9. Therapeutic targeting of pancreatic stroma

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is the fourth highest cause of cancer-related deaths in the United States, and for the vast majority of patients chemotherapy is the only course of treatment. The recent use FOLFIRINOX has provided the most significant survival benefit (~4 months) of any treatment brought to the clinic in nearly two decades. Despite recent advances, chemotherapeutics have proven woefully inadequate to treat this cancer. One of the hallmarks of PDAC is the presence of an extensive desmoplastic reaction consisting of pancreatic stellate cells, fibroblasts, immune cells, vasculature, and extracellular matrix. The complex interaction between cancer cells and the tumor microenvironment in PDAC is beginning to be understood, and the disruption of these interactions is a promising new avenue for therapeutic targeting of PDAC. In this chapter we will discuss a variety of therapies now entering the clinic that specifically target the tumor stroma. Among these are therapies that reduce the tumor stroma, allowing for a more effective delivery of conventional therapeutics to the cancer cells; antibodies that activate a macrophage-mediated immune response to the tumor and overcome its immunosuppressive environment; and inhibitors of signaling pathways that promote the vascularization of the tumor.
**Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is only the 14th most common cancer, but it is the fourth highest cause of cancer related deaths in the United States. Despite a dismally low five year survival rate (less than 5%), research on this disease has been limited due to the small number of people affected and an elderly patient population. Currently the only hope for patients diagnosed with PDAC is surgical resection. However, 80% of patients present with locally advanced or metastatic disease at the time of diagnosis, making them ineligible for surgical intervention. The standard of care for these patients for the past two decades has been chemotherapy with the nucleoside analog gemcitabine. However, the use of gemcitabine to treat these patients has resulted in only a five-week increase in median survival [1]. Combining gemcitabine treatment with platin-based agents (oxaliplatin and cisplatin) or a thymidylate synthetase inhibitor (capecitabine) has failed to enhance its therapeutic potential [2-4]. A phase III study has demonstrated that relative to treatment with gemcitabine alone, combinatorial treatment with erlotinib, an EGFR inhibitor, and gemcitabine results in a modest increase in median survival (6.24 vs. 5.91 months) for patients with unresectable PDAC [5]. More recently the use of FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin) in the treatment of metastatic PDAC has shown dramatic results. A phase II/III trial with 342 patients demonstrated that FOLFIRINOX improved median survival of patients by more than four months relative to gemcitabine (11.1 months vs. 6.8 months) [6].

Most, if not all, of the chemotherapeutic regimens for PDAC are directed at the cancer and not the microenvironment. The stroma of PDAC tumors is composed of abundant extracellular matrix and a complex set of cells including pancreatic stellate cells, fibroblasts, immune cells, and vasculature, each of which contributes to the survival and spread of the disease. There is a complex interplay between the cancer cells and the various components of the tumor microenvironment. Recently there has been a greater focus on defining how the stroma contributes to tumorigenesis; the results of these studies have begun to reveal novel therapeutic interventions for this cancer (Figure 1).

In this chapter we will review the efforts that have been made to develop therapeutics that specifically target the various components of the stroma. We will discuss classical approaches that target the tumor vasculature and early events in epithelial-to-mesenchymal transitions that promote the invasiveness of cancer cells. In addition, we will describe exciting new therapies that target paracrine pathways that promote angiogenesis, desmoplastic response and chemoresistance, as well as the host immune response.
Targeting the stroma to enhance conventional chemotherapeutics

PDAC is characterized by an extensive desmoplastic reaction which has recently been implicated in the chemoresistance of PDAC (Chapter 3). These tumors contain an abundant stroma and poor vascular perfusion, resulting in a tumor vasculature that rarely comes in close proximity to the adenomatous component of the tumor [7]. It has been proposed that the failure of chemotherapeutics to effectively treat this disease is not due to the inherent resistance of this cancer to the drugs, but rather to an inability of the drugs to reach the cancer cells. Recent work targeting the Sonic hedgehog (Shh) signaling pathway suggests that reducing desmoplasia in PDAC allows for a more effective delivery of chemotherapeutic agents to the tumor.

Sonic Hedgehog pathway

Shh is a lipid-modified protein that is secreted by cells and is involved in the regulation of developmental programs during embryogenesis; it is
described in detail in Chapter 6. Briefly, in the absence of Shh, the signaling pathway is kept inactive by Patch (Ptch) proteins, Ptch1 and Ptch2, which are localized at the base of primary cilia (Figure 2). These twelve-pass membrane-spanning proteins prevent the localization of the seven-pass membrane-spanning protein Smoothened (Smo) to the primary cilia of cells. The binding of the secreted Hh ligands to Ptch relieves this inhibition, allowing for the relocalization of Smo from vesicles to the primary cilia, a key step in the activation of downstream signaling. The activation of Smo results in the activation of the Gli family of transcription factors (Gli1, Gli2, and Gli3). Gli1 is believed to function as a transcriptional activator while Gli2/3 are transcriptional repressors. In the absence of Hh signaling Gli1 is transcriptionally silent and Gli2/3 undergoes proteolytic cleavage and functions to repress Hh-specific genes. Upon activation of the pathway, processing of Gli2/3 is reduced and there is a strong expression of Gli1, making

Figure 2. Model of the Shh signaling pathway. A. In the absence of Shh, the Ptch receptor inhibits Smo-containing vesicles from fusing to the cell membrane. Gli proteins are localized to the primary cilium by association with SuFu. Proteolytic processing of Gli creates a transcriptionally inactive form (Gli\textsuperscript{r}) which enters the nucleus to silence Shh target genes. B. The binding of Shh to Ptch allows for Smo localization to the primary cilium, resulting in the release of transcriptionally active Gli (Gli\textsuperscript{a}) from SuFu. Active Gli binds to the promoters of Shh responsive genes to activate transcription. Inhibitors that block the Shh signaling pathway are shown.
Gli1 one of the best characterized Hh responsive genes and one of the strongest predictors of Hh pathway activation.

Recent studies have demonstrated that Shh exerts its effect principally through a paracrine pathway in PDAC. These experiments have shown that Shh produced by tumor cells plays a key role in the recruitment and maintenance of the tumor mesenchyme. Mouse models employing orthotopic tumors of PDAC demonstrates a reduced desmoplasia in tumors from mice treated with neutralizing antibodies against Shh [8]. Further, tumors formed by transformed pancreatic ductal epithelial cells that overexpress Shh exhibit a greater desmoplastic reaction than tumors derived from control cells. In addition to their role in promoting the desmoplastic reaction observed in PDAC, the stromal cells of these tumors uniquely exhibit evidence of Hh pathway activation. Microarray analysis of microdissected human tumors reveals that cancer-associated fibroblasts express high levels of Smo [9]. Additional studies have demonstrated that Gli activity is restricted to the stroma [10] and that Smo is localized on the primary cilia of stromal fibroblasts and vessels [11].

Preclinical modeling with a genetically engineered mouse model of PDAC has provided evidence for the potential clinical benefit of targeting Shh in the treatment of PDAC [7]. In this study, mice treated with gemcitabine and the Shh inhibitor IPI-926 exhibited a greater than two-fold increase in survival relative to gemcitabine alone. Of particular interest was the mechanism for this enhanced survival. Shh inhibitor-treated mice (+/- gemcitabine) had tumors that were depleted of the stromal compartment after one week of treatment. Shh inhibitor-treated tumors had a higher mean vascular density than control tumors. Although this effect was transient, it correlated with better delivery of gemcitabine to the tumor cells. These results suggest that one of the mechanisms of the poor therapeutic response of PDAC to gemcitabine is the inefficient delivery of this drug to the cancer cells. Disruption of the tumor microenvironment with Shh inhibitors to allow for better drug delivery affords a novel treatment option.

The results of this preclinical study provide exciting evidence for the use of Shh inhibitors in the therapeutic treatment of PDAC. Due to the role of Hh pathway activation in the development of a wide variety of cancers, there have been extensive efforts to develop pharmacological agents to inhibit this pathway. Direct inhibition of Shh activity has been employed in preclinical animal testing and cell culture assays with neutralizing antibodies, such as 5E1 [11-13]. A recent report identified a small molecule antagonist of Shh, robotnikinin [14]. However, there are presently no therapies in the clinic that target Shh.
Drugs have been developed to inhibit Shh signaling at various points along the signaling pathway. Most of these therapeutics are directed at Smo and have been studied in several solid tumors, most recently PDAC. The first Hh inhibitor identified was the naturally occurring compound cyclopamine [15]. This teratogenic compound was discovered, in part, based on the induction of holoprosencephaly in lambs born to ewes that consume *Veratrum californicum* during pregnancy [16, 17]. Cyclopamine directly binds to the heptahelical bundle of Smo, altering its conformation to one that is incapable of downstream signaling [18]. The first synthetic molecule directed at Smo was developed by Curis, Cur61414 [19]. Since that time a number of chemical inhibitors targeting Smo have been developed and brought to the clinic, including GDC-0449 (Genentech/Curis), IPI-926 (Infinity Pharmaceuticals), LDE225 (Novartis), BMS-833923 (Exelixis/Bristol-Myers Squibb), and PF-04449913 (Pfizer) [20]. For all of these compounds there are no fewer than 49 clinical trials currently in progress in the United States. GDC-0449 and IPI-926 are the best studied among them; to date there are six clinical trials utilizing these two compounds in the treatment of PDAC (*Table 1*). Preliminary results from a Phase Ib/II trial using IPI-926 in combination with gemcitabine reveal that the drug is well tolerated in patients with metastatic pancreatic cancer; radiographic partial responses were seen in 3/9 patients [21].

In addition to targeting the upstream Shh signaling pathway, efforts have been made to target the final step of this pathway, the Gli family of transcription factors. This has become increasingly important as recent studies have demonstrated a growing number of signaling pathways that modulate the expression and activity of this family of zinc finger transcription factors. Due the complex regulation of Gli proteins, the identification of inhibitors that directly target these transcription factors has been challenging. The activity of Gli proteins is also affected by a variety of kinases including MAPK, PI3K, and PKA [22-24]. Many of the compounds identified as inhibitors of Gli may actually modulate one of these regulatory proteins and therefore likely have broader effects on the cell than the Hh pathway. A classic example of this can be found in forskolin, which through the activation of adenylate cyclase upregulates PKA, resulting in the maintenance of repressive Gli isoforms [24]. More recently identified inhibitors of Gli, acrylaflavin C and physalin F, indirectly antagonize Gli activity through the PKC/MAPK pathways [25]. Compounds known to directly affect Gli activity have now been identified. Two such molecules are GANT58 and GANT61. These compounds were identified in a cell-based screen for their ability to inhibit Gli1 transcriptional activity [26]. They appear to affect Gli activity in the nuclei of cells, as they are effective inhibitors
of a Gli protein with a mutant nuclear export signal. Targeted pathway analysis and microarray experiments did not detect additional pathways that were altered by these compounds, and cells treated with GANT61 exhibited reduced Gli DNA binding. A recent report has described the identification of four novel small molecule inhibitors of Gli, HPI-1, HPI-2, HPI-3, and HPI-4 [27]. Each of these compounds inhibits Hh signaling downstream of Smo, but employs unique strategies. HPI-1 is able to block the activity of both Gli1 and Gli2. The inhibition of Gli2 activation is likely accomplished by an indirect mechanism that prevents the conversion of Gli2 repressor to activator. In contrast, inhibition of Gli1 by HPI-1 is more likely direct, as it results in increased levels of Gli1 in cells. HPI-2 and HPI-3 both block Gli2 activator formation. Although their mechanism of action is not yet defined, these compounds result in Gli2 accumulation in the cilia, suggesting that they function through a cilia-dependent step. HPI-4 nonspecifically blocked Gli activation through the perturbation of cilia formation in cell lines. While the development of therapeutic compounds that inhibit Gli activity is still in its early stages, there appears to be a variety of druggable mechanisms to block the activity of these transcription factors.
Since PDAC cells require Smo-independent Gli activity for their survival, such compounds may have the added benefit of targeting both stromal and cancer cells [28].

The characterization of Shh in PDAC in recent years has focused on paracrine signaling to the cells of the stroma. This represents a change of experimental emphasis from earlier work which focused on an autocrine role for Shh. This shift resulted from experiments utilizing cell lines treated with the Smo inhibitor cyclopamine, which demonstrated altered biological effects on PDAC cell lines that did not correlate with Hh pathway inhibition. However, these results were not always clear, as observed in cyclopamine-treated cells that exhibited reduced proliferation and high levels of apoptosis [29]. Proliferation of these cells could be restored with an activator of Smo, but there was no protective effect observed for apoptosis, suggesting that only some of the biological changes were Hh-independent. Recent studies have demonstrated a number of signaling pathways that can activate Gli independently of Shh, including those found dysregulated in PDAC such as KRAS and TGF-β [28]. Additionally, recent work characterizing the cancer stem cell (or tumor initiating cell) population of PDAC has demonstrated that these cells express high levels of Shh [30]. Experiments with cells from PDAC and other cancers have shown that the cancer stem cell population is responsive to Shh inhibitors and that Shh makes these cells more susceptible to traditional chemotherapeutic drugs [31]. These studies demonstrate that in addition to playing a role in targeting the stroma, Shh inhibitors may exhibit direct effects on the cancer cells.

**Transforming growth factor-β pathway**

The observation that reducing the stromal component of the tumor by inhibiting Shh allows for a more effective delivery of conventional therapeutics opens the door for similar strategies. The transforming growth factor (TGF)-β pathway appears to have tremendous promise, as it has been implicated in the regulation of both the growth of cancer cells and the formation of the tumor microenvironment. This pathway is composed of three TGF-β ligands (TGF-β1, -β2, and -β3) and three TGF-β receptors [32]. The binding of ligand to the type II receptor allows the recruitment and phosphorylation of the Type I receptor. The TGF-β type I receptors contain intracellular kinase domains which phosphorylate and activate receptor-associated Smad proteins. These activated Smad proteins dimerize with Smad4 and translocate to the nucleus, where they act to regulate gene expression.
Elevated levels of TGF-β have been observed in PDAC and correlate with a reduced survival [33]. The mechanism by which TGF-β contributes to PDAC is likely multifaceted. TGF-β has been implicated in angiogenesis, immunosuppression, activation of pancreatic stellate cells, and promoting epithelial-to-mesenchymal transition and proliferation of PDAC cells. A role for TGF-β in promoting desmoplasia in PDAC is illustrated in studies demonstrating that primary cultures of pancreatic stellate cells exhibit an increased proliferation in response to media conditioned by PDAC cell lines [34]. These stimulated stellate cells exhibit an increase in extracellular matrix production that is dependent on TGF-β activity. The co-injection of PDAC cell lines and cultured pancreatic stellate cells into nude mice results in more rapid tumor growth and the formation of tumors with a greater desmoplastic response than observed with tumors formed from PDAC cell lines alone. Recent experiments characterizing fibrosis in a mouse model of pancreatitis implicate TGF-β in the activation of endogenous pancreatic stellate cells. A genetically engineered mouse that over-expressed Smad7 (an intracellular inhibitor of TGF-β signaling) in the pancreas exhibited reduced fibrosis and extracellular matrix production after induction of chronic pancreatitis [35]. These experiments further demonstrate that TGF-β signaling is required for the activation of pancreatic stellate cells, which have previously been implicated as the source of pancreatitis-induced fibrosis.

The TGF-β signaling pathway allows a variety of therapeutic mechanisms to be employed for its inhibition; examples of these may be found in clinical trials (Table 2). A number of approaches have been developed to prevent the binding of TGF-β to its cellular receptors, including antibodies that prevent signaling by binding to TGF-β or its receptors. A neutralizing antibody, fresolimumab (GC1008), which targets all three TGF-β molecules, is now in phase II trials for the treatment of metastatic breast cancer and malignant pleural mesothelioma [36]. Additionally, ligand trap approaches have been employed in preclinical models to inhibit TGF-β activity. Soluble forms of the TGF-β type II and III receptors have been employed to sequester TGF-β from the cellular receptors [37, 38]. Soluble forms of these receptors have been demonstrated to inhibit the invasiveness of PDAC cell lines and the formation of tumors in a xenograft mouse model.

The TGF-β type I and II receptors contain intracellular serine/threonine kinase domains which are responsible for downstream activation of Smad transcription factors. A number of inhibitors of these kinases have been developed, including LY2109761, which has shown efficacy in a preclinical model of PDAC [39]. Although an extensive analysis of the mechanism by
Table 2. Clinical Trials Investigating Inhibitors of the TGF-β Pathway.

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which these inhibitors affect tumorigenesis has not been performed, treatment of mice with orthotopic PDAC tumors with both LY2109761 and gemcitabine reduced both tumor burden and metastatic lesions relative to control mice. While this inhibitor has not been employed in patients, at least two kinase inhibitors (LY2157299 and LY573636) that target the TGF-β pathway have been developed by Eli Lilly and Company and are now in clinical trials for the treatment of cancers. LY2157299 is in phase Ib/II trials for the treatment of pancreatic cancer.

An additional approach that reduces the expression of TGF-β has been developed. Phosphorothioate antisense oligonucleotides that target TGF-β1 (AP 11014) and TGF-β2 (AP 12009) effectively reduce the secretion of TGF-β from tumor cells [40, 41]. In phase IIb clinical trials of patients with glioma, AP 12009 (trabedersen) provided a survival advantage relative to conventional treatments [42, 43]. Preclinical testing of AP 12009 in a mouse model of PDAC has demonstrated its therapeutic potential. AP 12009 dramatically reduced the secretion of TGF-β from PDAC cell lines and reduced the growth of orthotopically implanted xenograft tumors. Consistent with the varied roles of TGF-β in PDAC, AP 12009 diminished the immunosuppressive effects of PDAC cells and inhibited tumor angiogenesis in mice. Phase I/II clinical trials are now under way for adult patients with advanced melanoma, colorectal or pancreatic carcinoma [44]. An interim report reveals that 23 of the 33 patients treated to date had pancreatic adenocarcinoma. Median overall survival for pancreatic cancer patients in the 2nd schedule is 13.2 months. One of the patients in schedule 1 experienced a complete remission and is alive after 38 months, and two patients in schedule 2 have stable disease and are alive at 14.8 months after beginning AP 12009. Although a randomized phase II trial comparing this to standard treatment
remains to be done, these early reports suggest that AP 12009 has a good safety profile. Targeting the mesenchyme using TGF-β inhibitors may thus be a rational approach in the treatment of pancreatic cancer.

**Extracellular matrix**

A key feature of the microenvironment of PDAC is the extensive amount of extracellular matrix (ECM) found in the tumor. Pancreatic stellate cells are likely responsible for much of this extracellular matrix, as they have been shown to produce collagen type I and c-fibronectin in response to pancreatic cancer cells [34]. The breakdown of the ECM is critical for localized growth of cancer cells as well as their systemic dissemination. The matrix metalloproteinase family plays a key role in the regulation of the ECM and is a therapeutic target for the treatment of cancer.

**Matrix metalloproteinases**

The epithelial to mesenchymal (EMT) transition is one of the defining steps a cancer cell undergoes to become invasive and eventually metastatic [45]. One of the hallmarks of EMT is the upregulation of the matrix metalloproteinase (MMP) family of enzymes [46]. These enzymes play a key role in the breakdown of the basement membrane surrounding the cancer cells. The MMP family is composed of at least 24 structurally related proteins that have a diverse set of substrates including collagen, lamin, fibronectin, and gelatin [47, 48]. The enzymatic activity of these proteases can be regulated by a second family of proteins, tissue inhibitors of matrix metalloproteinases (TIMP 1, 2, 3, and 4) [49]. It is thought that the balance of MMP and TIMP proteins controls the integrity of the ECM and that alterations in the relative levels among these proteins potentiate malignant disease.

Elevated levels and/or activities of MMP1, 2, 3, 7, 9, and 11 have been observed in clinical samples of PDAC [50-55]. Likewise, elevated levels of TIMP 1 and 2 have been reported [51-53]. Recently the molecular mechanisms by which MMPs are activated in PDAC have begun to be elucidated. PDAC cell lines induce the expression of a membrane-bound MMP, MMP14, in response to Type I collagen [56]. The expression of MMP14 enhances the invasiveness and migration of these cells. Abundant levels of collagen are found in the stroma of PDAC, suggesting that the interaction of the cancer cells with this collagen initiates an invasive phenotype [57]. MMP2 is associated with the invasiveness of PDAC cells and the formation of the desmoplastic stroma [58]. Pancreatic stellate cells
are an additional source of MMP2 in PDAC tumors [59]. MMP9 has also been shown to enhance the invasiveness of PDAC cells as well as to play an important role in tumor angiogenesis [60]. Preclinical testing of inhibitors that target MMP family members has demonstrated potential efficacy of these drugs. In a chemically induced model of PDAC it was demonstrated that RO 28-2653, a specific inhibitor of MMP2 and MMP9, reduced the incidence of liver metastasis [61]. The use of a broad spectrum MMP inhibitor, batimastat, in an orthotopic mouse model of PDAC reduced the tumor volume and frequency of metastatic lesions relative to control mice [62, 63].

Because of these preclinical studies, two MMP inhibitors have been brought to clinical trials for the treatment of PDAC. A phase III trial enrolling 277 patients studied the efficacy of tanomastat (Bay 12-9566), an inhibitor of MMP-2, MMP-3, MMP-9, and MMP-13. A comparison of patients treated with tanomastat versus gemcitabine revealed that patients treated with gemcitabine exhibited a longer median survival (6.59 months vs. 3.74 months) as well as disease-free progression (3.5 months vs. 1.68 months) than those treated with tanomastat [64]. A second trial investigated the efficacy of marimistat, an inhibitor of MMP-1, MMP-2, MMP-7, MMP-9, and MMP-14 [65]. This study examined the combinatorial treatment of gemcitabine and marimistat compared with gemcitabine alone in a group of 239 patients. However, there was no difference in median survival, one-year survival, or disease-free progression. Despite the positive results of preclinical studies, there was no demonstrable benefit for the treatment of PDAC from the use of MMP inhibitors alone or in combination with traditional chemotherapy.

The failure of these MMP inhibitors to show clinical benefit was not limited to patients with PDAC. Despite the exciting promise of this class of drugs, there has been no benefit observed from these inhibitors in any cancer. There are likely many reasons for the failure of these drugs to elicit the desired biological change. The concept of targeting MMPs was based on the importance of these enzymes in the breakdown of the basement membrane allowing for invasion of the cancer cells. While this is an early event in tumorigenesis, the patients in these studies have advanced disease; perhaps these compounds would prove more effective in patients with earlier stage disease. Additionally, the lack of specificity in MMP inhibition by these drugs likely contributes to their ineffectiveness. Since their initial characterization in tumor progression, the large number of additional roles MMPs play has become more apparent. Not only have some MMPs (MMP-8 and MMP-12) been demonstrated to have anti-tumor effects, but there is a growing list of non-extracellular matrix proteins that are substrates for the
proteases [66]. Highly specific MMP inhibitors may prove to be a more effective approach to targeting this class of enzymes. MMP inhibitors that selectively target MMP-2, MMP-9, and MMP-14, alone or in combination, have demonstrated promising results in preclinical models [67-70]. It is hoped that selectively targeting those MMPs that play key roles in tumorigenesis may yield a more dramatic response in clinical trials.

Targeting the tumor vasculature

As a tumor grows beyond microscopic size it requires the formation of new blood vessels to provide nutrients and oxygen for growth and for the removal of metabolic byproducts. Tumors initiate the formation of new blood vessels through the process of angiogenesis. Unlike vasculogenesis, which forms new vessels de novo, angiogenesis forms new blood vessels through the remodeling of existing vasculature. With the exception of specialized conditions such as wound repair, this process is largely held in check after embryogenesis by the proper balance of pro- and anti-angiogenic factors [71]. In the tumor, the “angiogenic switch” is initiated by an increase in pro-angiogenic factors and/or a decrease in anti-angiogenic factors, allowing for the neovascularization of the tumor. While solid tumors efficiently promote angiogenesis, this event is not as orderly a process as occurs under normal conditions. Generally tumor vessels are composed of a single layer of both endothelial cells and basement membrane, and often lack surrounding smooth muscle [72, 73]. It is likely that these features of the tumor vasculature potentiate the migration of metastatic cells. The process of angiogenesis is complex and involves a large number of soluble and cellular factors [74]. Current efforts in targeting these pathways for drug development have focused on a few major pathways.

VEGF pathway

Some of the earliest factors studied in promoting angiogenesis are members of the vascular endothelial growth factor (VEGF) family. This family of secreted factors is composed of six members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF) [75]. Of these factors VEGF-A is the best studied and plays a key role in promoting the proliferation and migration of endothelial cells. Members of the VEGF family form dimers and bind to the VEGF receptor (VEGFR) family of receptor tyrosine kinases. This family is composed of VEGFR1, VEGFR2, and VEGFR3, which exhibit specificity with the VEGF factors they bind. VEGFR1 and VEGFR2 efficiently bind VEGF-A to promote the angiogenic
pathway, while VEGFR3 binds VEGF-C and VEGF-D and is mainly involved in lymphangiogenesis [76, 77]. The binding of VEGF to its cognate VEGFR initiates receptor dimerization, resulting in phosphorylation and activation of the intracellular kinase domain. Activated VEGFR2 exerts its angiogenic effects by stimulating phospholipase C gamma (PLCγ), resulting in higher levels of protein kinase C (PKC) activity [78-80]. While VEGF-A binds to both VEGFR1 and VEGFR2, the intracellular kinase activity of VEGFR1 is dispensable for the development of animals as well as tumor angiogenesis [81]. It appears that the role of VEGFR1 is to bind and recruit VEGFA from the microenvironment, facilitating its interaction with VEGFR2.

The elevated expression of VEGF has been observed in a wide range of solid tumors, and preclinical studies have demonstrated an important role for the VEGF pathway in tumorigenesis. Furthermore, there is evidence to suggest an important role for VEGF in angiogenesis of PDAC. PDAC cell lines secrete active VEGF-A [82]. Studies have also shown a correlation between levels of VEGF-A and the density of blood vessels in tumors [83, 84]. Elevated levels of VEGF-A in patients also correlate with greater disease progression. In addition to these expression studies, VEGF-A activity has been demonstrated to be important in the development of tumors in a mouse model [85]. Given the potential for VEGF inhibitors in a broad spectrum of cancers, there has been tremendous interest in developing inhibitors of this pathway, a number of which have been employed in clinical trial of PDAC.

Two strategies have been used to disrupt this pathway, inhibiting the binding of VEGF-A to its receptor and blocking the kinase activity of VEGFRs (Table 3). A number of monoclonal antibodies have been developed that specifically bind to VEGF-A, preventing its binding to VEGFR1 and VEGFR2. One such therapy is bevacizumab (Avastin), which was developed by Genentech and Roche [86]. Bevacizumab was the first clinically available therapy in the United States to block angiogenesis. More recently, ranibizumab, an Fab fragment of this antibody, was developed and approved for the clinic. Monoclonal antibodies that bind to VEGFR and prevent VEGF binding have also been developed. IMC-1121b specifically recognizes VEGFR2, preventing VEGF-A binding and subsequent VEGF signaling. Alternative methods to physically prevent VEGF-A function are also utilized. Aflibercept was developed by joining the extracellular domains of VEGFR1 and VEGFR2 with an IgG1 Fc region, creating VEGF-Trap [87]. Aflibercept sequesters VEGF-A, preventing its signaling through VEGFR. There are also a number of broad-spectrum RTK inhibitors that target the
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<td>Phase I/II</td>
<td>NCT0260364</td>
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<td>Bevacizumab</td>
<td>Gemcitabine and external beam radiotherapy</td>
<td>Efficacy of Neoadjuvant Chemoradiation for Potentially Resectable Pancreas Cancer</td>
<td>Phase II</td>
<td>NCT00557492</td>
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<td>Bevacizumab</td>
<td>Gemcitabine and Radiation Therapy</td>
<td>GDC-0449 and Erlotinib Hydrochloride With or Without Gemcitabine Hydrochloride in Treating Patients With Metastatic Pancreatic Cancer or Solid Tumors That Cannot Be Removed by Surgery</td>
<td>Phase II</td>
<td>NCT00878163</td>
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<td>Bevacizumab</td>
<td>Erlotinib, Cephitabine and Radiotherapy</td>
<td>Bevacizumab, Erlotinib and Cephitabine for Advanced Pancreatic Cancer</td>
<td>Phase I</td>
<td>NCT00614653</td>
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<td>Bevacizumab</td>
<td>Gemcitabine and Radiotherapy</td>
<td>Phase II Pilot Study of Preoperative Gemcitabine and Bevacizumab-Based Chemoradiation for Patients With Resectable Adenocarcinoma of the Pancreas</td>
<td>Phase II</td>
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<td>Bevacizumab and Soraferib</td>
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<td>Soraferib and Bevacizumab in Treating Patients With Refractory, Metastatic, or Unresectable Solid Tumors</td>
<td>Phase I</td>
<td>NCT00098592</td>
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<td>Soraferib</td>
<td>Gemcitabine and Radiotherapy</td>
<td>Phase I Study of Gemcitabine, Soraferib and Radiotherapy in Patients With Unresectable Pancreatic Cancer</td>
<td>Phase I</td>
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<td>Soraferib and Gemcitabine in Treating Patients With Locally Advanced or Metastatic Pancreatic Cancer</td>
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<td>PTK787/ZK 222584</td>
<td>RAD001</td>
<td>Everolimus and Vatalanib in Treating Patients With Advanced Solid Tumors</td>
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<td>A Phase I/II Study of the VEGF Receptor Tyrosine Kinase Inhibitor PTK787/ZK 222584 and Gemcitabine in Patients With Advanced Pancreatic Cancer</td>
<td>Phase I/II</td>
<td>NCT0185588</td>
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<td>Lenalidomide</td>
<td>Bevacizumab, Soraferib, Temsirolimus, or FOLFOX</td>
<td>A Phase I Study of Lenalidomide in Combination With Bevacizumab, Soraferib, Temsirolimus, or 5-fluorouracil, Leucovorin, Oxaliplatin (FOLFOX) in Patients With Advanced Cancers</td>
<td>Phase I</td>
<td>NCT01183663</td>
<td>Recruiting</td>
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tyrosine kinase activity of the VEGFRs. Sunitiab targets all three VEGFRs as well as other RTKs such as PDGFRs, FLT3, RET and c-Kit [88]. Sorafonib inhibits the activity of VEGFR2 and VEGFR3 as well as PDGFRs, FGFR1, FLT3, c-Kit, and MAPK [89-91]. Axitinib inhibits the activity of VEGFR1, VEGFR2, and VEGFR3 as well as PDGFRs and c-Kit [92].

The use of inhibitors of the VEGF pathway for therapeutic treatment of PDAC has not yielded promising results. A number of phase II and III studies have recently been published that evaluated the efficacy of anti-angiogenic therapies alone or in combination with conventional chemotherapy. A phase III trial was performed to evaluate the efficacy of axitinib treatment in combination with gemcitabine in patients with metastatic PDAC [93]. Despite promising results of earlier studies with this compound, no significant survival was observed in patients treated with axitinib with gemcitabine relative to those treated with gemcitabine plus placebo (mean survival 8.5 months vs. 8.3 months) [94, 95]. Similarly, a phase III trial of bevacizumab in combination with gemcitabine did not demonstrate any significant advantage over treatment with gemcitabine alone [96]. Preclinical studies that demonstrated a synergistic effect of anti-angiogenic agents with drugs that target the EGFR pathway have led to clinical trials that target both of these pathways [97, 98]. However, a phase II trial testing the EGFR inhibitor cetuximab with bevacizumab in 61 patients showed no clinical benefit with or without combinatorial treatment of gemcitabine [99]. A recently published phase III study evaluated the use of bevacizumab and erlotinib (an EGFR inhibitor) in 301 patients with advanced cases of PDAC [100]. No meaningful change in overall patient survival was observed in this study. The cumulative results of these clinical trials support the idea that the VEGF pathway in PDAC, although an attractive therapeutic target, has failed to show clinical benefit. The mechanism of the failure remains unknown, as there are no reports that anti-VEGF therapy changes the tumor vasculature in patients from these trials.

**Angiopoietin pathway**

A large number of mitogenic factors which can contribute to angiogenesis are overexpressed in PDAC, including members of the FGF family, EGF, PDGF-beta, and TGF-α [101]. In addition, angiopoietins are now thought to play a role in PDAC angiogenesis [102, 103]. There are four secreted angiopoietins, Ang1, Ang2, Ang3, and Ang4, which can bind to the receptor tyrosine kinase Tie2 to activate downstream signaling. There is an additional Tie receptor, Tie1, which does not bind Ang proteins but rather heterodimerizes with activated Tie2 to regulate signaling. While this pathway was initially described as inhibitory towards angiogenesis, recent studies
have revealed a context-dependent role for promoting angiogenesis. Experiments demonstrated that there are unique Ang1/Tie2 complexes formed depending on the presence of cell-cell contact or extracellular matrix [104, 105]. Tie2 is localized to the site of cell-cell contact through Tie2-Ang1-Tie2 interactions that bridge the two cells. Activation of Tie2 signaling by this mechanism results in the activation of the AKT pathway, promoting endothelial cell quiescence. However, Tie2 activation in the absence of cell-cell contact occurs by the binding of ECM-bound Ang1. Tie2 activated by this mechanism promotes at least two downstream pathways. The adaptor protein Dok-R is phosphorylated by Tie2, promoting endothelial cell migration [106]. In addition, ECM-bound Ang-1 activation of Tie2 results in stimulation of the ERK pathway, which promotes angiogenesis by inducing endothelial cell migration [107].

Drugs developed to inhibit signaling of the Ang-Tie pathway are now undergoing clinical testing. Therapeutic antibodies that block Ang1 and Ang2 (AMG 386, Amgen) or specifically block Ang2 interaction with Tie2 (CVX-241 and CVX-060) are being evaluated for a variety of advanced solid tumors including ovarian, lung, and colorectal cancer as well as glioblastomas [108, 109]. While these clinical studies are not yet mature, these drugs have clinical activity and are well tolerated, making them appropriate for future studies in patients with PDAC.

Notch pathway

The Notch signaling pathway is a key developmental pathway that is involved in the regulation of cell proliferation and apoptosis. Studies characterizing the role of Notch during embryogenesis reveal a role for this pathway in the formation of blood vessels [110-113]. The mammalian Notch signaling pathway is composed of four single-pass integral membrane Notch receptors (Notch 1-4) and five Notch ligands (DLL1, DLL3, DLL4, Jagged 1 and Jagged 2). Notch receptors bind to membrane-bound Notch ligands on neighboring cells, resulting in a two-step proteolytic activation of the receptor. Notch receptors are initially cleaved by members of the ADAM family of metalloproteinases, allowing for further processing by γ-secretase. The final cleavage event releases the intracellular domain of the Notch receptor, allowing for its nuclear translocation, where it cooperates with additional transcription factors (such as CSL and Mastermind) to regulate gene expression.

Like other developmental pathways, the activation of Notch has been linked to tumorigenesis, including pancreatic cancer [114]. Preventing Notch activation with an inhibitor of γ-secretase inhibited the growth of 13/26
PDAC cell lines in vitro [115]. Furthermore, in a genetically engineered model of PDAC, inhibition of γ-secretase activity prevented the progression of premalignant PanIN lesions to PDAC in 100% of treated animals. Emerging evidence suggests a role for this pathway in PDAC tumor angiogenesis. Downregulation of Notch-1 in BxPC3 cells resulted in a decrease in VEGF mRNA levels and a corresponding decrease in secreted VEGF [116]. High levels of the Notch ligand Delta-like ligand-4 (DLL4) have been correlated with poor prognosis in patients who underwent surgical resection for PDAC [117]. A xenograft mouse model employing PK-1 cells demonstrated that employing a neutralizing antibody to disrupt signaling of DLL4 greatly reduced vascular density of tumors. Likewise, a role for DLL4 in tumor angiogenesis has been observed in other systems [118, 119].

Pharmaceutical inhibition of Notch signaling has focused on the proteolytic activation of Notch receptors. A number of γ-secretase inhibitors (GSIs) have been developed that prevent the final cleavage event required for Notch activation. The potential clinical effectiveness of this class of compound in the treatment of PDAC was first demonstrated in a mouse model of PDAC [115]. Two GSIs, R04929097 and MK0752, are now in clinical trials for the treatment of PDAC (Table 4). RO4929097 was able to inhibit tumor growth by 50-80% in preclinical xenograft mouse models of PDAC [120]. While clinical data on the efficacy of this drug in the treatment of PDAC is not yet available, early phase I trials have demonstrated that it is well tolerated in patients [121].

**Table 4.** Clinical Trials Employing Notch Inhibitors in the Treatment of Pancreatic Ductal Adenocarcinoma.

<table>
<thead>
<tr>
<th>Hh Inhibitor</th>
<th>Other Interventions</th>
<th>Study Title</th>
<th>Phase</th>
<th>Trial Identifier</th>
</tr>
</thead>
<tbody>
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<td>MK0752</td>
<td>Gemcitabine</td>
<td>A Cancer Research UK Phase I/IIa Trial of an Oral Notch Inhibitor (MK-0752) in Combination With Gemcitabine in Patients With Stage IV Pancreatic Cancer</td>
<td>Phase I/II</td>
<td>NCT01098344</td>
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<tr>
<td>RO4929097</td>
<td>none</td>
<td>A Neoadjuvant Pharmacodynamic Study Of RO4929097 (RO) in Pancreas Cancer</td>
<td>Phase I</td>
<td>NCT01192763</td>
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<td>RO4929097</td>
<td>none</td>
<td>A Phase II Study of the Gamma Secretase Inhibitor RO4929097 in Previously Treated Metastatic Pancreas Cancer</td>
<td>Phase II</td>
<td>NCT01232829</td>
</tr>
<tr>
<td>RO4929097</td>
<td>Cediranib maleate</td>
<td>A Phase 1, Pharmacokinetic and Pharmacodynamic Study of the Combination of RO4929097 and Cediranib in Patients With Advanced Solid Tumors</td>
<td>Phase I</td>
<td>NCT01131234</td>
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<tr>
<td>RO4929097</td>
<td>Gemcitabine</td>
<td>A Phase I Study of RO4929097 in Combination With Gemcitabine in Patients With Advanced Solid Tumors</td>
<td>Phase I</td>
<td>NCT01145456</td>
</tr>
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</table>

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Activation of the immune response

A dense inflammatory infiltrate is found throughout the desmoplastic stroma of PDAC (Chapter 9). It is believed that most cancer cells are identified and cleared by the immune system at a very early stage in their development. For many years it was thought that the tumor growth was kept in check by the immune system, that advanced disease occurred as a breakdown in the immune response, and that the successful formation of a tumor involved the establishment of a localized immunosuppressed environment [122]. Paradoxically, the immunoprivileged tumor is not characterized by the absence of immune cells, but rather exhibits an extensive immune infiltrate. This infiltrate is largely composed of immune cells that under normal conditions the body uses to dampen the immune response. These cells include myeloid-derived suppressor cells (MDSCs), regulatory T-cells (Treg cells), and macrophages [123]. The active suppression of the immune system at the site of the tumor is a common event in tumor development [124].

Recently it was demonstrated that alterations in the immune response to PDAC occur at a very early stage in the development of the disease [125]. Experiments utilizing a genetically engineered mouse model of PDAC expressing an activated KRAS allele (Kras\textsuperscript{G12D}) indicated that an immunosuppressive environment is established as early as preinvasive PanIN lesions. PanINs, as well as invasive PDAC, were characterized by the infiltration of immune cells that dampen the immune response, including macrophages and MDSCs. There was an inverse correlation between the number of effector T-cells and MDSCs in tumors. Suppression of T-cell function by MDSCs has been attributed to a number of mechanisms. MDSCs exhibit high levels of Arg1 and iNOS [126, 127]. These enzymes both utilize L-arginine as a substrate in their metabolic cycle, resulting in the depletion of L-arginine from the microenvironment. Interestingly, the lack of L-arginine results in the downregulation of the CD3zeta chain on T-cells and subsequent inhibition of T-cell proliferation [128, 129]. In addition, elevated iNOS activity results in increased NO production, which can inhibit signaling downstream of the IL2 receptor [130]. In addition to elevated levels of MDSCs, these pancreata also had significant numbers of Treg cells. A major function of Treg cells is to end the T-cell-mediated immune response, and these cells have been implicated in limiting the immune response in tumors [131]. Due to the early establishment of an immunosuppressive state, therapeutics directed at activating the immune response against PDAC are an attractive target of research.
The development of a T-cell-dependent anti-tumor response is largely dependent on the activation of the cell surface protein CD40 [132]. During the normal immune response, naïve CD8+ cells are stimulated to become cytotoxic T-lymphocytes (CTLs) upon binding to antigen-presenting cells (APCs). However, in order for APCs to efficiently promote CTL formation, they must first be primed by the binding of CD4+ T-helper cells. This priming occurs through the binding of the CD40 molecule on the surface of the APC by CD154 on the surface of the CD4+ T-helper cell [133]. It has been thought that a limiting step in the development of an effective CTL response against tumors was the effective priming of APCs. Key experiments demonstrated that the binding of T-helper cells could be efficiently substituted by the addition of an antibody specific to CD40 [134-136]. The activation of CD40 licenses antigen-presenting cells for tumor-specific T-cell priming and activation. Based on these experiments, a number of antibodies have been developed for the activation of CD40 [137].

A clinical trial was recently performed to determine whether activation of CD40 could overcome the immunosuppressive environment in PDAC and induce T-cell-mediated antitumor activity [138]. This trial enrolled 21 patients who were given a combinatorial therapy of a CD40 monoclonal antibody, CP-870,893 and gemcitabine. These patients had an overall survival of 7.4 months with a median disease-free survival of 5.6 months. While this study size was small, the observed results are an improvement over the use of gemcitabine alone, which had an overall survival of 5.7 months with a median disease-free survival of 2.3 months.

The mechanism of the antitumor effects of this therapy is predominantly mediated by macrophages. Despite eliciting a strong T-cell response in a mouse model, these cells were not responsible for the observed anti-tumor activity. Interestingly, the authors observed that the CD40-specific antibody bound to macrophages in the peripheral blood and that these macrophages specifically migrated to the tumor. Antibodies to CD40 did not activate macrophages already residing in the tumor, but rather recruited macrophages from the peripheral blood to tumor. The mechanism by which the activation of CD40 results in the migration of macrophages to PDAC tumors has not been defined.

Conclusion

Therapeutic targeting of PDAC has historically focused on the cancer cells. Scientific investigation over the past decade has focused on the cancer genome, and has failed or been slow to identify more effective targets for this cancer. Over the past few years, however, it has become increasingly evident
that the tumor and its stromal microenvironment have a dynamic and reciprocal interaction that plays a critical role in tumor initiation, progression, metastasis and chemoresistance, and that these interactions can be exploited for novel therapeutic targets. We are just beginning to understand these complex interactions and to discover that the stroma not only is composed of cancer-associated fibroblasts with an extensive ECM which contributes to chemoresistance, but is also composed of inflammatory cells and mediators which may cause a local immunosuppressive environment, as well as blood and lymphatic vessels important in the initiation, growth and metastatic spread of this cancer. Much more work remains to be done before the promise of preclinical models is realized in the clinic. The picture that is clearly evolving, however, suggests that future strategies targeting both pancreatic ductal adenocarcinoma and its microenvironment will be needed in order to effectively treat this cancer.

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