Reshaping the tumor stroma: emerging therapies in pancreatic cancer

Short title: Stromal therapies for pancreatic cancer

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Abstract

Pancreatic cancer is accompanied with a fibrotic reaction that alters interactions between tumor cells and the stroma to promote tumor progression. Consequently, strategies to target the tumor stroma might be used to treat patients with pancreatic cancer. We review recently developed approaches for re-shaping the pancreatic tumor stroma and discuss how these might improve patient outcomes. We also describe relationships between the pancreatic tumor extracellular matrix, the vasculature, the immune system, and metabolism and discuss the implications for the development of stromal compartment-specific therapies.

Keywords: pancreatic cancer, stromal remodeling, invasion, metastasis, diagnostic tool, patient-derived models

List of abbreviations:

AMP: Adenosine Monophosphate-Activated Protein

ACTA: Alpha-Smooth Muscle Actin

ATRA: All-Trans Retinoic Acid

CAF: Cancer-Associated Fibroblasts

CTC: Circulating Tumor Cell

ECM: Extracellular Matrix

EMT: Epithelial to Mesenchymal Transition

FAP: Fibroblast Activated Protein

HA: Hyaluronic Acid

LOX: Lysyl Oxidase

MMP: Matrix Metallo-Proteinase

PET: Positron Emission Tomography

PDX: Patient Derived Xenograft

PSC: Pancreatic Stellate Cell

TAM: Tumor Associated Macrophage

VEGF: Vascular Endothelial Growth Factor

Pancreatic cancer is predicted to be the second-largest cause of cancer-related death by 2030 and fewer than 7% of patients survive for 5 years[1]. We therefore need to identify new therapeutic targets and radically rethink our approach to developing treatments for patients with pancreatic cancer. Pancreatic cancer progression is accompanied with a fibrotic stromal (desmoplastic) reaction, characterized by extensive deposition of extracellular matrix (ECM) components, recruitment and activation of cancer-associated fibroblasts (CAFs), decreased vasculature patency and altered immune-surveillance[2,3]. Stromal remodeling leads to altered interactions between tumor cells and stromal compartments, which can promote tumor progression[4]. Studies have shown that the stroma can promote and prevent pancreatic cancer progression, highlighting that multiple considerations should be taken into account when clinically translating stromal-based therapies[5].

We review the importance of the different stromal compartments, strategies for targeting them or re-shaping the pancreatic tumor stroma, and we explore their potential to improve outcomes of patients with pancreatic cancer. In particular, we outline how short-term, fine-tuned manipulation of interactions between cancer cells and the stroma, both in primary and metastatic sites (such as the liver), can improve the efficacy of chemotherapy and reduce growth of metastases while maintaining normal tissue functions. We discuss findings from studies reporting the intricate interactions between different elements of the stroma (such as the ECM, CAFs, immune cells, blood, and the lymphatic vasculature) and how these affect the development of new stromal-based treatments for pancreatic cancer. We also summarize recently developed diagnostic tools and pre-clinical models that can be used to assess individualized stromal-based therapies. Lastly, we discuss how discoveries from research on other types of tumors with high levels of fibrosis could be repurposed in pancreatic cancer.

Fine-Tuned Manipulation of the Interactions Between Tumor Cells and ECM

Within pancreatic tumors, extensive remodeling of the ECM can increase tissue stiffness to mechanically induce intracellular signaling that promotes disease progression[6]. Remodeling of the ECM does not occur evenly throughout the tumor—it was recently shown to be heterogeneous and spatially well-defined within pancreatic tumor tissues and to correlate with clinical and pathology features of patient tumors[7]. Although ECM remodeling has been proposed to be predominantly mediated by activated stromal cells such as CAFs[8], cancer cell

tension, mediated for instance by JAK signaling via STAT and ROCK, can tune the pancreatic ECM and thereby mechanically activate signaling pathways that regulate survival and metastasis in pancreatic cancer cells[7-9]. Similarly, increased stiffening of the ECM has been reported to promote the epithelial-to-mesenchymal transition (EMT) in pancreatic tumor cells, a key step of the metastatic cascade, and to reduce their response to chemotherapy[10-12].

Targeting mechanical features of the ECM

The mechanical features of the ECM can determine pancreatic cancer aggressiveness. Consequently, disruption of the mechanical feedback between tumor cells and the ECM, or mechano-reciprocity[6], has been evaluated as an approach to impair pancreatic cancer progression. Initial studies assessing ECM targeting have demonstrated that reducing fibrosis in pancreatic tumors is possible by inhibiting the fibrotic Hedgehog (Hh) signaling pathway[13] or by targeting hyaluronic acid (HA) with PEGPH20 (PEGylated hyaluronidase)[14,15] in *Pdx1-cre; Kras^{G12D}; p53$^{R/+}$* (KPC) mice bearing primary tumors[16,17]. These strategies led to reduced intra-tumor pressure, increased vasculature patency, and longer survival times of KPC mice.

The efficacy of anti-ECM agents in combination with chemotherapy has also been assessed in clinical trials of patients with pancreatic cancer. For instance, vismodegib, IPI-926 (hedgehog inhibitors, NSC74769[18] and NCT01383538[19]) or PEGPH20 (NCT01839487[20], Table 1) have been tested in combination with chemotherapy. In addition, PEGPH20 is also currently being tested in patients where high HA deposition in their tumors is assessed as a marker for response to treatment (HaLo 109-202, Table 1). The promising interim results from these trials led to a Phase 3 trial for PEGPH20 in combination with gemcitabine and abraxane (NCT02715804, HaLo 301). Moreover, a phase 1b/2 study of PEGPH20 in combination with anti-PDL1 cancer immunotherapy is also underway[21] (Table 1).

Inhibition of lysyl oxidase (LOX), an enzyme required for collagen biogenesis and crosslinking, which is overexpressed in hypoxic tumor environments, was also assessed in KPC mice. In KPC mice with pancreatic primary tumors, the combination of a LOX blocking antibody with gemcitabine reduced ECM crosslinking, blocked metastasis, and increased survival times, compared to gemcitabine alone[22]. The increased efficacy of gemcitabine upon LOX inhibition was not due to increased vasculature patency or drug delivery, suggesting that manipulation of the ECM and of mechano-reciprocity using LOX inhibitors might

deprive cancer cells of mechanical survival cues that promote metastasis and resistance to treatment[22]. Interestingly, inhibition of LOX in combination with gemcitabine in mice with locally advanced tumors, with a well-established matrix, did not significantly increase survival[22]. As LOX inhibition blocks only progressive cross-linking of the ECM and does not reverse previous LOX-induced changes to the ECM, these findings indicate that agents to manipulate the ECM are likely to have tumor stage-dependent effects. In light of this, ECM biomarkers could be used to identify tumors most likely to respond to these agents.

Studies of stroma-targeting agents in mouse models of pancreatic tumors have mainly been tested in mice with early-stage (primary) tumors, and have provided insights into the effects of long-term stromal manipulation[14, 15, 23-25]. However, most patients present with late-stage pancreatic cancer and metastases. Consequently, there are valid arguments for testing anti-stroma agents in mice with early- or late-stage tumors to optimize stromal agents in combination with standard-of-care therapies in both settings[26, 27] (Fig. 1A). In addition, studies using human pancreatic tumor tissues and mathematical modeling have shown that these tumors do not always progress in a linear or gradual manner, but rather can be a result of fast and simultaneous accumulation of genetic alterations that lead to early dissemination of tumor cells[28]. This finding suggests that testing anti-stroma agents in mice with localized primary tumors and with metastatic tumors, rather than optimizing the timing of anti-storma agent administration, could be beneficial.

Tissue stiffening is also mediated by cell contractility, which is in part regulated by Rho kinase (ROCK) signaling [29-31]. Expression of ROCK1 and ROCK2 were recently found to be increased in human pancreatic tumors with stage and grade, and genomic alterations in *ROCK1* and *ROCK2* correlated with shorter survival times of patients[32]. Interestingly, ROCK2 activation in non-invasive pancreatic cancer cells promoted their invasion of a collagen matrix and increased ECM remodeling, potentially via an increased release of matrix metalloproteinases (MMPs) into the surrounding environment[32]. In line with this, short-term inhibition of ROCK activity, via oral administration of fasudil as a priming agent before administration of a chemotherapeutic reduced fibrosis in pancreatic tumors[33]. Intravital imaging analyses of single cells in primary and metastatic pancreatic tumors showed that pulsed and iterative priming with fasudil, rather than chronic exposure to anti-ECM drugs (Fig. 1B), reduced ECM crosslinking, increased vasculature patency and enhanced the effects of chemotherapeutic agents [33]. Survival and proliferative stimuli provided by the ECM are

153 partly mediated by integrins and Src signaling, and these were also reduced in tumors primed
154 with fasudil. Src can promote progression of pancreatic tumors by reducing their responses to
155 chemotherapy[34] and increasing their invasive activities [35]. Because fasudil priming reduces Src
156 activity, anti-stromal priming agents such as these could potentially be employed as an anti-
157 invasive approach in pancreatic cancer. This is in line with recent assessment of Src
158 inhibition post-surgery in pancreatic cancer[36, 37]. Fine-tuned ROCK inhibition also reduced
159 cancer cell resistance to shear stress in the blood circulation, decreased cancer cell seeding in
160 the liver, and inhibited the establishment of a fibrotic environment that supports growth of
161 metastases [33], as recently reported in models of melanoma[38].
162 Together, these findings indicate that short-term, sequential and pulsed administration
163 of anti-fibrotic agents allows subtle manipulation of the ECM and deprives cancer cells of a
164 supportive mechanical niche[3, 6, 31, 33]. This is an important advantage of fine-tuned ECM
165 targeting, since chronic, systematic ablation of fibrosis can be accompanied with enhanced
166 metastasis and increased tumor infiltration by immune cells that support tumor progression [23, 24]
167 (Figs. 1B and 2A).
168
169 *Inhibiting the ability of the ECM to promote metastasis*
170 Although remodeling of the ECM accompanies primary tumor progression, alterations of the
171 tumor ECM can also mediate metastasis[39]. Changes of the ECM in distant organs before
172 seeding of metastatic cells can be mediated by exosomes released by primary tumor cells. For
173 instance, pancreatic cancer cell-derived exosomes can accumulate in other tissues, such as
174 the liver, to create a pre-metastatic niche by activating hepatic stellate cells and Kupffer
175 cells[39-41]. This was shown to induce remodeling of the host ECM and to facilitate cancer cell
176 invasion and growth in the liver.
177 Surgical resection of primary tumors has also been reported to alter the ECM in other
178 tissues such as the lungs[42], and to thereby increase the ability of circulating tumor cells to
179 form metastases at these sites compared to mice that were not undergoing surgery[42]. Given
180 that approximately 20% of patients with pancreatic cancer are eligible for surgical resection
181 of primary tumors, this may have implications in this disease too. In addition, local changes
182 to the ECM in secondary organs can reawaken disseminated and dormant tumor cells
183 (identified as single, non proliferative tumor cells), which could then form metastases.
184 Activation of dormant tumor cells by a fibrotic matrix can be prevented by blocking

mechanical interactions between tumor cells and the ECM[43]. Conversely, cell quiescence and dormancy can be induced by ECM components such as lumican—further highlighting how the ECM can promote and impair tumor progression at multiple stages[44]. FOXO4 was recently identified as a regulator of cell senescence and dormancy, and inhibiting interactions between FOXO4 and p53 with a FOXO4 peptide caused apoptosis specifically in senescent and dormant cells [45]. Combinations of such pharmacological agents that induce death of dormant cells and anti-stroma agents could be repurposed to prevent cancer recurrence caused by dormant pancreatic cancer cells [46,47].

Collectively, these studies show how primary tumor can induce early stromal alterations in other tissues to promote cancer spread. This highlights the need for assessing anti-stromal agents in combination with chemotherapy in the neo-adjuvant and adjuvant settings to reduce the risk of tumor dissemination[22,39-41,48-50] (Fig. 1A). In addition, in patients with late-stage pancreatic cancer, tissues containing metastases or pre-metastatic lesions have already recruited CAFs, increased the density of collagen I fibers and HA, become hypovascular, and induced changes in the anti-tumor immune response [51]. Consequently, strategies aimed at reversing stromal alterations in secondary sites to restore normal tissue homeostasis and mechanical properties might also impair pancreatic cancer progression (Fig. 1A).

Stromal Targets for Fine-Tuning the ECM in Pancreatic Cancer

Additional regulators of ECM stiffening and mechano-signaling, such as FAK, MMPs, SerpinB2, RhoA, JAK/STAT, YAP/TAZ, CDK4 and PAK, which are known to play vital roles in mechano-reciprocity and cancer development, might also be targeted to prevent pancreatic tumor progression [7,32,52-57] (Fig. 2A). For example, the activity of RhoA was recently demonstrated to switch during pancreatic cancer development and metastasis[53]. Given the role of RhoA in regulating the interactions between tumor cells and the surrounding stroma, this calls for careful consideration for the development of fine-tuned targeting of RhoGTPases in pancreatic cancer[58-60]. In addition, in pancreatic cancer cells that have lost p53 function (*TP53* is frequently mutated in pancreatic cancer cells)[61], JAK2 signaling via STAT3 has been shown to promote activation of pancreatic stellate cells (PSCs) and to increase ECM remodeling[9]. Similarly, mechanically induced FAK activity can help establish a fibrotic and immunosuppressive environment, and FAK inhibition in combination with immunotherapy

was shown to double survival times of mice with pancreatic tumors[54]. These observations led to studies of the efficacy of the FAK inhibitor defactinib in combination with anti-PD1 antibody and gemcitabine in patients with advanced pancreatic cancer (phase 1, NCT02546531; study still recruiting, see Table 1).

Importantly, these signaling pathways are not active in only pancreatic cancer cells. Indeed, changes in tumor–stroma interactions have been reported to affect the mechanical features of liver tumors [55], melanomas[62], breast tumors[49, 63], and glioblastoma[64]. Consequently agents designed to alter mechanical feedback from the ECM could also be beneficial in these contexts.

Simultaneous manipulation of distinct stromal compartments in pancreatic cancer

Although manipulation of the mechanical features of the ECM disrupts intracellular signaling and thereby promotes pancreatic tumor progression, alterations of the ECM can also induce changes in the intra-tumor vasculature. For instance, increases in matrix stiffness were shown to induce invasion of endothelial cells and formation of new vessels, potentially via upregulation of MMPs in endothelial cells. These changes also reduced vessel barrier function, as demonstrated in experiments in which Evans Blue was injected into mice, and could be reversed by blocking collagen crosslinking[65]. Similarly, increased tissue stiffness has been shown to induce cadherin 2 (CDHN) presentation on the surface of endothelial cells, thereby facilitating cancer cell interactions with the endothelium and metastasis[66].

Conversely, changes in tissue vascularity can modulate the properties of the ECM. For example, proteomic analyses of the ECM in decellularized pancreatic tissues undergoing angiogenesis revealed that numerous ECM proteins are differentially regulated during angiogenesis. These include fibrillin 1, Von Willebrand factor A domain containing 5a, and hemicentin; none of these had previously been associated with pancreatic cancer progression[67].

Due to the intricate interactions between the tumor ECM and vasculature, manipulation of one compartment might affect another stromal component. This was recently assessed in a mouse model of metastatic colorectal cancer, in which administration of blocking antibodies against VEGF to impair angiogenesis was associated with increased ECM remodeling and enhanced deposition of HA in liver metastases[68]. Enzymatic depletion of HA following anti-

VEGF administration increased tissue perfusion and thereby prolonged survival of mice, compared to mice that received only anti-VEGF agents [68], demonstrating the increased benefit of sequential targeting of both compartments (Fig. 1B and 2B). In line with this, manipulation of the ECM in fibrosarcoma increased the permeability of the tumor vasculature and response to anti-VEGF agents [69]. In addition, Frentzas et al reported interactions between the ECM and vasculature in liver metastases in human and in mice. Here, the authors demonstrated that metastatic emboli surrounded by a fibrotic capsule respond well to anti-angiogenic agents, whereas metastases progressing without a fibrotic tissue were resistant to these drugs [70]. These findings indicate the potential benefits of dual, or sequential, targeting of the ECM and vasculature and such approaches might be used in the treatment of pancreatic cancer (Fig. 1B). These studies also suggest that combinations of markers of the tumor ECM and vasculature might be used to identify patients most likely to benefit from dual manipulation of the ECM and vasculature.

Manipulating Pancreatic Tumor Immune Response

While pancreatic cancer cells often display oncogenic mutations that affect anti-tumor immunity, the fibrotic reaction also affects the immune response in pancreatic tumors [61, 71, 72]. For instance, low infiltration of tumors by T cells has been correlated with poor outcomes [73], and PSCs and CAFs have been reported to reduce T-cell infiltration of the tumor site[74]. Treating KPC mice with all-trans retinoic acid (ATRA) to render PSCs and CAFs more quiescent increased T-cell infiltration into pancreatic tumors, and prolonged survival [74, 75]. Furthermore, CAFs can secrete CXCL12, which binds to cancer cells and protects them from T-cell induced apoptosis[76]. Depletion of CAFs that express fibroblast-activation protein (FAP) increased T-cell infiltration of tumors and enhanced the efficacy of anti-PD-L1 [76, 77]. This suggests that manipulating, rather than eliminating CAFs (for which increased infiltration of tumors with immune cell that promote cancer progression has been reported) might increase the efficacy of immune-based therapies for pancreatic cancer (Fig. 2C). Such strategies could be achieved using pharmacologic agents, such as osteopontin-neutralizing antibodies or vitamin D, which have both been shown to deactivate CAFs[78, 79] (Table 1). In addition, ATRA is being tested in combination with gemcitabine and abraxane in a phase 1 clinical trial in pancreatic cancer (the STARPAC study); and given the interactions between CAFs and T

cells within tumors, ATRA could also be tested in combination with immunotherapy (Fig. 2C and D, Table 1).

Immune cells recruited to tumor tissues can also affect some features of the tumor ECM. Tumor-associated macrophages (TAMs) have been reported to promote deposition and crosslinking of ECM components such as collagens and fibronectin[80]. Immune cells might therefore also be involved in shaping the tumor ECM, and thus could be targeted to not only improve the anti-tumor immune response but also to reduce ECM stiffness in pancreatic tumors (Fig. 2D).

Although the work described above report that CAFs potentially reduce T-cell infiltration of tumors[74, 75], studies that mapped T cells within mouse and human pancreatic cancer tissues using multiplex immune-labelling and computational imaging did not correlate T-cell infiltration with the abundance of collagen I or alpha-smooth muscle actin (ACTA)-positive CAFs[81]. This implies that the relationships between T cells, CAFs, and fibrosis might be more complicated and heterogeneous than previously reported. A potential explanation of these results may be that rather than homogenous fibroblastic population, heterogeneous subtypes of CAFs co-exist in tumor tissues, and have distinct roles in promoting ECM remodeling, recruitment of immune cells, and response to therapy[82-84] (Fig. 2C). Researchers have described 2 populations of CAFs which are spatially separated in pancreatic tumor tissues: $ACTA^{High}$ CAFs and $ACTA^{Low}/IL6^{High}$ CAFs[84]. $ACTA^{High}$ CAFs were shown to promote ECM remodeling, whereas $ACTA^{Low}/IL6^{High}$ CAFs secreted higher levels of cytokines[84]. Moreover, CDHN-expressing CAFs have been shown to promote cancer cell collective invasion. Here, heterotypic interactions between CDHN on CAFs and cadherin 1 on cancer cells allowed the transmission of mechanical forces and induced collective cell movement[85] (Fig. 2C). Similarly, CAFs have been shown to have a heterogenous epigenetic signature and varying patterns of gene expression, and these are associated with ECM remodeling, angiogenesis, inflammation, and metastasis[86, 87]. In addition, although cancer cells can induce epigenetic changes in CAFs, targeted therapies might also affect their epigenetic regulation. Consequently, assigning CAFs to subgroups based on their histologic, epigenetic, mechanical and/or immunologic profiles could be used to target specific sub-populations while leaving other fibroblast populations intact (Fig. 2C, D). Together, these studies suggest that subtle, context-dependent targeting of specific CAF populations, rather than complete ablation of

CAFs, could be beneficial in pancreatic cancer (Fig. 2C, D). This aligns with observations discussed above for subtle manipulation of ECM stiffness.

During tumor progression, the vasculature also interacts with the immune system, and this has implications for the development of anti-stroma agents. For example, chronic stress was shown to induce dissemination of pancreatic cancer cells via the lymphatic vasculature network, and this was supported by TAMs. Interestingly, blocking the recruitment of macrophages to the tumor site reduced lymphatic network remodeling and the subsequent dissemination of cancer cells[88] (Fig. 2D). In addition, targeting beta-adrenergic stress-responsive signaling using beta-blockers reverted stress-induced lymphatic changes and reduced metastasis (Fig. 2B). Beta-blockers are already used in the clinic to control blood pressure, so their effects on pancreatic tumor metastases should be evaluated. Beta-blockers are being tested in a phase 2 trial of patients with breast cancer (ACTRN12615000889550, Table 1). In addition, in a phase 2 trial of patients with pancreatic cancer, beta-blockers will be combined with non-steroidal anti-inflammatory drugs, as a perioperative therapy (personal communication, M. Diener, E. Sloan, and I. Rooman).

Analyses of gene expression patterns in human breast cancer tissues demonstrated a positive correlation between expression of genes that regulate vessel normalization with immune-stimulatory signaling pathways[89]. In mice bearing tumors, disruption of vessel normalization reduced T-cell infiltration, while blockade of T-cell activity reduced tumor vessel pericyte coverage. In addition, immune checkpoint blockade increased vessel patency and reduced hypoxia in patient-derived xenografts (PDXs)[89]. Similarly, normalization of the vasculature using A2V, an inhibitor of angiopoietin 2 and VEGFA, led to recruitment of TAMs, dendritic cells, and T cells to different types of tumors, including neuroendocrine pancreatic tumors [90,91]. The studies also revealed that administration of A2V combined with PD1 blockade significantly increased T-cell activation and prolonged survival of mice [90,91] (Fig. 2B and D). Together, findings from these studies indicate that manipulation of the tumor vasculature and tumor immune response might be more beneficial than targeting a single stromal compartment (Fig. 1B); these findings could be applied to pancreatic cancer, in which both the tumor vasculature and immunity are compromised.

The development of immune-based therapies for pancreatic cancer has proven to be challenging, because of the tumor's poor antigenicity, dense fibrotic stroma and

immunosuppressive environment, leading to a paucity of infiltrating T cells [3]. Recently, immune-based targets have been identified and have shown promising results in mice. In patients with pancreatic cancer, expression of the neutrophil-homing receptor CXCR2 and its ligands[92,93] correlated with lower survival times [48]. Inhibition of CXCR2 using a small molecule inhibitor in KPC mice reduced ECM remodeling and increased infiltration of neutrophils, macrophages, and T cells into the tumor, while also reducing metastases. In addition, sequential blocking of CXCR2 to increase T-cell infiltration followed by administration of anti-PD1 significantly prolonged survival in mice with established tumors. This may be a promising treatment for pancreatic cancer and is being assessed in a phase 1 clinical trial (NCT02583477, Fig. 2A, D, Table 1). Similarly, TAMs can also affect response to treatment[94], and manipulating their effects has been suggested to increase the efficacy of immune checkpoint inhibitors. Indeed, TAMs can capture anti-PD1 antibody, potentially via Fcγ receptor, and prevent activation of T cells. Blockade of Fcγ receptor before administration of anti-PD1 antibody significantly increased the efficacy of immunotherapy, and such approach could be used for the development of immune-based therapies in pancreatic cancer[95] (Fig. 2D). The efficacy of anti-PD1 agents can be increased by administration of anti-OX40 agents. For instance, sequential administration of anti-OX40 to increase T-cell activation, followed by administration of anti-PD1 agents, delayed tumor growth and increased survival compared to anti-PD1 alone[96]. Importantly, concurrent administration of anti-OX40 and anti-PD1 agents, rather than sequential administration, did not increase survival times of mice but instead provoked a cytokine storm-like event. In addition, sequential administration of anti-PD1 agent first followed by OX40 blockade failed to increase survival, demonstrating that timing and order are crucial for the combination of anti-OX40 plus anti-PD-1 agents[96,97] (Fig. 2D).

These findings might guide clinical studies of the efficacy of anti-OX40 antibodies such as MOXR0916 and GSK3174998, in combination with anti-PD1/PDL1 agents for patients with solid tumors[98] (NCT02410512; NCT02528357, Table 1). Given the subtle balance of the tumor immune landscape in pancreatic cancer, sequential administration of immune-based agents, rather than concurrent administration should be considered for the development of immunotherapies[72] (Fig. 1B).

Effects of the Stroma on Cancer Cell Metabolism

Alterations in the stroma during pancreatic tumor progression have also been shown to affect cancer cell metabolism[99]. Given the metabolic switch occuring in cancer cells during tumor progression, this may have implications for the development of anti-stroma agents. Signaling from activated PSCs can induce metabolic changes in cancer cells, such as secretion of non-essential amino-acids, which fuel the tricarboxylic acid cycle and mitochondria metabolism in cancer cells[100, 101]. Similarly, patient-derived CAFs were shown to release exosomes, which can be taken in by cancer cells and inhibit mitochondrial oxidative phosphorylation, increase glycolysis and glutamine-dependent reduction of carbon in cancer cells[102]. On the other hand, cancer cell metabolic pathways may also shape some features of the ECM. As such, AMP-kinase, a metabolic sensor, was recently shown to regulate the activity of β1-integrin and to affect fibronectin remodeling induced by fibroblasts[103, 104]. Here, fibroblasts derived from AMPK knock-out mice assembled more fibronectin and had higher mechano-reciprocity, indicating a connection between metabolic activity and cell stiffness[103, 104]. Moreover, pancreatic tumor cells can scavenge and degrade extracellular proteins through macropinocytosis and thereby acquire nutrients that support their metabolic activity[105]. For instance, using a micro-device to deliver labeled extracellular proteins into pancreatic tumors, Davidson et al monitored albumin and fibronectin uptake and catabolic degradation specifically by cancer cells and not by adjacent non-cancerous pancreatic tissue[106]. In addition, blocking macropinocytosis in pancreatic tumors of mice via administration of 5-(N-Ethyl-N-isopropyl)-amiloride led to reduced levels of amino acids in pancreatic cancer cells. This could be an interesting approach to deprive cancer cells of metabolic factors provided by the extracellular compartment (Fig. 2E).

Obesity, a factor for pancreatic cancer[107], has been shown to trigger inflammation and fibrosis within pancreatic tumor tissues. As such, growth of pancreatic primary tumors, orthotopic xenograft tumors, and metastases were all accelerated in obese mice compared with lean mice [108]. Obesity was also reported to promote remodeling of the tumor ECM and vasculature and to reduce drug diffusion into tumor tissue. In addition, tumor infiltration by $Ly6G^+$ lymphocytes was increased in obese mice compared to lean mice and inhibiting these lymphocytes reduced activation of PSCs, decreased fibrosis, and increased vasculature patency[108]. This study demonstrates complex, subtle crosstalk between obesity, ECM remodeling, the vascular network, and the immune response within pancreatic tumors. It also

suggests that stromal intervention could be fine-tuned based on the metabolic profile of patients or pancreatic tumors.

Lastly, metabolic agents such as metformin (used to treat metabolic diseases)[109], lactate dehydrogenase[110], or glutaminase inhibitors[111] were recently suggested to be beneficial for patients with pancreatic cancer and could be combined with other anti-stromal drugs to slow development of pancreatic cancer (Fig. 2E). Serine and glycine were also recently shown to promote cancer progression in genetically engineered mouse models of intestinal cancer and lymphoma[112]. Restriction of serine and glycine prolonged survival of *Apc*$^{Min/+}$ mice, which develop intestinal adenomas. However, the anti-tumor effects of serine and glycine starvation were moderate in KPC mice, so although dietary changes appear to potentially impair cancer progression in some cases, specific genetic features of patients and their tumors may need to be factored in to predict response to serine and glycine starvation in combination with stromal therapies (Fig. 2E).

This body of work suggests that concomitant manipulation of multiple stromal compartments before administration of standard-of-care therapies can be more beneficial than single targeting alone (Fig. 1B). However, the timing of administration of stroma-targeting agents must be carefully optimized and balanced to maximize the effects of anti-cancer drugs. Recently, more than 10,000 sequential drug combinations were screened using systematic cell imaging and global Bayesian analysis and this was employed for melanoma and pancreatic cancer cell lines[113]. The authors identified multiple time-dependent, sequential drug combinations which may be relevant for the treatment of pancreatic cancer cells[113]. Screening platforms such as these could be combined with 3-dimensional tumor stroma models described below to optimize sequential administration of anti-stroma agents. In addition, chemotherapies not only kill cancer cells but can also have unintended negative effects on stromal compartments[114-116]. Therefore, the addition of the anti-stromal effects of chemotherapy with those of anti-ECM agents needs to be fine-balanced in order to maximize anti-tumor effects and minimize negative effects.

Technologies for Detection of Pancreatic Cancer

Although strategies to target the tumor stroma have the potential to improve outcomes of pancreatic cancer, the lack of sensitive diagnostic tools poses a critical challenge to early treatment. However, recently developed technologies such as liquid biopsies and live

imaging techniques could facilitate identification of early-stage tumors in patients (Fig. 3). Markers in the circulation, such as CTCs, circulating tumor DNA, carcinoembryonic antigen, and cancer antigen 19-9 (CA 19-9), have been used to detect pancreatic tumors in humans and in mice, in a non-invasive and cost-effective manner. However, tests for these markers sometimes lack sensitivity and yield high false-positive rates[117,118].

Recently, circulating extracellular vesicles (cEVs) in the blood of patients with cancer or of mice bearing tumors have been reported to successfully determine prognosis (Fig. 3A). Specifically, molecules carried by cEVs, such as microRNA23b3p3, microRNA10b, microRNA30c, mutant Kras, CD44v5, Tspan8, MET, and CD104 have been suggested to facilitate detection of pancreatic cancer [119-122].

Isolation of tumor-derived cEVs requires large volumes of blood and can be technically challenging, time-consuming, and costly. Platforms are being developed for faster and more accurate detection of cEVs from patients' blood. One such example is a plasmonic-sensing system that has been developed for high-throughput and cost-effective detection of cEVs[123] (Fig. 3A). This platform identified a cEV-based signature composed of 5 markers that correlate with presence of pancreatic cancer in patients[123]. Similarly, a nanoparticle-based chip was engineered for high-throughput identification of cEVs in small quantities in plasma samples[124] (Fig. 3A). Using this platform, EPHA2 was identified as a potential biomarker of early-stages pancreatic cancer and response to treatment in patients[124]. Alternative approaches for isolation of cEVs in patients, such as double-filtration microfluidics, sequential filtration, or surface plasmon resonance, which have been used for detection of tumor lesions in glioblastoma, prostate, ovarian, and breast cancer, might also be repurposed for detection of early-stage pancreatic cancer [125,126]. In addition, because of the role of cEVs in facilitating establish the pre-metastatic niche[39], cEVs could be monitored to detect tumor metastasis.

Given the extent of the stromal alteration during solid tumor progression, circulating stroma-derived markers have also been tested as potential diagnostic biomarkers. For example, circulating collagens fragments and thrombospondin-2 have been identified in serum and plasma of cancer patients [127,128], and persistence of collagen in the blood following surgery have been suggested to predict disease relapse or poor outcomes of patients with pancreatic cancer,[129] as well as metastatic disease in patients with colorectal cancer[130]. Similarly, circulating markers of collagen turnover such as MMPs and tissue inhibitor of metalloproteinases have been found in the serum of cancer patients and might be used to

detect fibrotic changes occurring during pancreatic cancer progression[131, 132]. Circulating CAFs (cCAFs) have also been detected in mouse models of breast and lung cancer as well as in blood from cancer patients, and this correlated with metastatic disease[133, 134] (Fig. 3A). This could be used to detect pancreatic tumors or to monitor tumor response to treatment. Similarly, immune cells that recognize tumor antigens, such as monocytes and neoantigen-specific lymphocytes, were found in blood of patients with melanoma or colorectal cancer[135, 136] (Fig. 3A). Given the immune reaction occurring during pancreatic cancer, circulating immune cells that recognize tumor antigens might represent additional markers to identify patients with early-stage pancreatic cancer[72].

In patients, imaging technologies such as computed tomography, endoscopic ultrasound or positron emission tomography (PET), have been used to detect pancreatic tumors and to monitor cancer progression and response to treatments (Fig. 3B). Given the stromal alterations occurring during pancreatic cancer development, imaging technologies can be developed that might allow clinicians to detect changes in the pancreatic stroma in a non-invasive manner, while also providing information about the tumor response to stroma manipulation.

Technologies have been developed to image changes in pulmonary and liver fibrosis in patients, and might be used to detect fibrotic alterations in patients with pancreatic cancer. A PET-based probe was recently developed for the detection of young and fibrotic collagen in patients with idiopathic pulmonary fibrosis[137]. The probe allowed for sensitive detection of fibrotic tissue in the lungs, staging of disease development, and monitoring the efficacy of anti-fibrosis agents in mice and patients [137] (Fig. 3B). Similarly, cathepsin protease probes and PET probes to detect $\alpha_v\beta_6$-integrin were engineered for non-invasive imaging of fibrotic tissue in lungs and liver[138, 139]. In addition, features of the gut microbiome were also shown to serve as markers of fibrotic changes in the liver[140].Together, tools assessing gastro-intestinal cancers could be repurposed for detection of fibrosis in early-stage pancreatic tumors and other types of cancer, at primary and metastatic sites. Measurements of tissue stiffness could also be achieved by revisiting techniques such as elastography, previously used to detect fibrotic tissues following liver transplantation[141], or using magnetically responsive ferrofluid microdroplets, which have recently been used to assess mechanical events that promote organ development[142] (Fig. 3B).

Tumors might also be stratified based on their fibrotic signature for instance using automated second harmonic generation imaging (SHG)[53], which provides label-free imaging of non-centrosymmetric entities such as crosslinked collagen fibers, or using immunohistochemical staining of HA content in patient biopsies (clinical trial HaLo 109-202 and HaLo 301[143], Table 1). These approaches might guide the development of personalized anti-stroma manipulation for patients with pancreatic cancer. Importantly, although the molecular profiles of pancreatic tumors are highly heterogeneous[61, 144-148], these studies suggest that stratifying patients based on a tumor's stromal signature, rather than solely that of the cancer cells, might provide the most useful information for the development of precision stromal medicine in combination with chemotherapy.

The metabolic switch that occurs in cancer cells during tumor development has been used as the standard for detection of a tumor mass in an organism. Our increasing understanding of tumor metabolism has led to the development of tools for detection of precursor pancreatic cancer lesions[89]. For instance, 18-FDG PET imaging relies upon imaging tumor's avidity for glucose, whereas glutamine-based PET probes have been developed for detection of tumors in animals and have been tested in patients with glioblastoma[149, 150]. Similarly, pancreatic cancer progression has been shown to be accompanied by a decrease in the ratio of alanine:lactate in primary tumors of mice, and this can be imaged using ^{13}C magnetic resonance[151] (Fig. 3B). This approach could be used for early, non-invasive, radiation-free detection of pancreatic cancer. Finally, subtle changes in the tumor vasculature were recently detected in mice with ovarian or breast tumors using non-invasive techniques such as ultrasound imaging[152-154], Doppler ultrasonography,[155] and optical coherence tomography[156]; these approaches could also be used for patients with pancreatic cancer (Fig. 3B).

The recent development of sensitive, cost-effective, and faster diagnostic tools could facilitate early detection of tumor lesions. This would allow for earlier therapeutic intervention and/or surgery to be offered to a larger number of patients.

Modeling the stroma and patient's response to stromal manipulation

Testing the efficacy of anti-stroma agents can be facilitated using in vitro and in vivo tools that mimic some stromal compartments of human tumors. These tools allow researchers to optimize manipulation of the stroma before clinical assessments. Each of these models is

specifically designed to study specific events occurring during cancer progression (see Figs. 4 and 5). More detailed information on the current and future applications as well as caveats of these models can be found in Fig. 4 and Fig. 5. For instance, tumors that develop in genetically engineered mice and PDXs recapitulate many of the stromal and genomic features found in the tumor environment[157-161], and are powerful tools for development of strategies for precision cancer medicine [33, 56, 162-167] (Fig. 4A). Organoids are also emerging as miniature platform for studying tumor development [168-170]—stem cells, cultured under specific conditions, spontaneously generate structures that contain much of the architecture, functions, and genetic features of the tissue of origin[171-174]. Organoids display some features of the tissue stroma, and might be used to study the effects of anti-stromal agents in combination with chemotherapy[84, 175]. In addition, the generation of organoids derived from patient tissues may facilitate the development of individualized therapies in pancreatic cancer [84, 176-178] (Fig. 4B).

Three-dimensional organotypic matrices, designed based on specific features of individual pancreatic tumors, have been developed to optimize selection of anti-stroma agents [33, 56, 179]. In this system, patients' cancer cells and fibroblasts that have been exposed to the cancer cells are used to mimic tumor–ECM interactions in a collagen matrix[33, 56]. This approach can be used for faster testing of stromal manipulation before assessment in vivo (Fig. 4C). This is relevant to pancreatic cancer pre-clinical research because of the need and interest for precision medicines, due to pancreatic tumor heterogeneity [61, 144-147].

More insights into the properties and functions of the ECM were also provided by de-cellularization protocols, which allow the complete removal of cells from tissues, leaving the native ECM intact[180-183]. This technique was recently used to catalogue matrix alterations that occur during breast cancer development in multiple sites and has been used to catalogue ECM and angiogenic changes in pancreatic tumors [67, 180] (Fig. 5A). Lastly, bioengineered scaffolds with adjustable properties enable researchers to generate 3-dimensional tailored matrices with controlled mechanical and biochemical features. These tools have been used to investigate how the ECM promotes angiogenesis, cancer cell intravasation, drug diffusion, migration of endothelial cells[184, 185], EMT [10] and the metabolic activity of pancreatic cancer cells[186, 187] (Fig. 5B). Together, these approaches could provide important insights into tumor–stroma interactions occurring during tumor progression and could guide the development of stroma-targeting agents for patient-specific treatment of pancreatic cancer.

Future Directions

Agents and fine-tuned strategies designed to target the specific stromal features of pancreatic tumors offer new opportunities for the development of stromal-based therapies in this disease. Fine-tuned manipulation of the tumor stroma, using carefully timed, sequential targeting of multiple stromal compartments can deprive cancer cells of the supportive stromal niche in primary tumors and metastases, without disrupting most normal tissue functions. The stromal fingerprint of pancreatic cancer, like its epithelial counterpart, is heterogeneous, and as such the development of stromal-based biomarkers may facilitate identification of patients that could benefit from subtle manipulation of the stroma prior to, and in addition to standard-of-care therapy. Pancreatic cancer treatment is in an exciting phase, where fine-tuned, sequential treatment regimens as well as targeting of specific stromal compartments are set to improve patients' outcome in this devastating disease.

Figure 1. Manipulating the tumor stroma reduces progression and metastasis

A) Pre-clinical assessment of stromal manipulation in the context of primary tumors and metastatic disease.

B) Limitations and benefits of long-term exposure to stromal agents (i), pulsed and iterative administration of anti-stromal agents (ii) and sequential targeting of multiple stromal compartments (iii).

Figure 2. Stromal targets in pancreatic tumors

A) Agents and targets for manipulation of the ECM. Left: SHG imaging of the ECM in subcutaneous xenografts; right hand image: polarized image of picrosirius red staining in subcutaneous xenografts. Adapted from[33].

B) Strategies for normalization of the tumor vasculature. Red: blood vessel (quantum dot); blue: collagen (SHG signal).

C) Approaches to induce quiescence in CAFs. Fluorescent image of a spheroid containing A341-EcadKO cancer cells (magenta) and CAFs (blue). Scale bar: 100 μm. Adapted from[85].

D) Immune-based therapies for pancreatic cancer. Infiltration of $CD45^{+}$ immune cells (red) in pancreatic tumor tissues, containing cancer cells (blue) and CAFs (green). Adapted from[134].

E) Approaches for blocking the metabolic switch associated with pancreatic cancer progression. Images show the mass-spectrometry signal used to detect aspartate and glutamate (left) and overlaid onto a bright-field image of the tissue section (right). Adapted from[106].

Figure 3. Tools for early detection of pancreatic cancer

A) Liquid biopsies can be used to identify patients with pancreatic cancer. (i) Circulating extracellular vesicles can be detected using high-throughput plasmon sensor chip (left, adapted from[123]) or nanoplasmonic technologies (right, adapted from[124], scale bar=2 μm). (ii) Detection of circulating CAFs (green) clustered with CTCs (red) (adapted from[134]). (iii) Isolation of circulating, immune cells (lymphocytes) from patients' tumors and matched peripheral blood mononuclear cells (PBMCs) by flow cytometry (adapted from[135]).

B) Imaging and detection of stromal alterations occurring during pancreatic cancer progression in patients (i) Detection of fibrotic tissue using PET-based probes. Image represents detection of fibrotic tissue in the liver using PET-based probe, adapted from[137]. (ii) Vizualisation of liver fibrosis and of changes in the mechanical properties of tissue using acoustic radiation force impulse elastography (adapted from [141]). (iii) Detection of aberrant metabolic activity by monitoring alanine:lactate ratio using ^{13}C magnetic resonance imaging in mice with pancreatic tumors and metastases (adapted from[151]). (iv) Mapping of subtle changes of the tumor vasculature using acoustic angiography (adapted from[154], left panel: acoustic angiography image of the tumor and surrounding tissue; right: vessel segmentation following acoustic angiography) and via micro-ultrasound and photoacoustic imaging. Image represents heat map of wash-in of gas-filled micromarker in pancreatic tumor tissue. Blue: low-wash-in; red, high wash-in. Adapted from [153].

Figure 4. Three-dimensional in vitro and in vivo models of tumor–stroma interactions for development of personalized treatment

Description, applications, limitations and future directions of (A) patient-derived xenografts; (B) organoids (image adapted from[178] and representing pancreatic organoids cultured for 2 weeks in human complete media. hN1: organoid derived from human normal pancreas, hT1: organoid derived from human pancreatic tumor) and (C) personalized organotypic matrices (adapted from[33]).

Figure 5. Three-dimensional tools for studying tumor–stroma interactions and testing anti-stroma agents

Description, applications, limitations and future directions of (A) decellularization protocols (image adapted from[180], top: image of polymer casting in the vascular compartment of pulmonary ECM, lower: fibril-orientation analysis overlay of SHG in decellularized tissue) and (B) 3-dimensional bioengineered scaffolds (image adapted from[187] and representing scaffold engineered using melt electrospun).

Table 1: Clinical trials in pancreatic cancer assessing stroma manipulation.

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Conflict of interest statement

No competing or financial interests declared.

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