which is dramatically visualized in cases of acute or chronic pancreatitis[33–37]. However, more detailed analyses of fibroblast subtypes and functions have not been performed in these settings. Interestingly, while fibrosis in acute pancreatitis may be resolve after removal of the inciting insult, fibroblast activation may also paradoxically decrease in more advanced stages of CP, perhaps due to complete resorption of necrotic debris, and/or depletion of inciting pancreatic enzymes and subsequent loss of paracrine signaling from nearby macrophages[34]. Pre-invasive lesions (i.e. PanIN[38], MCN[39] or IPMN[40]) in humans and mice[19–25] also induce PSC activation, either as a result of microscopic structural deformations and ductal obstruction (inducing injury and obstructive lobular atrophy)[41] or by direct paracrine signaling from transformed epithelia.

Activated fibroblasts constitute a significant fraction of the PDA bulk and may represent an organizing feature of the overall tumor architecture since they arrange themselves concentrically around epithelial clusters and deposit aligned structural components of the ECM[42]. As a whole, activated CAF are equipped to play many structural, biophysical and signaling roles in PDA, but some markers, including alpha smooth muscle actin (αSMA), fibroblast activation protein alpha (FAPα) and platelet-derived growth factor receptor (PDGFR), have been proposed to help delineate subpopulations of activated fibroblasts with more circumscribed features and functions. While αSMA positivity has long been the gold standard for identifying an activated fibroblast, FAPα[43] has more recently risen to prominence as a marker that defines a more pro-tumorigenic fibroblast population (see below). Differing reports suggest that these two markers may describe distinct but overlapping populations or, alternatively, be mutually exclusive[14, 16]. There is the additional, intriguing suggestion that such populations inhabit distinct strata of the fibrotic response surrounding tumor epithelial structures, with αSMA+ myofibroblasts tightly surrounding ductal structures and FAPα+ fibroblasts more distal, indicating that spatial cues direct their activation or *vice versa*[44, 45]. PDGFR expression appears to further define a subset of αSMA + fibroblasts, though FAPα+ fibroblasts may also express PDGFR[46]. Taken together, even just these three markers can potentially describe up to eight distinct populations, and although they provide a starting point to define distinct CAF populations, further discrimination with additional markers such as those found on mesenchymal stem cells[47] may prove necessary.

Further complicating these attempts at classification, fibroblasts in the pancreas appear to be remarkably plastic and dynamic[48]. The observation of activated fibroblasts in the settings of acute and chronic pancreatitis, as well as with PDA precursors suggest a stepwise genesis of CAF, from quiescent PSC to injury-activated fibroblasts to CAF. However, it remains unclear whether these fibroblast subpopulations are wholly distinct, organized into a hierarchy or represent reversible activation states. Regardless, that both the accumulation

and the activation of fibroblasts resolve after extinguishing mutant *Kras* expression in reversibly inducible GEMM of PDA[49, 50] hints that CAF can potentially be normalized and cleared once invasive disease is eradicated. Targeted depletion of αSMA+ fibroblasts has no effect on FAPα+ populations[16], whereas depletion of FAPα+ fibroblasts simultaneously reduced αSMA+ fibroblasts by 70%[14]. These findings, too, could result from sequential activation through hierarchical states or by asymmetric crosstalk between distinct fibroblast populations.

Paracrine signaling from and to PDA-associated fibroblast populations

CAF participate in a coordinated signaling network that is shared by tumor cells, the immune infiltrate and other cell populations. Certain critical cytokines and other signaling molecules have been identified that have principal roles in defining cell behavior and modulating the TME. At early stages of tumor development, tissue-resident PSC become activated after receiving stimuli from the nascent lesion. These signals include diverse growth factors[51], inflammatory cytokines[52], oxidative stress, autophagy[53] and/or paracrine Hedgehog (Hh) signaling from transformed epithelial cells (see below). Ultimately, PSC activation leads to a change in phenotype wherein these quiescent fibroblasts lose their lipid droplets, express markers of activation such as αSMA and FAPα, and ramp up secretion of their own signaling molecules and ECM constituents. One model suggests that a gradient of cytokines, perhaps primarily Tgfβ and Il-1, radiating outward from epithelial lesions influences fibroblast intracellular signaling to drive the distinct subtypes as defined by αSMA or FAPα expression[54].

Global analyses of the PSC secretome have identified hundreds of signaling proteins that are further enriched after PSC activation[46, 55]. Distinct activated fibroblast subtypes have been primarily defined by the presence or absence of αSMA expression (and in at least one study, with the αSMA- population expressed more than an order of magnitude greater FAPα at an adjusted p-value of $<10^{-14}$). These subtypes express highly distinct secretomes[45]. Of the many notable differences, the upregulation of *Il6*, *Lif*, *Csf3*, *Cxcl1* and *Cxcl2* in αSMA-/FAPα+ versus αSMA+/FAPα- CAF undoubtedly elicit widespread effects: 1) Il6 may bolster chemoresistance of PDA through inflammatory monocytes and STAT3 signaling[56, 57]; 2) Lif mediates remodeling of intratumoral nerves in PDA[58] and may be a key enforcer of the αSMA-/FAPα+ phenotype through autocrine JAK/STAT signaling[54]; 3) Csf3 may sustain granulocytic myeloid-derived suppressor cells (Gr-MDSC)[10] and thereby enhance tumor growth[59]; and 4) the Cxcr2 ligands, Cxcl1 and Cxcl2, mediate the influx of immunosuppressive cells in PDA and support metastatic outgrowth[9, 60]. FAPα+ CAF in other cancer types similarly promote immunosuppression through increased expression of *Il6, Cxcl2*, *Cxcl12* and *Ccl2*, suggesting the conservation of this distinction between αSMA-/FAPα+ and αSMA+/FAPα- CAF subpopulations[61]. Yet another example of subtype-specific paracrine signaling occurs through GM-CSF, a well-known modulator of immunosuppressive cells, and which is specifically expressed by a population of stem-like fibroblasts marked by CD90, CD49α, CD44 and CD73[47]. Fibroblast signaling can also affect tumor epithelial gene expression: IGF1 and HGF were highly expressed by stromal cells (although not necessarily fibroblasts or specific fibroblast subtypes) but their cognate receptors IGF1R and MET, respectively, were preferentially expressed on tumor epithelial

cells in patient-derived xenograft (PDX) models of PDA[62]. Finally, in addition to cytokines and chemokines, secretion of metabolites such as alanine from activated, αSMA+ PSC can bolster tumor growth metabolically by supplying a carbon source for the TCA cycle and supporting biosynthesis of non-essential amino acids in tumor epithelia[63, 64].

While NF-κB and JAK/STAT signaling have been shown to help define CAF subtypes[45, 54], Hh signaling from tumor epithelial cells to fibroblasts is also likely among the most critical pathways in differentiating fibroblast subtypes. Sonic hedgehog (Shh) and Indian hedgehog (Ihh) are highly expressed by tumor epithelia (as opposed to stromal cells)[62] and the effects of Hh signaling in stromal fibroblasts (i.e. myofibroblasts) have been extensively studied, mostly those defined by αSMA positivity[65–68]. Whereas short-term, pharmacological inhibition of Hh signaling in GEMM of PDA increased tumor perfusion and chemotherapeutic effect by arrest of myofibroblast proliferation[15], depletion of desmoplastic stroma and stimulation of angiogenesis, long-term inhibition of Hh signaling or pancreas-specific genetic ablation of *Shh* in GEMM shortens survival and increases disease aggressiveness[17, 69]. Deletion of the surface receptor, *Smoothened* (*Smo*), that mediates Hh signaling in fibroblasts had a similar effect[67]. These results suggest that Hh signaling from tumor epithelia to fibroblasts may also support tumor-suppressive activities, though the exact mechanism is unknown. One intriguing possibility is that inhibition of Hh signaling selectively depletes a tumor-suppressing population of fibroblasts, with lesser effect on tumor-promoting fibroblasts. This hypothesis is supported by the observation that the expression of Hh signaling regulators, *Ptch1*/*2* and *Smo*, are significantly different between αSMA+/FAPα- (putatively tumor-suppressing) and αSMA-/FAPα+ (putatively tumor-promoting) fibroblast populations[45]: αSMA+/FAPα- fibroblasts are also physically closer to tumor epithelia, the source of Hh ligand, and they express less *Ptch1/2* and more *Smo* (negative and positive regulators of Hh signaling, respectively) than the more distal αSMA-/FAPα+ fibroblasts, suggesting higher overall Hh pathway activity in the former (and therefore greater sensitivity to inhibition) than in the latter population (Figure 1).

Remodeling TEM structure and function by CAF subtypes

Although fibroblasts and immune cells comprise much of the cellular mass in PDA, ECM proteins and glycosaminoglycans (GAGs) secreted by activated fibroblasts also contribute substantially to the tumor bulk. Co-culture experiments have shown that fibroblasts can also stimulate tumor epithelia to contribute to ECM remodeling (e.g. by upregulated expression of *hyaluronan synthase 2* (*Has2*), an enzyme that catalyzes the extension of HA polymers)[70]. Collagens, fibronectin, versican and many other proteins are secreted by activated fibroblasts[62, 71], in addition to GAGs such as HA and chondroitin sulfate[4]. Fibroblast-mediated secretion of these structural components of the ECM can greatly influence tumor biology by altering the biophysical properties of the TME and activating additional signaling pathways in both tumor epithelia and stromal cells. The ECM proteins can be assembled and aligned in tethered networks, assigning polarity to the ECM[42]. The GAGs can bind water and swell against these restraints, combining to induce high interstitial pressures that collapse blood vessels and isolate the tumor from circulating therapeutics and anti-tumor immune cells[4]. Moreover, the aligned protein network can also provide tracks along which tumor cells can migrate[42], and the degree of collagen alignment has been shown to be a

negative prognostic factor for patients with resected PDA[72]. These matrix proteins and GAGs also engage diverse intracellular signaling pathways through cell surface receptors, such as integrins, discoidin domain receptors and CD44, that can further influence tumor phenotypes[73, 74].

Many of the above described protein factors, including collagens 1a1, 1a2, 2a1, 3a1, 5a1, 11a1, 18a1, fibronectin and versican are differentially expressed by distinct fibroblast populations[45], which may have important prognostic implications (see below). Furthermore, expression levels of hyaluronan synthases, *Has1* and *Has2*, are significantly lower in αSMA + fibroblasts[45], suggesting that fibroblast subpopulations may also differentially contribute to GAG deposition. These comparative gene expression analyses are supported by experiments with targeted depletion of a single fibroblast subtype. When αSMA+ fibroblasts are depleted, collagen levels are decreased along with the elastic modulus and tissue stiffness, but HA is unchanged[16]; depletion of FAPα+ fibroblasts similarly reduces collagen content but also results in loss of HA deposition[14] and additionally reduces versican and fibronectin levels. Thus, the distinct fibroblast subpopulations may also disparately influence distinct contributors to intratumoral biophysics, including free and gel-fluid pressures as well as solid stress.

In addition to the differences in secretion of collagens and HA described above, fibroblast subtypes exhibit different states of tension. αSMA+ fibroblasts, often called "myofibroblasts" because of their resemblance to smooth muscle cells, anchor to ECM components (i.e. collagen) via integrin receptors and propagate tension through contraction of intracellular smooth muscle actin[75, 76]; conversely αSMA- (FAPα+?) fibroblasts appear to be less rigid and may not contribute substantively to tissue tension. Solid stress and fluid pressures contribute to the overall forces exerted on cells in the tumor, but there are indications that tension itself can affect tumor progression, drug response and patient survival[77, 78]. Abrogation of these forces either by PEGPH20-mediated degradation of HA and removal of the swelling pressure that loads tethered collagen fibers or by reducing solid stress through inhibition of collagen synthesis[79] or depletion of αSMA+ fibroblasts through Hh pathway inhibition, can open blood vessels in PDA GEMM and increase perfusion[4, 15]. Ultimately, in this respect, it will be important to determine whether targeted depletion of specific fibroblast subpopulations differentially affects free fluid pressures, gel-fluid pressures, tension or solid stress, as well as measuring the impact on drug perfusion.

Do fibroblast subtypes correlate to PDA subtypes?

In addition to the subpopulations of activated fibroblasts described above, subtypes of PDA have been identified based on the genomic analyses of the tumor epithelium[80–82]. Profiles of each of these compartments from the same tumor specimen have even been dichotomized virtually to test the hypothesis that epithelial and stromal subtypes evolve coordinately[71]. The genes used to delineate subtypes in these large-scale genomic studies often code for secreted factors, implicating the secretome as a defining feature. For example, *LGALS4* and *TFF1/2/3* are upregulated in the "Classical" PDA subtype across multiple studies[71, 81, 82]; in the stromal compartment, *SPARC*, *COL1A2*, *MMP11* and *POSTN* contribute to the "Activated stroma" signature, while *DES*, *IGF1* and *OGN* are expressed by "Normal

stroma”[71]. The tumor (epithelial) subtype and the stromal subtype can independently influence patient prognosis. However, specific tumor epithelial subtypes did not correspond to stromal subtypes in these studies, suggesting somewhat counterintuitively that the epithelial and stromal compartments progress independently. The four cardinal genetic abnormalities found in PDA epithelial cells (*KRAS* mutation, *P53* mutation, *SMAD4* loss and *CDKN2A* loss) for the most part do not significantly associate with particular subtypes as defined by such global gene expression analyses, although *SMAD4* loss and *TP53* mutation trend with basal-like[71] and squamous[80] subtypes, respectively.

Despite this apparent disconnect between epithelial and stromal subtypes, discrete mutations or epigenetic alterations (such as the cardinal events in PDA progression described above) can alter intracellular signaling to modify the cancer cell secretome and subsequently influence fibroblast phenotype; while this hypothesis has not been directly tested, several studies support its merit. First, each genomic alteration is associated with distinct clinicopathologic features and patient prognosis[83, 84], demonstrating not surprisingly that tumor phenotypes are influenced by their mutational repertoire. Oncogenic activation of *KRAS*, the common initiating event in pancreatic carcinogenesis, stimulates *Il6* and *Shh* expression and secretion to activate fibroblasts[49, 85]. Since *KRAS* activation occurs in >95% of PDA, oncogenic *KRAS*-dependent secreted factors can be considered foundational influences on fibroblast activation, whereas subsequent acquired mutations may shape the diversity and phenotypes of fibroblast subpopulations (Figure 2). For example, compared to the background of GEMM with pancreas-specific *Kras* activation (*KC*), the additional heterozygous deletion of *Tgfbr2* in the epithelium resulted in increased collagen deposition, tissue stiffness, fibronectin and galectin-4 in the tumor stroma[77, 86]. Mutation of *Trp53* in the same *KC* model[77] or in culture systems[87] also altered the epithelial secretome and paracrine signaling to influence ECM composition. Loss of *Cdkn2a* expression similarly induced widespread changes to gene expression[88], including upregulation of chondroitin sulfate synthase 3 (*CHSY3*). Thus, as PDA progress and evolve, successive genomic alterations in these cardinal and other passenger genes may dynamically perturb the cancer cell secretome to influence fibroblast heterogeneity and activity across time and space[86, 89].

Fibroblasts in MCN-PDA

One particularly notable example of PDA genotype driving fibroblast phenotype (or perhaps the reverse) is the clinically and histopathologically distinctive MCN. These PDA precursors, which are commonly discovered incidentally and often do not progress to invasive or metastatic disease[90], exhibit a distinct fibroblast phenotype wherein estrogen receptor (ER) and/or progesterone receptor (PR) are expressed in peri-cystic, “ovarian-type” fibroblasts[91]. In fact, it is the hormone receptor positivity in these fibroblasts along with the characteristic wavy nuclei of an “ovarian stroma”, and not any molecular or phenotypic feature of the tumor epithelia, that provide the conclusive and requisite diagnostic criteria by which MCN are defined (Figure 3). Together with the >20:1 female:male incidence ratio and the frequent presentation at peri-menopausal age, these features strongly suggest that circulating hormones influence the development of the tumor and stroma in MCN. Studies in GEMM and human patients further suggest that MCN can be directed to arise after specific sequelae of genomic alterations: for example, when pancreatic epithelial cells undergo loss

of *Smad4* after *Kras* activation but before *Trp53* mutation or *Cdkn2a* silencing MCNs are favored over PanINs[20, 92]. Immunohistochemical assessment of SMAD4 expression in human MCN shows that nearly 90% of invasive MCN lack SMAD4 (i.e. have lost expression from both alleles) obscuring the possibility that an initial heterozygous mutation in *SMAD4* represents a (relatively) early event[92]. This can occur either by stepwise deletion of one allele and subsequent loss of heterozygosity or epigenetic silencing or some combination, which may or may not be detected by selective genomic techniques[93]. In other GEMM, virally-induced overexpression of *Wnt1* or targeted expression of *Hif2α* in the context of pancreatic *Kras* activation enhanced the development of MCN lesions[24, 25], indicating potential roles for Wnt signaling and hypoxia in MCN evolution.

The prognosis for patients with PDA derived from MCN is substantially better than for those with conventional PDA derived from PanIN[94, 95], despite a highly conserved mutational repertoire between these two forms of invasive disease[96]. Since the unique fibroblast features are a defining property of MCN, it is not unreasonable to suggest that these fibroblasts may be, at least partially, responsible for this difference in prognosis. Detailed comparisons of fibroblasts from conventional and MCN-derived PDA have not been performed, although αSMA expression has been shown in one study to be roughly comparable[97]. Thus, many important questions remain regarding this hormone receptor-positive fibroblast phenotype, including: 1) how tumoral SMAD4 loss (or other canonical genomic alterations), Wnt signaling, hypoxia and hormonal factors integrate to create a ER/PR+ ovarian stroma; 2) whether the ER/PR positive fibroblasts overlap with any of the previously defined fibroblast subtypes; 3) how the expression of secreted factors or other genes differs in the ER/PR+ population of fibroblasts; and 4) whether targeting ER/PR+ stromal fibroblasts would influence progression of MCN to PDA.

Is targeting fibroblasts a viable strategy for PDA therapy?

Fibroblasts may support tumor growth by several mechanisms, including: 1) secretion of ECM components that contribute to elevated force generation and transmission[76], interstitial pressures, vascular collapse and drug exclusion[4], 2) recruitment and programming of immune populations to sustain an immunosuppressive environment[16, 18], 3) sequestration of intratumoral gemcitabine[98] and/or 4) radioprotection of tumor cells[99]. Not surprisingly, given the complexity of fibroblast populations and functions in the tumor stroma, experiments addressing the question of whether to target fibroblasts in PDA have given mixed results. Several studies have arrived at divergent conclusions regarding whether a highly dense tumor stroma (or low cellularity) is a positive or negative prognostic factor for patient survival and metastasis[100–103]. When focusing on fibroblasts specifically, retrospective analyses have shown that high stromal levels of either αSMA or FAPα correlate with poorer survival[44, 104–107]. Nevertheless, targeted depletion of specific fibroblast subpopulations in PDA indicates that the distinctions between fibroblast populations are important (Table 1). In studies where depletion of fibroblasts was detrimental, αSMA was considered the primary fibroblast marker, suggesting that αSMA+ fibroblasts are anti-tumorigenic[16, 17]. In contrast, in experiments where fibroblast depletion improved outcomes, FAPα was generally the targeted marker and consequently FAPα+ fibroblasts may be predominantly protumorigenic[14, 108].

In the current state of the art, the standard treatments for PDA generally include some combination of surgery, radiation and/or chemotherapy depending on clinical parameters such as disease stage, location of the tumor and its relationship to critical vessels, and performance status of the patient. No targeted therapies that account for mutational status or fibroblast heterogeneity are currently in use, though some clinical trials have incorporated the former and more global inhibition/modulation of fibroblasts has been tested. As we integrate the amassing data regarding fibroblast subpopulations and their effects on the ECM and tumor biology, novel targets such as FAPα+ fibroblasts or ER/PR+ fibroblasts may emerge more clearly. Undoubtedly, if fibroblasts are to be targeted in PDA, the treatment must precisely target specific fibroblast populations, since depletion of the anti-tumorigenic fibroblast subpopulation could (and has[109]) shortened patient survival. Accurate targeting could theoretically be accomplished through cutting-edge immunologic strategies such as designer CAR-T cells[14, 110], but much more preclinical work is needed to characterize fibroblast subpopulations and the fates of other subpopulation(s) when one is specifically depleted.

To add to the complexity, inter- and intra-tumoral heterogeneity with respect to fibroblast subpopulations is completely unexplored; it may be that patients need to be screened by biopsy and assessed for the presence of certain fibroblast subpopulations to identify patient groups most likely to benefit from specific therapies. The ability to retrospectively stratify patients based on fibroblast markers and observe differences in survival supports the notion that, as with many other stromal targeted therapies[5], anti-fibroblast therapies may have very select utility, with some patients receiving little to no benefit. Fibroblast-targeted therapies may also be less effective in metastases, eliminating the majority of patients, who present with Stage IV disease and are in most dire need of novel therapies. Conflicting reports propose that PDA metastases are either relatively astromal[71] or similarly desmoplastic[111] compared to the primary tumor, possibly depending on the size of metastatic lesion[112]. Certainly, there are at least some activated fibroblasts that associate with metastatic deposits and they may originate from local reservoirs[113] or traverse tissues along with metastasizing tumor cells[114]. Although the inception of liver metastases seems to be independent of activated fibroblasts, metastatic outgrowth is slowed when recruitment/activation of αSMA+ fibroblasts is prevented[113], which would not be predicted by the studies mentioned above describing the impact on the primary tumor of targeting αSMA+ cells. Since most PDA patients present with metastatic disease and nearly all patients eventually succumb with if not because of metastatic disease burden, a deeper comparison of fibroblast subtypes and secretomes between primary and metastatic sites may, therefore, have large ramifications for the deployment and success of fibroblast-targeted therapies in the clinic.

Overall, we should not be discouraged by the complexity of fibroblast heterogeneity in PDA. Rather, the complexity likely reflects both critical importance and therapeutic opportunity. Within the diverse ecosystem of the TME, fibroblasts are an important, influential and heterogeneous population of cells that have outsized role(s) in defining ECM composition and tumor biophysics. Anti-fibroblast therapies, even those targeting very specific subpopulations of pro-tumorigenic fibroblasts, may not be the proverbial silver bullet against PDA, but curative therapies will absolutely require a multi-faceted, multi-compartment

approach, and consideration of fibroblast functions and effects will likely be of great benefit in this pursuit.

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Biography

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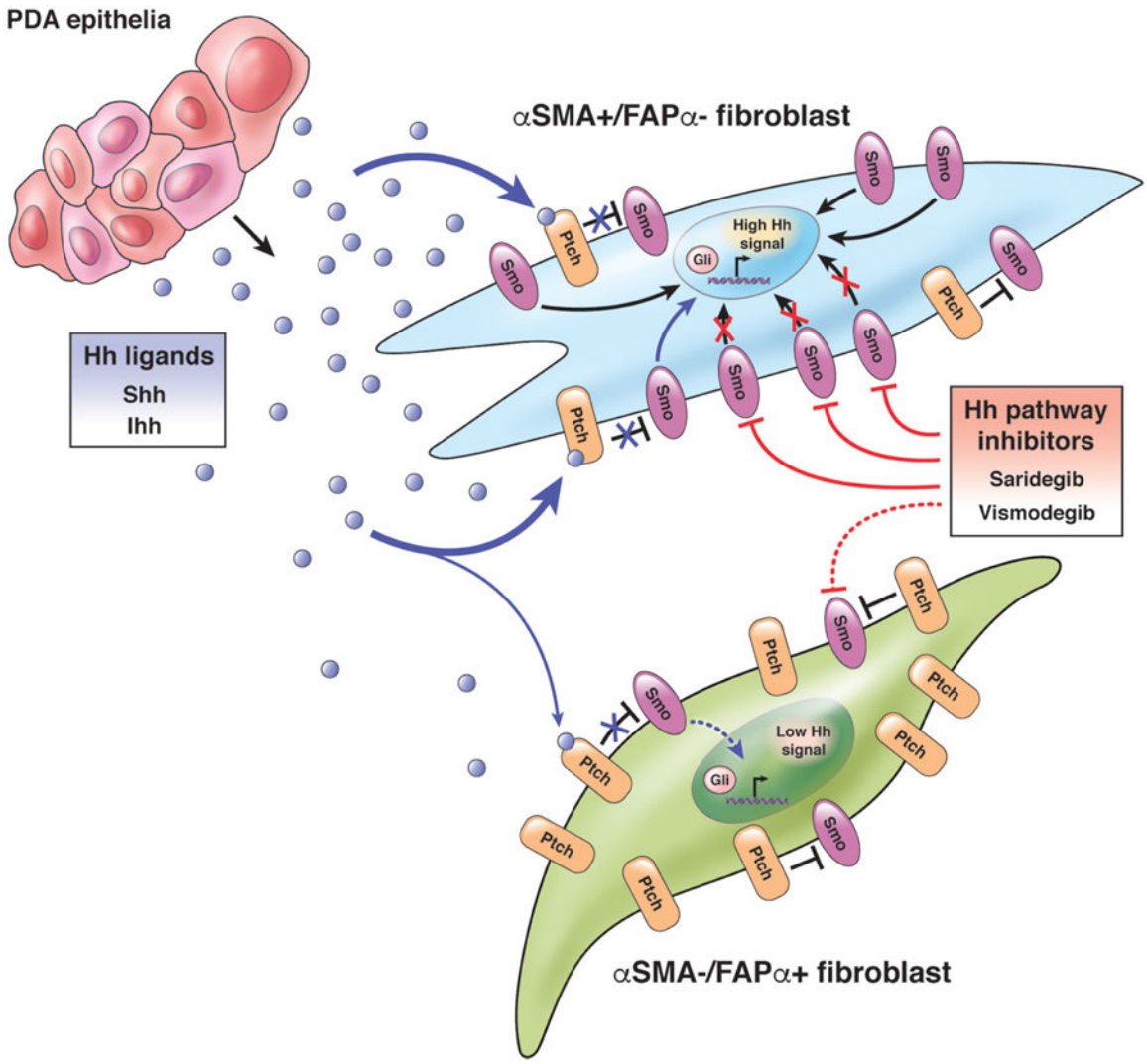

Figure 1. Differential Hh signaling in αSMA+ and FAPα+ fibroblast subpopulations.

αSMA+/FAPα-fibroblasts are physically closer to PDA epithelia and likely have higher overall Hh signaling than αSMA-/FAPα+ fibroblasts due to greater exposure to epithelial-derived Hh ligand, lower expression of *Ptch1/2* and higher expression of *Smo*. For these reasons, inhibitors of *Smo*, such as saridegib and vismodegib, may preferentially target this αSMA+/FAPα- fibroblast subpopulation.

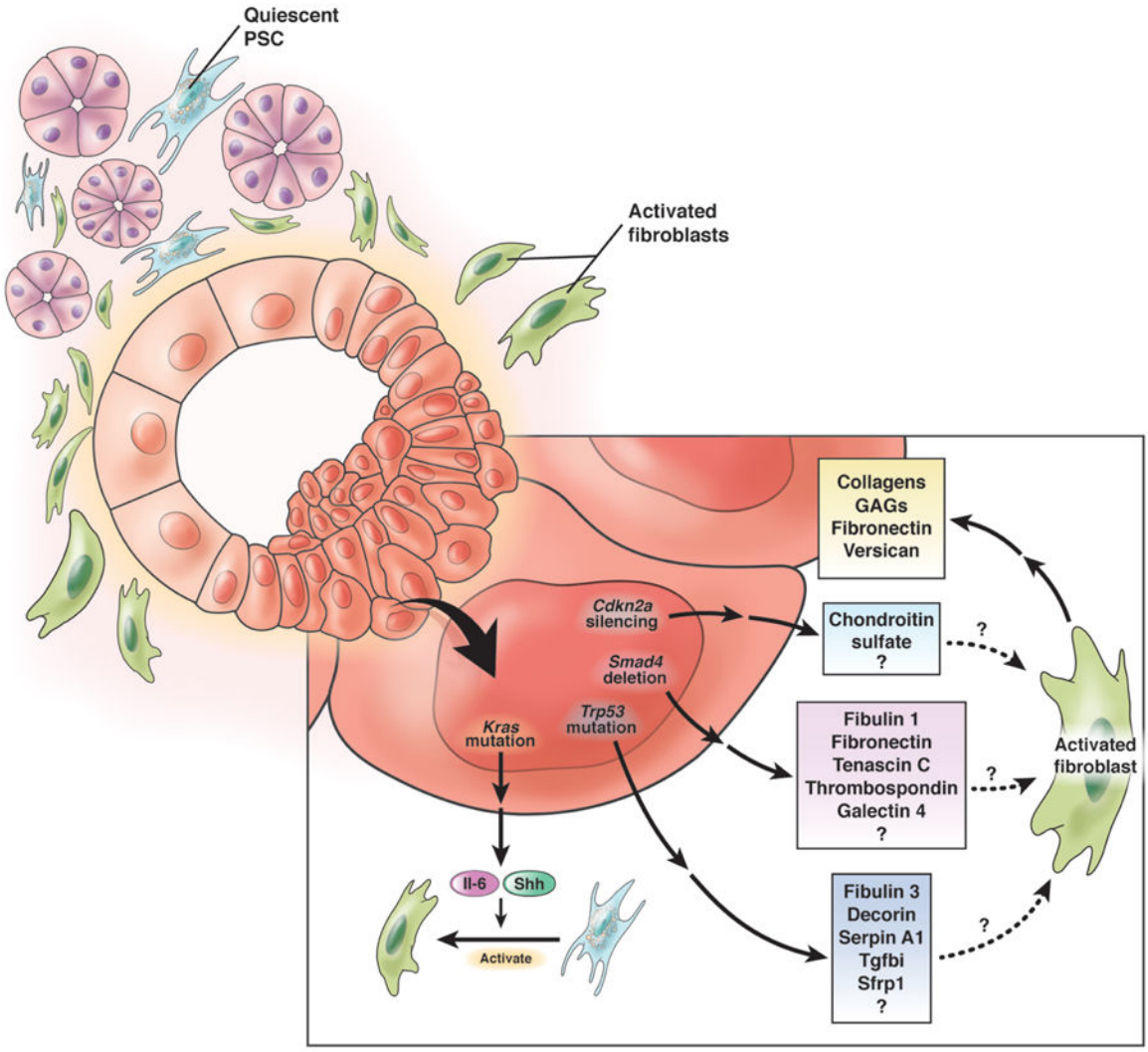

Figure 2. Effects of cardinal genomic alterations in PDA may disparately influence fibroblast context, function and heterogeneity.

Activating *Kras* mutation in pancreatic epithelial cells induces production of Il-6 and Shh, which help convert quiescent PSC to activated fibroblasts. Subsequent mutation of *Trp53*, loss of *Smad4* and/or silencing of *Cdkn2a* in the neoplastic epithelium may differentially shape the ECM through distinct mechanisms of paracrine signaling to fibroblasts.

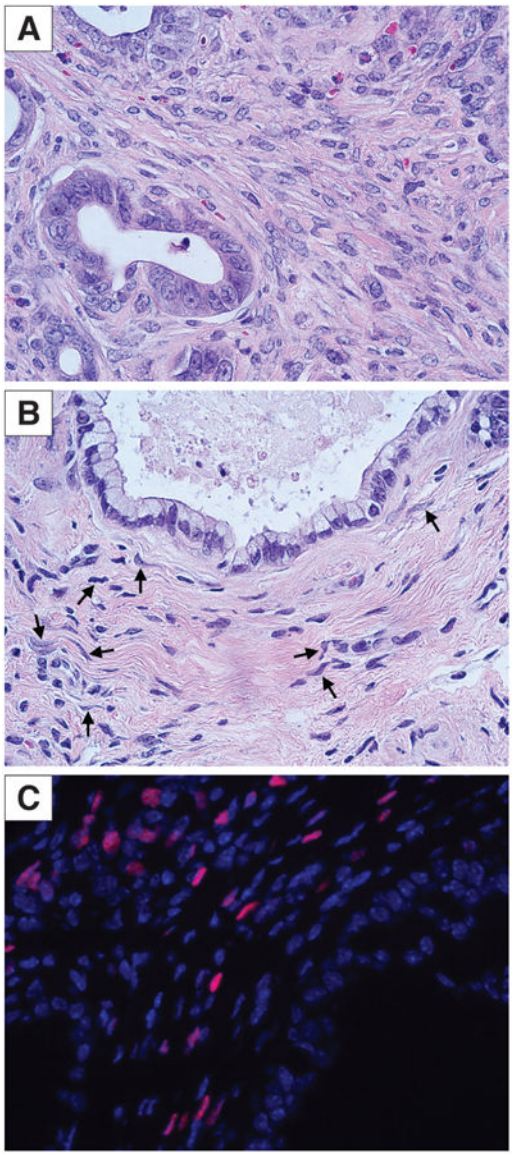

Figure 3. MCN are characterized by unique fibroblast subpopulations.

A) Stromal fibroblasts in murine PanIN. B) Ovarian-type stromal fibroblasts with characteristic wavy nuclei (arrows) in murine MCN. C) Nuclear ER positivity (red) in fibroblasts of murine MCN.

Table 1.

Representative examples of fibroblast subtype-specific depletion strategies

Fibroblast marker	Depletion strategies	Effect of depletion					Therapeutic potential
		Fibroblasts	ECM	Epithelial cells	Immune cells	Outcomes	
αSMA	αSMA-directed TK/ Ganciclovir suicide gene (Özdemir, et al. 2014)	80% depletion of αSMA+ fibroblasts Unchanged FAPα+ fibroblasts	Loss of collagen content and organization Decreased elastic modulus and tissue stiffness Unchanged HA content	Decreased differentiation Increased expression of epithelial-to-mesynchymal transition markers	Reduced CD45+ immune infiltrate Increased immunosuppressive T-regulatory cells Decreased T-effector cells	Increased hypoxia Increased metastasis Decreased survival Does not improve gemcitabine therapy	Unlikely[1]
	Hh pathway inhibition (Rhim, et al. 2014)	80% depletion of αSMA+ fibroblasts	N/A	Decreased differentiation Increased expression of epithelial-to-mesynchymal transition markers Increased proliferation	Reduced CD45+ immune infiltrate Decreased F4/80+ monocytes	Increased vascular density and perfusion Increased metastasis Decreased survival Does not improve gemcitabine therapy	Acute treatment: Perhaps Chronic treatment: No
FAPα	CAR-T (Lo, et al. 2015)	91% depletion of FAPα+ fibroblasts 70% depletion of αSMA+ fibroblasts	Decreased collagen content Decreased HA content Decreased versican and fibronectin	Decreased proliferation Increased apoptosis	Decreased Gr-MDSC	Decreased vascular density Reduced tumor growth Improves gemcitabine therapy	Yes

[1]There was an apparent benefit when this approach was used with anti-T_{Reg} cell agent suggesting a potential for combinatorial strategies.