14 Smad4-TGF-β Pathways in Pancreatic Cancer: Translational Implications

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Abstract  Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive disease with dismal survival statistics. Extensive research efforts have focused on the elucidation of the specific molecular alterations behind pancreatic cancer, with the goals of understanding PDAC pathobiology and devising new and effective targeted therapies. These studies have yielded surprisingly consistent results, indicating that key genetic alterations include a high frequency of mutations in the K-ras, p53, p16 and Smad4 genes. In addition, there is excessive activation of mitogenic pathways, overexpression of TGF-β isoforms, and an intense desmoplastic reaction that is driven, in part, by the proliferation of pancreatic stellate cells, and marked apoptosis resistance. This chapter focuses on the potential role of the TGF-β signaling pathway in PDAC progression and metastasis while highlighting the importance of Smad4 in TGF-β signal transduction.

1  Pancreatic Ductal Adenocarcinoma

1.2 Disease Description

Pancreatic ductal adenocarcinoma (PDAC) is the deadliest form of pancreatic cancer and is presently the fourth leading cause of cancer-related mortality in the United States. Patients have an extremely poor prognosis, with a 5-year survival less than 5% [1] and a median survival of 6 months [2]. These dismal statistics are due to a combination of a low rate of resectability at presentation [3,4] and inherently aggressive tumor behavior. The poor survival of this malignancy has initiated an impetus of research efforts to understand the molecular mechanisms driving pancreatic cancer.

1.3 Overview of Molecular Alterations in PDAC

A number of common pathways are known to be frequently altered in PDAC. Often, it is the somatic mutation of only one or a few key regulatory genes within a pathway that leads to its signaling dysfunction. As shown in Table 14-1, the most common alterations include mutations of the K-ras oncogene (~90%), the p53 (~85%) and SMAD4 (~50%) tumor suppressor genes and the p16 cell cycle inhibitory gene (~85% mutated and ~15% epigenetically silenced) [5,6]. Conversely, elevated expression of multiple tyrosine kinase receptors and/or their ligands is documented in PDAC as well as over-activation of the src, NFkB and Stat3 signaling pathways [7,8]. The somatic alterations outlined here have the potential to increase cell proliferation while reducing normal apoptotic mechanisms that protect against tumor development, thereby laying the groundwork for cancer initiation.

Molecular alterations have also been documented in PDAC which are likely contributors to the inherently aggressive cancer phenotype. For instance, the KAI1 tetraspan receptor and NK4 are often lost [9], leading to increased cancer cell motility. Additional contributing factors may include increased activation of proangiogenic factors, altered epithelial-mesenchymal interactions and the alteration of transforming growth factor-β (TGF-β) signaling [10–12]. While a combination of these molecular alterations is likely to contribute to cancer cell invasion and metastatic potential, the remainder of this chapter will focus on the specific role of the TGF-β signaling pathway in pancreatic cancer.
2 TGF-β Background

2.1 TGF-β

TGF-β is a cytokine that has been implicated in a diverse range of biological processes. After its initial discovery in 1983, it was shown to have transforming ability in rat fibroblasts [13] and was initially named “sarcoma growth factor.” Following further study, it became clear that TGF-β is one of the most potent biological regulators of proliferation in normal cells. In addition to proliferation, the TGF-β signaling pathway has been implicated in numerous cellular and biological processes including embryogenesis, differentiation, apoptosis, angiogenesis, immunosuppression and wound healing.

TGF-β is a member of a large family of structurally related polypeptide growth factors. The TGF-β superfamily comprises nearly thirty members in human, mouse, Xenopus and other vertebrates [14,15]. Seven members are known to exist in Drosophila [16] and four in C. elegans [17]. The proteins within this family are divided into two main branches as defined by sequence similarity: the BMP/GDF/MIS branch and the TGF-β/activin/nodal branch [18,19]. There are three mammalian TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) that are encoded by different genes with different expression patterns [20]. All three isoforms are highly conserved and all are expressed in epithelial, endothelial, mesenchymal and hematopoietic cells, with TGF-β1 being the most abundantly expressed isoform. The TGF-β1 protein is made and secreted into the extracellular matrix where it forms a complex comprised of a TGF-β1 dimer and one of many latent TGF-β1 binding proteins. Upon release from this complex, the TGF-β1 ligand is activated and is free to propagate signaling by binding to defined receptors.

2.2 TGF-β Receptors

As depicted in Fig. 14-1, TGF-β ligands initiate signaling by acting through specific cell surface receptors that belong to a family of transmembrane serine/threonine kinase receptors.
Thus, TGF-βs act through two receptors, designated as type I (TβRI) and type II TGF-β (TβRII) [19,21,22]. In addition, there is a type III TGF-β receptor (TβRIII) which differs from the other two receptors in that it has no intrinsic signaling function and instead serves to present activated TGF-β to the other two receptors [15]. Both TβRI and TβRII exist as homodimers and consist of an extracellular ligand binding domain, a transmembrane domain, and an intracellular serine/threonine kinase domain. In the presence of TGF-β ligand and following binding to the TβRI homodimer, TβRII complexes with and phosphorylates TβRI within a conserved 30 amino acid segment known as the GS region (GSGS) [23]. Phosphorylation at this GS site results in the activation of TβRI kinase activity and subsequent phosphorylation of TGF-β signal transducers: the Smad family proteins [19,24–27].

2.3 Smad Proteins

Smad proteins are a family of transcription factors that are divided into three structure/function subcategories: the receptor-regulated Smads (R-Smads), the common-partner...
Smad (Co-Smad) and the inhibitory Smads (I-Smads). In total, there are eight Smad family members: Smad1–8 [28]. Smads1, 5 and 8 mediate bone morphogenic protein (BMP) signals, however, and are generally not relevant to TGF-β signaling. Smad proteins have a highly conserved MH1 domain at the N-terminus and a highly conserved MH2 domain at the C-terminus. The MH1 domain facilitates Smad binding to DNA, namely the promoters of target genes (Fig. 14-2). The MH2 domain has been shown to mediate Smad transcriptional activity, oligomerization and protein-protein interactions with receptors and nuclear co-factors [19,23,26,27]. Smads2 and 3 have been shown to have intrinsic nuclear import activity in the MH2 domain [29]. In a dormant state, the Smads are primarily localized to the cytoplasm, which ensures their active response to activated receptors. The cytoplasmic retention of Smads2 and 3 is facilitated by the binding of the protein to the Smad anchor for receptor activation (SARA) protein [30]. In addition to tethering the Smads in the cytoplasm, bound SARA prevents exposure of the nuclear import signal in the Smad MH2 domain [29] and aids in the presentation of Smads to activated receptors [30].

Following binding with and activation by TβRII, TβRI directly phosphorylates the R-Smads, Smad2 and Smad3, at their C-terminal SSXS motif [10,12,31–34]. The phosphorylated R-Smads then heterodimerize with the Co-Smad, Smad4, and the resulting complex is translocated to the nucleus. The exact mechanisms behind Smad nuclear translocation are still unknown, however one report by Xu et al. (2002) suggests the shuttling is dependent on nucleoporins [35]. Once in the nucleus, the complex is free to modulate gene transcription in conjunction with co-activators and co-repressors such as AP-1, FAST, TFE3, p300/CREB and Ski [19,22,24,25,27,36]. It is the specific interactions of the Smad complex with these nuclear factors that facilitates the specificity and complexity of TGF-β signaling (Fig. 14-1). These
nuclear factors are required for Smad genetic regulation because, although Smads are able to bind to DNA on their own, their affinity for the Smad cognate sequence is too low to achieve unassisted binding to DNA [37].

The I-Smads (Smad6 and Smad7) act to inhibit activation of Smad2 and Smad3 phosphorylation [38]. This inhibition is enhanced by Smad7 associating proteins such as STRAP, p300, the Yes-Associated Protein 65 (YAP65), Smurf1/2 and GADD34/PP1c [39–43]. I-Smads have been shown to be transcriptional targets of the TGF-β pathway, suggesting they also function in a negative-feedback loop to modulate TGF-β signaling.

2.4 Smad4

The Co-Smads associate with the R-Smads after TGF-β receptor activation and prior to Smad complex nuclear accumulation. Smad4 is the only known member of the Co-Smad family in humans and mice. Despite being structurally similar to the R-Smads, Smad4 is unable to become phosphorylated by the TβRI receptor and contains a nuclear export signal that prevents nuclear localization in the absence of agonist stimulation [44]. Smad4 is not required for the nuclear accumulation of Smad complexes, but it is required for the formation of active transcriptional complexes [45]. Gene activation is mediated by the presence of a Smad binding motif (CAGAC) and nuclear Smad-interacting DNA binding proteins mentioned above. Ultimately, Smad4 is essential for the specific binding of these nuclear proteins to their consensus DNA binding sites and subsequent TGF-β-induced gene regulation.

2.5 Consequences of Normal TGF-β Signaling

TGF-β has been shown to affect cell growth and differentiation by enhancing the proliferation of mesenchymal cells while inhibiting the proliferation of epithelial cells [34,46]. The TGF-β-mediated growth inhibition is due to suppression of the G1 phase of the cell cycle via several mechanisms [47–49]. One mechanism is by the TGF-β-dependent up-regulation of cyclin-dependent kinase (CDK) inhibitors (Fig. 14-3). The CDK inhibitors known to be affected by TGF-β include p16, p21Cip1, p27Kip1 and p15Ink4b [18,19,23]. p15Ink4b directly binds to CDK4/6 and interferes with cyclin D-CDK4/6 complex formation, while simultaneously inducing the redistribution of p27Kip1 from the cyclin D-CDK4 complex to the cyclin E-CDK2 complex, leading also to CDK2 inhibition. p21Cip1 directly inhibits the activity of cyclin E-CDK2. Induction of these CDK inhibitors by TGF-β contributes to the accumulation of a hypophosphorylated (active) form of the retinoblastoma protein (pRb), a key regulator of the G1-S transition [50,51].

A second mechanism of TGF-β-dependent cell cycle arrest is by the suppression of the cell cycle machinery. The list of suppressed cell cycle players includes c-Myc, Cdc25A [52,53], cyclin E [54,55], cyclin A [56–58], Cdc2 [59,60] CDK2 and CDK4 [47–49,54,61–63]. Also, in one report by Kornmann et al. (1999), TGF-β1-dependent growth inhibition was shown to be associated with an increase in cyclin D1 levels [64] but this observation may be a peculiarity of that particular cell line. Some of these effects on the cell cycle machinery are likely cell-type specific and/or secondary events to global G1 inhibition [18].
2.6 TGF-β in Normal Development

TGF-β1 is thought to be an important regulator of pancreatic organogenesis, due to the effects on both exocrine and endocrine pancreas when it is altered during development [65]. At embryonic day 12.5, TGF-β1 is expressed solely in the embryonic pancreatic epithelium and is devoid of expression in the mesenchyme. Approaching embryonic day 15.5, TGF-β1 mRNA begins to localize to the developing acini at modest levels. Towards the end of gestation, TGF-β1 is upregulated and then becomes essential for terminal acinar differentiation. This upregulation may also be important for islet formation and inhibition of proliferation of pluripotent cell growth [66].

To determine the specific roles for TGF-β in pancreatic development, transgenic mice were developed which expressed a dominant-negative form of TβRII, thereby inactivating TGF-β.
signaling [67]. The mice developed increased proliferation in the acinar cells combined with reduced acinar differentiation. The mice also developed fibrosis, inflammatory infiltration into the pancreas and acute neo-angiogenesis. These results indicate that TGF-β negatively controls the growth of acinar cells and is essential for acinar differentiation in the developing exocrine pancreas.

Another important role for TGF-β is in the regulation of epithelial-mesenchymal interactions. Treatment of cells with follistatin, a TGF-β and activin antagonist, was shown to decrease the differentiation of endocrine cells and promote embryonic exocrine cell differentiation [68]. Conversely, the induction of TGF-β signaling in embryonic mouse pancreas led to the formation of endocrine cells [69], the disruption of epithelial branching and the reduced formation of acinar cells [68]. Thus, TGF-β is a key player in the developing pancreas due to its ability to regulate cross-talk between the epithelium and mesenchyme. This function becomes important in tumorigenesis, as well.

### 2.7 TGF-Beta Signaling in the Adult Pancreas

TGF-βs are known to be expressed at low levels in both the exocrine and endocrine compartments of the normal pancreas [22]. TGF-β1 is specifically expressed in both the developing and adult pancreas. The endocrine islets show expression of both TGF-β2 and TGF-β3. The ductal cells are equally positive for all three TGF-β isoforms, while the acinar cells also stain for all three isoforms but show predominance towards TGF-β1 [70]. Additionally, TGF-β signaling is known to elicit an immunosuppressive response. Thus, TGF-βs may also act to inhibit harmful immune-mediated attacks against the endocrine or exocrine pancreas.

### 2.8 Smad-Independent Pathways of TGF-β

In addition to its canonical roles, TGF-β can signal independently of Smad-mediated transcription (Fig. 14-4). Some of the pathways affected include the ERK, JNK and p38 MAPK kinase pathways. Cells that are deficient in Smad4 or express mutated TβRII (that are deficient in Smad signaling) were able to activate p38 signaling in response to TGF-β [71,72]. Kinetics studies suggest that the activation of pathways with slow kinetics may depend on Smad-dependent transcription while rapid activation may occur independently of transcription [73].

The specific mechanisms guiding Smad-independent pathway signaling by TGF-β are not well understood. In vitro studies suggest that Ras, MAPK kinase kinases, TGF-β-activated kinase 1 (TAK1), X-linked inhibitor of apoptosis (XIAP), MEKK1, and NF-κB may all be players in TGF-β-mediated Smad-independent signaling [74].

These signals may also be important feedback loops for the canonical TGF-β pathway. Activation of the ERK and JNK pathways by TGF-β results in the regulation of the Smad family proteins [71,75]. Smad4 is activated in response to TGF-β-dependent signaling through the MAPK pathway [76]. MAPK effectors were also shown to interact with Smad-interacting nuclear transcription factors (e.g., c-Jun and ATF-2) following TGF-β [73,77].

Smad4-independent signaling is an important factor in the overall cellular response to TGF-β. Signaling through the p38/MAPK pathway allows TGF-β to regulate epithelial-to-mesenchymal differentiation and enhances its pro-invasion effects. The associations
between the TGF-β and the mitogenic pathways can also be counteractive. Smad6 can downregulate the activity of TAK1 [78] while Smad7 can promote the activation of JNK [79]. Conversely, c-Jun is known to inhibit Smad2 signaling (through interaction with Smad co-repressors) in a JNK-dependent manner [80]. Therefore, it is ultimately the balance between the Smad and MAPK signaling pathways that ultimately defines the outcome of TGF-β signaling in a cell.

3  TGF-β and Pancreatic Cancer

3.1  Noted Alterations

Both precursor and malignant lesions of the pancreas express TGF-β1, suggesting a role for it in pancreatic tumorigenesis. Similarly to normal epithelium, TGF-βs act as tumor suppressors
in the early stages of pancreatic tumorigenesis [18]. Cultured pancreatic cancer cells, on the other hand, demonstrate an attenuated response towards TGF-β-mediated growth inhibition [49,81–84] and the expression of TGF-β at later stages of cancer progression fosters a more aggressive phenotype. This apparent dichotomy is the subject of much debate and the detailed mechanisms contributing to this functional “switch” remain to be elucidated. A number of alterations in the TGF-β signaling pathway are suggested to contribute to the resistance to TGF-β-mediated growth inhibition by cancer cells.

The overexpression of TGF-β correlates with pancreatic cancer progression and other malignancies [85,86]. TGF-β1 was shown to be differentially expressed in increasing grades of PanIN lesions and in PDAC, all three mammalian TGF-β isoforms have been shown to be expressed at high levels in the cancer cells by both protein and RNA. That elevated expression also associated with advanced stage and poor survival of PDAC patients [87].

The expression of these isoforms is capable of exerting paracrine growth-promoting properties that enhance tumor angiogenesis, growth, and metastasis. Additionally, cancer cells have been shown to secrete higher amounts of TGF-β than their normal cell counterparts resulting in high levels of the TGF-β ligand in the tumor-associated microenvironment and tumor stroma.

The reduced levels of circulating TGF-β isoforms in patient serum was also shown to be associated with prolonged survival [88]. These data suggest a possible role for altered epithelial-mesenchymal interactions by TGF-β signaling in pancreatic tumorigenesis. TβRII is also known to be overexpressed in PDAC and correlate with advanced tumor stage [89], decreased patient survival [90] and increased expression of genes known to promote angiogenesis and invasion (e.g., plasminogen activator 1 and matrix-metalloproteinase-9) [91]. Additionally, high levels of Smad2 have been documented in PDAC [91], leading to a more potent response to TGF-β signals.

In addition to overexpression of TGF-β components, loss-of-function or deletion alterations in the TGF-β signaling pathway have been documented. Smad4 mutations are the most frequent TGF-β alteration in PDAC [92], followed by decreased TβRI expression [84,93], increased TβRII expression, overexpression of I-Smads [94,95] and rarely mutations in TβRI/TβRII [96]. The net result of these alterations is a loss of the negative growth constraints imposed by TGF-β signaling at later stages in PDAC progression and may prove to be the basis for the dichotomy behind TGF-β.

3.2 Smad4 and Pancreatic Cancer

The mutation or deletion of Smad4 is one of the best characterized disruptions of TGF-β signaling in pancreatic cancers [92,97,98]. It has been estimated that 50–60% of all pancreatic cancer patients have alterations in Smad4, leading to aberrant cell cycle regulation by TGF-β [99,100]. As one of the first novel candidate tumor suppressors identified in pancreatic cancer, the original name for Smad4 was DPC4 (deleted in pancreatic carcinoma locus 4) [97].

Homzygous deletion of Smad4 has been estimated for approximately 30% of cases while allelic loss of the Smad4 chromosome (18q) is found in about 90% of all pancreatic cancers [101]. Inactivating mutations of Smad4 occurs in approximately 20% of all pancreatic cancer and are typically within either the MH1 (DNA binding) or MH2 (transcriptional activation) domains of the protein. Documented mutations include deletion of the entire chromosome and a combination of point, frame-shift, nonsense and missense mutations. Missense mutations found within the MH2 domain typically result in the loss of stability and disruption of the dimerization ability of the Smads [102]. Further, a study by Xu et al. (2000) found that
mutated Smad4 proteins with an arginine mutation in the MH1 domain are translated at similar rates as wild type proteins, but are degraded more rapidly by a ubiquitin-mediated pathway [102].

A juvenile polyposis syndrome (JPS) co-segregates with the transmission of germline defects in Smad4. JPS is an autosomal dominant disorder in which patients have an increased risk of gastrointestinal cancers and have widespread intestinal polyps [103]. Occasionally, Smad4 mutations have been found in conjunction with TβRI and TβRII mutations in biliary [96] and colon cancer [104], respectively. Deletion of Smad4 in pancreatic cancer cell lines leads to the alteration of genes that modulate multiple biological functions, including ECM remodeling, cell adhesion, membrane transport, signaling transduction, intracellular transport, metabolism and transcriptional regulation [105]. These observations suggest that Smad4 may have nonoverlapping tumor suppressive functions with the TGF-β receptors.

Murine knockout studies have been performed for Smad4. Homozygous deletion of Smad4 was embryonic lethal, with mutants dying before day 7.5 of embryogenesis [106]. Mutant embryos were shown to be smaller, to not express a mesodermal marker and to have abnormal visceral endoderm development. Further, it was concluded that the Smad4 knockout embryos had reduced cellular proliferation (not increased apoptosis). These results suggested that Smad4 is specifically required for the differentiation of the visceral endoderm. Additionally, it was determined that Smad4 has an important role in anterior patterning during embryogenesis, as rescue experiments resulted in embryos with severe anterior truncations. In contrast, Smad4 heterozygotes are viable and developed gastric polyps that progress into full tumors later in life [107].

Despite its prevalence in pancreatic cancer, Smad4 re-expression may not be a viable therapeutic option. The presence of Smad4 in vitro was associated with a prolonged doubling time and an enhanced sensitivity to TGF-β-mediated growth inhibition [108]. Smad4 re-expression in vitro was also shown to induce a TGF-β-independent angiogenic response which correlated with a decrease in vascular endothelial growth factor (VEGF) and increase in thrombospondin-1, leading to reduced tumor formation and vascular density [109]. Also, experiments in human cervical cancer cell lines showed that Smad4 re-expression led to transcriptional induction of ECM-associated genes in response to TGF-β, without alteration of classical TGF-β cell cycle targets (e.g., p21, p15 and c-myc) [110]. Similarly, in a nude mouse model of PDAC, the initial re-expression of Smad4 in Smad4 deficient tumor cells was found to be associated with an immediate elongation of the lag phase of in vivo tumor growth [108]. The prolonged lag phase was attributed to restoration of the TGF-β signaling pathway and reduced proliferative capacity in Smad4 expressing cells. Following the initial delay, however, the Smad4-expressing tumors exhibited renewed growth and proliferation, indicating that cells are able to escape the growth suppressive effects of a reactivated TGF-β pathway. Taken together, these observations suggest that Smad4 re-expression may not necessarily be sufficient to inhibit tumor growth in the pancreatic setting and that Smad4 growth inhibitory actions are circumvented in later stages of pancreatic tumorigenesis.

3.3 TGF-β and Acute Pancreatitis

There is enhanced expression of TGF-βs in acute pancreatitis in humans [111] as well as in rodent models [112]. Interestingly, the administration of the pancreatic secretagogue caerulein, which binds and activates the cholecystokinin (CCK) receptor, to transgenic mice that are heterozygous for a dominant negative TβRII (called FVB) results in a markedly attenuated
inflammatory response in comparison to that observed in wild type mice [113]. Caerulein injection in wild type mice resulted in 6- and 36-fold increases in serum amylase and lipase levels, respectively, as well as increased serum trypsinogen activation peptide (TAP) levels, gross edema and a marked inflammatory response in the pancreas that consisted mainly of neutrophils and macrophages. There was an associated increase in TGF-β1 mRNA levels in pancreas of these mice [113]. By contrast, FVB heterozygous mice exhibited minimal alterations in response to caerulein, with attenuated neutrophil-macrophage infiltrates and a blunted increase in TGF-β1 mRNA levels [113]. Moreover, pancreatic acini from FVB heterozygotes did not exhibit restricted stimulation at high caerulein concentrations, even though CCK receptor mRNA levels were not decreased. Thus, a functional TGF-β signaling pathway may be required for caerulein to induce acute pancreatitis, for the CCK receptor to induce acinar cell damage at high ligand concentrations and for the injury response to lead to TGF-β1 up-regulation.

3.4 TGF-β and Chronic Pancreatitis

Several studies have emphasized the potential role of chronic pancreatitis, which may occur in the context of repeated episodes of acute pancreatitis, in the pathobiology of PDAC in humans [114–116], as well as in mouse models of this malignancy [117]. Given the abundance of TGF-β in chronic pancreatitis and PDAC, its marked up-regulation in acute pancreatitis, and the important role of TGF-β in stem and progenitor self renewal, it is not surprising that aberrant TGF-β signaling pathways could be viewed as contributing to the genesis of PDAC. In addition, activated pancreatic stellate cells (PSCs) are known to be key contributors to stroma formation and fibrosis in both chronic pancreatitis and PDAC. In the normal pancreas, PSCs consist of approximately 4% of the total cell population and are located in the inter-acinar spaces. Recent studies have drawn correlations between PSCs and progenitor cells. For instance, stellate cells were shown to express the stem cell markers nestin and CD133 [118] and rat pancreatic stellate cells were able to differentiate in vitro into lineages from all three germ layers [119]. PSCs require a progenitor phenotype because their normal role is as part of a healing process after pancreatic injury. Activated PSCs also inhibit matrix metalioproteases-3 and -9, thereby enhancing fibrogenesis by reducing collagen degradation [120]. It is the perpetuation of these activated PSCs in response to CP and PDAC that are thought to promote tumor pathobiology.

The TGF-β pathway is thought to be a key activator of PSC activation. Thus, the elevated TGF-β levels in the pancreas of patients with CP lead to PSC activation and proliferation, functioning in both autocrine and paracrine pathways to activate Smads2 and 3 in these cells [121]. Additionally, a potential mediator of PSC activation and well-established target gene of TGF-β is connective tissue growth factor (CTGF). CTGF is upregulated in PDAC [122] and binds to α5β1 integrin and heparan sulphate proteoglycan receptors [123], thereby stimulating PSC adhesion and migration.

4 Translational Implications

4.1 Overview

The use of new and emerging therapies is crucial in the battle against PDAC. The Food and Drug Administration recently approved the use of erlontinib, an inhibitor of the tyrosine
kinase activity of the epidermal growth factor (EGF) receptor, in combination with gemcitabine, a nucleoside analogue. Gemcitabine is converted intracellularly to active metabolites difluorodeoxycytidine di- and triphosphate (dFdCDP, dFdCTP), which both inhibit ribonucleotide reductase and decrease the amount of deoxynucleotide that is available for DNA synthesis. In addition, dFdCTP is incorporated into DNA, resulting in DNA strand termination and apoptosis. It is currently used as a first-line therapy and radiosensitizer in the treatment of pancreatic cancer [124]. The combined use of gemcitabine and elontinib improved the median survival by 0.5 month (5.9 mo. of gemcitabine alone vs. 6.4 mo. of combination) [125]. Due to the prevalence of TGF-β alterations in pancreatic cancer, similar targeting of TGF-β pathways at the receptor and ligand level, or at the level of their downstream gene targets, may yield more promising results.

4.2 Blocking TGF-β Actions in Models of PDAC

Several approaches have been used to suppress the paracrine actions of TGF-βs. These approaches include the use of antisense strategies to inhibit TGF-β synthesis [126,127] and anti-TGF-β neutralizing antibodies to block the action of TGF-β [128]. Efforts have also been made to express a mutated form of the TGF-β1 precursor, thereby inhibiting the mature processing of all three TGF-β isofoms [129]. In addition, small molecule inhibitors that target the kinase activity of TβRI have been tested [130]. In all cases, the blockade of TGF-β actions was sufficient to reduce cellular proliferation in vitro, and attenuate tumor growth and metastasis in vivo.

Investigators have also used the expression of soluble TβRII or TβRIII to sequester free TGF-β ligand [131,132]. When use of a soluble TβRII was explored, tumor growth and metastasis was found to be attenuated in both a subcutaneous or orthotopic model of pancreatic cancer [133,134]. The treated tumors were also found to have less angiogenesis and impaired expression of genes associated with growth and metastasis (e.g., plasminogen activator inhibitor 1 and urokinase plasminogen activator) [133,134]. These results suggest that the observed TGF-β overexpression in pancreatic cancer has both proliferative and angiogenic paracrine effects in vivo. Due to the far-spanning biology of the TGF-β pathway, it has been suggested that additional paracrine effects might include the modification of the composition of the extracellular matrix (ECM), stimulation of fibroblast and stellate cell proliferation and the suppression of cancer-directed immune mechanisms.

Another mechanism by which TGF-β signaling can be implemented to attack PDAC would be the manipulation of known TGF-β gene targets. One such attempt was made by blocking the action of connective tissue growth factor (CTGF). As mentioned above, CTGF is upregulated by TGF-β and is known to be overexpressed in PDAC [122]. In vitro, CTGF increases pancreatic cell proliferation and invasion [135]. In vivo, blocking CTGF by an antibody (e.g., FG-3019) reduces tumor growth, metastasis and angiogenesis in an orthotopic mouse model of pancreatic cancer [136]. These results indicate that the blockage of TGF-β molecular targets may prove to be therapeutic in the treatment of PDAC.

4.3 TGF-β in the Clinic?

Due to its complexity, the TGF-β signaling pathway contains multiple options for intervention by targeted therapies in patients [37,137–139]. For instance, interference with the physical
binding of active TGF-β ligand to its receptor could be achieved by preventing the formation of processed and active TGF-β ligand, by scavenging circulating TGF-β with excess binding proteins (e.g., latency-associated protein), or by blocking receptor binding using an inhibitory antibody. Small molecule inhibitors might be targeted to the intracellular portions of TGF-β receptors, thus inhibiting signal transduction, or degradation of TGF-β isoforms using antisense technology could be employed. Pharmacologic and/or biological inhibitors (e.g., FKBP12 and TRAP-1) could be used to inhibit the kinase activity of activated TGF-β receptors. Alternatively, elevated expression of the I-Smads would prevent phosphorylation of Smads2 and 3, thereby eliminating TGF-β signaling. As all of these options are viable approaches to the inhibition of the pro-cancer effects of TGF-β, continued investigation is needed to evaluate clinical relevance.

Currently, several efforts are underway to define the potential for TGF-β inhibition in PDAC patients to improve survival. New TGF-β inhibitors are already being tested preclinically and, in a few instances, in human clinical trials. These new therapies are designed to block activity of or interrupt signaling by TGF-β in tumor cells or activated immune cell populations [128,140,141]. While early results are encouraging, the dichotomous role of TGF-β in tumorigenesis complicates the facile implementation of TGF-β inhibitors into clinical practice. The global suppression of TGF-β, while beneficial in terms of tumor reduction, has the potential to also affect TGF-β signaling in normal (or close-to-normal) tissue and thereby contribute to the formation of new tumors or hyperplasia. To circumvent this outcome, novel strategies should seek to target specific members of the TGF-β pathway which are known to play a role in tumor progression, while avoiding members that are involved in growth inhibition and cell cycle arrest. Continued research into the intricacies of TGF-β associated proteins will help to elucidate specific mechanisms for future targeted therapies. The possibility of devising therapies that target specific pathways that are known to be altered in pancreatic cancer may also lead to individualized therapies that are based on the specific alterations in the cancer of PDAC patients, thereby presenting new hope for efficient therapeutic modalities that minimize potential side effects.

4.4 The Future of TGF-β

As regulators of global cellular biology in virtually every cell type, maintenance of the TGF-β pathway is crucial to maintaining healthy growth. Therefore, it is not surprising that altered expression or regulation of TGF-β family members is a predisposition for aberrant physiological behavior and pathology [18]. New and exciting research is exploring the epigenetic aspects of TGF-β expression and signaling. Epigenetic modifications are emerging as important modulators of cellular biology and include a diverse set of regulators and mechanisms. The variability in epigenetics can be used to partly explain the discrete differences between cells that otherwise have identical genomes. The epigenetic regulation of the TGF-β signaling pathway and its downstream targets is currently poorly described and future studies in this area will surely reveal potential therapeutic targets in PDAC.

The complexity of TGF-β signaling is also the target of ongoing research. In addition to the canonical TGF-β/Smad pathways presented in this review, there is intricate and undefined cross-talk between TGF-β and other signaling pathways within the cell and surrounding microenvironment [74]. The mechanisms of regulation and downstream biological consequences of these signaling networks underscores the complex influences of the TGF-β superfamily in both
normal development and tumorigenesis. Additionally, evidence has suggested that there are TGF-β-independent functions for the Smad proteins. The delineation of these functions is lacking and is likely an area of future research. All of these network and TGF-β-independent interactions are likely the reason for the acute toxicity witnessed after TGF-β-targeted therapy and will need to be better understood before more efficacious and specific therapies can be designed.

Key Research Points

- The TGF-β pathway is responsible for a diverse range of physiological and cellular processes.
- TGF-β is a key regulator of normal pancreatic development and organogenesis.
- TGF-β signaling is aberrant in pancreatic cancer and associates with a more aggressive phenotype.
- Smad4 mutations are one of the most common and well-documented alterations of the TGF-β pathway in pancreatic cancer.

Future Scientific Directions

- The use of animal models of pancreatic cancer to delineate the specific roles of all members of the canonical TGF-β/Smad family in cancer initiation and development
- The characterization of epigenetic modifications regulating TGF-β signaling
- The continued elucidation of the intricate interactions between the TGF-β pathway and a multitude of other cellular signaling networks
- The investigation into TGF-β-independent functions of the Smad proteins

Clinical Implications

- Continued research into the biology governing TGF-β regulation will unveil new and more specific avenues for TGF-β targeted therapy in pancreatic cancer.
- Compounds and molecular therapeutics will need to be chosen based on their specificity for targeting the pro-tumorigenic properties of TGF-β signaling while sparing the “good” tumor suppressive outcomes.
- Knowledge of the spectrum of TGF-β alterations in an individualized setting may be used to help predict patient response to current anti-cancer therapy and/or the stage of their disease progression.

References

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