

Perspectives of TGF- β inhibition in pancreatic and hepatocellular carcinomas

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ABSTRACT:

Advanced pancreatic ductal adenocarcinoma (PDAC) and hepatocellular carcinoma (HCC) are non-curable diseases with a particularly poor prognosis. Over the last decade, research has increasingly focused on the microenvironment surrounding cancer cells, and its role in tumour development and progression. PDAC and HCC differ markedly regarding their pathological features: PDAC are typically stromal-predominant, desmoplastic, poorly vascularized tumours, whereas HCC are cellular and highly vascularized. Despite these very different settings, PDAC and HCC share transforming growth factor- β (TGF- β) as a common key-signalling mediator, involved in epithelial-to-mesenchymal transition, invasion, and stroma-tumour dialogue. Recently, novel drugs blocking the TGF- β pathway have entered clinical evaluation demonstrating activity in patients with advanced PDAC and HCC. TGF- β signalling is complex and mediates both pro- and anti-tumoural activities in cancer cells depending on their context, in space and time, and their microenvironment. In this review we provide a comprehensive overview of the role of the TGF- β pathway and its deregulation in PDAC and HCC development and progression at the cellular and microenvironment levels. We also summarize key preclinical and clinical data on the role of TGF- β as a target for therapeutic intervention in PDAC and HCC, and explore perspectives to optimize TGF- β inhibition therapy

INTRODUCTION

Advanced pancreatic ductal adenocarcinoma (PDAC) and hepatocellular carcinoma (HCC) have remarkably poor prognosis. Synchronous metastases are identified in 50% of PDAC patients at diagnosis[1] and preclinical models suggest that metastatic dissemination, the leading cause of PDAC-related death, might exist even before the primary tumour is detectable[2]. Unlike PDAC, HCCs are mostly locoregional-spreading tumours, with extra-hepatic metastases being a late event. Mortality is closely related to liver dysfunction or portal hypertension complications due to underlying

liver disease, portal thrombosis and/or massive tumour involvement[3]. Treatment options are limited for both malignancies with only a minority of PDAC and HCC patients being candidates for surgery due to disease extent and/or liver dysfunction. Advanced PDAC is a contender for cytotoxic-based therapies (gemcitabine, nab-paclitaxel, or combined 5-FU/irinotecan/oxaliplatin as the FOLFIRINOX regimen), while sorafenib, an oral multi-tyrosine kinase inhibitor targeting the VEGFR, PDGFR and Raf pathways is the only approved systemic therapy for advanced HCC patients[4, 5]. Both PDAC and HCC are clearly therapeutically challenging digestive cancers and new therapeutic options are urgently needed.

Over the last decade, research has increasingly focused on the microenvironment surrounding cancer cells, and its role in tumour development and progression. PDAC and HCC differ markedly regarding their pathological features: PDAC are typically stromal-predominant, desmoplastic, poorly vascularized tumours, whereas HCC are cellular and highly vascularized[1, 6]. Despite these contrasting microenvironment settings, PDAC and HCC share transforming growth factor- β (TGF- β) as a common key signalling mediator. TGF- β is involved in epithelial-to-mesenchymal transition (EMT), invasion, and stroma-tumour dialogue in both tumour types.

In the first part of this review, we provide a comprehensive overview of the roles played by the TGF- β pathway and its deregulation in PDAC and HCC development and progression, at the cellular and microenvironment levels. We then go on to summarize key preclinical and clinical data describing the role of

TGF- β as a target for therapeutic intervention in PDAC and HCC, and explore perspectives to optimize TGF- β inhibition therapy.

2.ROLE OF TGF-B AT THE CELLULAR LEVEL

2.1. TGF- β pathway in a nutshell

TGF- β is a well-recognised actor of development and is involved in the regulation of cell proliferation, differentiation, invasion, and inflammation. Key features of the TGF- β signalling pathway are depicted in figure 1. Deletion of the TGF β 1 or TGF β RII gene in mice resulted in defects in haematopoiesis, vasculogenesis, and endothelial differentiation of extra-embryonic tissues, while knockout mice for SMAD2 or SMAD4 displayed

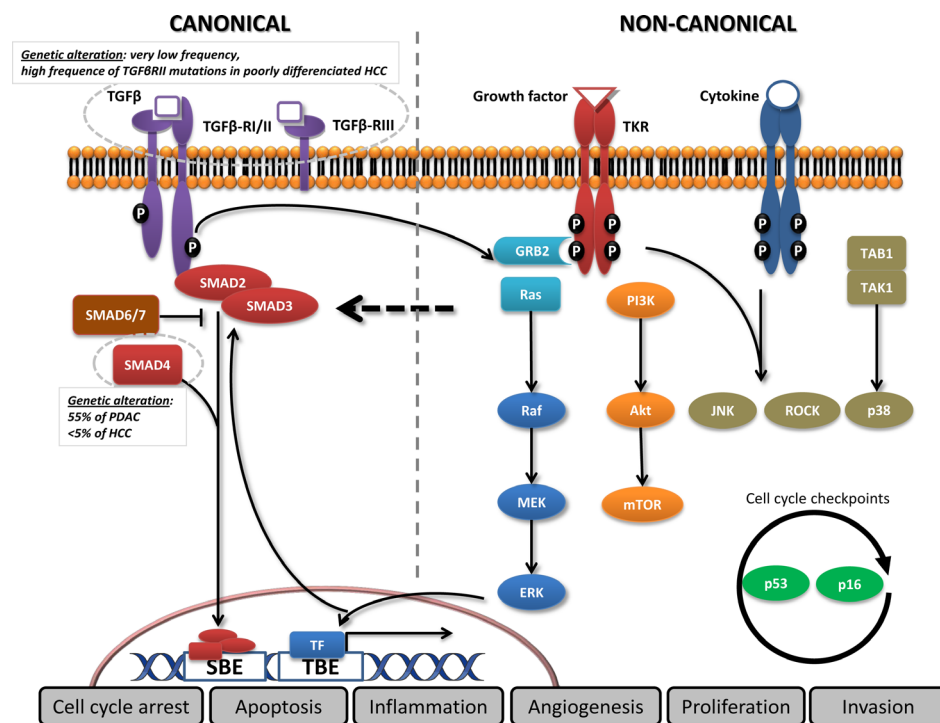


Figure 1: Canonical and non-canonical TGF- β pathways. In the canonical pathway, the three TGF- β ligand isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, are synthesized as precursors and bind to form the latent TGF- β complex before being secreted[138]. After extracellular activation, TGF- β ligands bind to the membranous TGF- β type III receptor or the TGF- β type II receptor (TGF- β RII) homodimers with high affinity. TGF- β RII binding allows dimerization with TGF- β type I receptor (TGF- β R1) homodimers, activation of the TGF- β R1 kinase domain and signal transduction via phosphorylation of the C-terminus of receptor-regulated SMADs (R-SMAD), SMAD2 and SMAD3. The TGF- β R dimer then forms a heterotrimeric complex with SMAD4 which translocates and accumulates in the nucleus[139, 140]. TGF- β dependent signalling can activate or repress hundreds of target genes through the interaction of SMADs with various transcription factors (TF). SMAD activities are regulated through several mechanisms: SMAD2/3 nucleocytoplasmic shuttling, binding to anchor proteins such as SARA, phosphorylation (e.g., by ERK, JNK, and p38 MAPK), Smurf (SMAD-ubiquitination-regulatory factor)-dependent degradation, or via expression of inhibitory SMAD6 and SMAD7[141]. In the non-canonical pathway, TGF- β signalling activates SMAD-independent pathways such as PI3K/AKT, MAPK pathways (ERK, JNK, and p38 MAPK) as well as NF- κ B, Rho/Rac1, Cdc42, FAK, Src, Abl[142]. Moreover, transversal signalling, especially at the SMAD level, allows TGF- β pathway activation to integrate signals from integrins, Notch, Wnt, TNF- α , or EGF-dependent pathways as well as signals from cellular processes such as the cell cycle or apoptosis machineries[143]. The TGF- β signalling pathway thus has pleiotropic functions regulating cell growth, differentiation, apoptosis, cell motility, extracellular matrix production, angiogenesis and cellular immune response[144].

abnormal mesoderm formation[7]. Mice knockout for TGF β 1, TGF β RII, or SMAD4 genes are more likely to have spontaneous tumour development and excessive inflammatory responses, confirming the tumour suppressor properties of the TGF- β pathway[7]. In humans, mutations in the TGF β RII gene have been associated with multiple syndromes, and SMAD4 mutation is genetically responsible for familial juvenile polyposis, an autosomal dominant disease characterized by predisposition to gastrointestinal polyps and cancers.

Hijacking crucial biological functions by deregulating the TGF- β signalling pathway has recently emerged as a leading area of preclinical and clinical cancer research. The TGF- β pathway has both pro- and anti-tumoural activities[8-10]. On one hand, the TGF- β pathway promotes cell cycle arrest, apoptosis, and autophagy in epithelial cells, and also inhibits inflammation[8-10]. On the other hand, by promoting angiogenesis, cell motility, invasion, EMT, or cell stemness, the TGF- β pathway promotes tumour progression[8-10]. The current paradigm for the role of TGF- β in carcinogenesis is that accumulation of genetic alterations in the TGF- β pathway drives the pathway's evolution from tumour-suppressive to tumour-promoting functions[9]. However, as we discuss in this review, it is critical not to isolate the role of TGF- β in tumour cells from their context in space and time, and from their microenvironment.

2.2. TGF- β pathway alterations in tumour cells

Alterations in TGF- β signalling are common in cancer. The TGF- β pathway is one of the 12 central cellular signalling pathways and processes that are frequently genetically altered in PDAC[11]. Regarding the TGF- β ligand, TGF- β mutations are very uncommon and to date none have been reported in PDAC or HCC [COSMIC Database]. TGF- β 1 expression is detected immunohistochemically in about 40% of PDACs, and high TGF- β 1 plasma levels have been measured in HCC patients compared to patients with cirrhosis only, with decreasing levels in patients who underwent effective HCC therapy[12-15]. Several studies have evaluated the prognostic significance of TGF- β levels in both PDAC and HCC[15-23]. However, published results are conflicting and no real consensus currently exists. For TGF- β receptors, TGF β R1 gene alterations are uncommon (< 2%), whereas mutations in the TGF β R2 gene are present at high frequencies in some cancers including HCC. Higher rates are found in poorly differentiated HCC tumours (53% of cases), but mutations are infrequent in PDAC (4%) [COSMIC Database][24, 25]. Low levels of TGF- β R1, TGF- β R2 and TGF- β R3 have been observed and may be associated with poor prognosis in various cancers[26]. However, similar to TGF- β ligand levels,

analyses of their prognostic value are conflicting and no consensus has been reached[27].

Downstream of the TGF- β receptors, mutations in SMAD2 and SMAD3 genes are infrequent and reports on the prognostic value of their expression level in PDAC and HCC are sparse [COSMIC Database]. In patients with hepatitis B and hepatitis C virus chronic infection, the phospho-SMAD2/3 L isoform was associated with a higher risk of developing HCC[28, 29]. SMAD4 inactivation is one of the most common alterations in PDAC (50-60%), caused by deletion, mutation or epigenetic modification, and many studies have shown that loss of SMAD4 expression is associated with a worse prognosis[30-32]. For instance, in a genetic analysis of 39 frequent mutations in 89 patients with resected PDAC, SMAD4 mutations were significantly associated with shorter survival[33]. In the largest study to date by Bachet et al.[34], loss of SMAD4 expression had no prognostic value but was predictive of adjuvant gemcitabine benefit. These apparently conflicting results may arise from the different techniques used to evaluate SMAD4 alterations and differences in patient populations and treatments. In HCC, SMAD4 mutations are uncommon (> 2%) [COSMIC Database]. However, there are also reports of reduced SMAD4 expression in HCC cells compared to surrounding liver tissue[35]. In contrast, increased expression was reported in subsets of patients with chronic viral hepatitis infection or in association with increased TGF- β R2 overexpression, and was linked with poor prognosis[35-37]. Emerging data regarding inhibitory SMADs (I-SMAD) showed that low levels of SMAD7 correlated with increased recurrence and shorter survival in PDAC and HCC patients[38, 39].

2.3. TGF- β pathway in early events of carcinogenesis

The role of the TGF- β pathway in carcinogenesis is currently controversial. In its primary function, TGF- β has major tumour-suppressive properties, suggesting that TGF- β pathway inactivation is mandatory for tumour cell growth[9, 10]. In PDAC, mutation-driven KRAS over-activation is a very early alteration present in almost all tumours. Isolated expression of activated KRAS in mice drives the formation of pancreatic intraepithelial neoplasia (PanIN) and PDAC, however lesion progression is slow, and TGF- β pathway activation is sustained[40, 41]. SMAD4 or TGF β R2 deletion in KRASG12D transgenic mice dramatically increased tumour aggressiveness, with accelerated PanIN and PDAC development, suggesting cooperation between these genetic alterations and mutant KRAS[42-44]. The importance of SMAD4 loss-of-function in PDAC carcinogenesis is supported by the fact that SMAD4 inactivation is observed in most tumours.

In the context of tumour progression, SMAD4 loss-of-function may not only be necessary for counteracting TGF- β anti-proliferative effects, but may also contribute to rewiring the cells' processing system, translating TGF- β input signals into different outputs. In *in vitro* and *in vivo* experiments, adding TGF- β to SMAD4-null cell lines resulted in increased proliferation rather than tumour suppression[45].

In HCC, the low frequency of SMAD4 inactivation implies that different mechanisms are engaged in eliciting TGF- β inhibitory functions. The current paradigm, supported by studies involving expression of TGF- β pathway components in patient tissues, is that early carcinogenesis relies on low TGF- β pathway activity or "early TGF- β signature"[46, 47]. Early TGF- β pathway attenuation is characterized by activation of a negative feedback loop through increased expression of the I-SMAD SMAD7, and expression of two negative regulators of the TGF- β pathway, the SKI-like and TGF- β -induced factor genes, which are both co-repressors of the SMAD2/3-dependent transcription complex[46]. This early TGF- β signature is also characterized by expression of the DNA damage gene family Gadd45, which is involved in cell cycle arrest and apoptosis[46, 48]. Given this strong induction of anti-tumourigenic genes, early tumour promoting activity of TGF- β requires a cellular context with imbalanced sensitivity towards pro- and anti-growth signals. For example, p16INK4 gene alterations are present in up to 90% of HCCs. They favour insensitivity to anti-growth signals by affecting both the cell cycle through relieving cyclin D/CDK4,6 complex inhibition, and the apoptotic machinery by lowering p53 activation[32]. Similarly, over-activation of pro-mitogenic pathways such as EGFR, or TGF- β -dependent cytokine expression (EGF, PDGF, IGF-1, HGF, FGF, etc.) may modify TGF- β response. For example, the Ras-ERK pathway can transduce signals downstream of TGF- β R, and ERK may modify SMAD-dependent signalling by modulating SMAD2/3 phosphorylation. SMADs are differentially phosphorylated by TGF- β R (C-terminal region) and ERK (linker L region), resulting in various phosphoisoforms (C, L, L/C) with distinct localization and cellular effects[49]. Hyperactivation of the PI3K/AKT pathway also cooperates with the TGF- β pathway in hepatocarcinogenesis in cirrhotic livers[50].

To summarize, in early steps of carcinogenesis, TGF- β displays tumour-suppressive properties, with mechanistic differences between PDAC and HCC models. In PDAC, the TGF- β pathway initially drives anti-proliferative signals until SMAD4 silencing occurs, modifying the outputs of TGF- β pathway activation. In HCC, the anti-proliferative effects of TGF- β are bypassed via mitogenic signals or impaired sensitivity to anti-growth signals.

2.4. TGF- β pathway in promoting metastasis

Most tumours acquire a metastatic phenotype during progression, developing the capacity to invade surrounding tissues, migrate and grow at distant sites via an EMT-dependent process. This process is linked to dedifferentiation, and epithelial cells undergo a phenotypic shift from having tight cell-cell junctions, clear basal and apical polarity, sheet-like growth architecture, with expression of epithelial markers such as E-cadherin, into spindle-like fusiform, motile cells expressing mesenchymal markers such as vimentin and N-cadherin. This change in morphology and remodelling of the extracellular matrix (ECM), notably through the expression of matrix metalloproteinases (MMPs), confers them with invading potential. Accumulating evidence shows that TGF- β has late-stage tumour effects particularly in promoting EMT and, as a consequence, cancer dissemination[9, 10].

Coulouarn et al.[46] showed that high TGF- β pathway activity with the "late TGF- β signature" favours late tumourigenic evolution in HCC, notably in terms of metastatic spreading. It is characterized by modulation of genes involved in cytoskeleton organization (e.g., vimentin and supervillin), cell adhesion (e.g., integrin- α 6 and activated leucocyte cell adhesion molecule), and matrix remodelling and migration, along with expression of connective tissue growth factor (CTGF) and Rhob[46, 48, 51]. Interestingly, this late TGF- β signature is also characterized by the expression of the SNAI1 gene which encodes Snail, an E-cadherin transcription repressor[46, 48]. In addition, TGF- β is a major actor in EMT in PDAC, and cooperates with the activated Ras-ERK pathway to activate EMT transcription factors such as Snail and ZEB[32, 52].

Moreover, in both PDAC and HCC, TGF- β promotes tumour invasiveness through MMP induction. For example, MMP-2, MMP-9, MT-MMP1 and urokinase-like plasminogen activator are up-regulated *in vitro* by TGF- β 1[53-55]. TGF- β 1 inhibition reduces MMP production and cell invasiveness, possibly via partial EMT reversion, inhibition of integrin signalling, and reduced CTGF production[51, 56, 57].

In both PDAC and HCC, mesenchymal differentiation of tumour cells has been associated with poor prognosis, while TGF- β expression or TGF- β pathway activation correlates with the EMT status of tumour cells. In a recent analysis of a series of resected HCC specimens, a mesenchymal phenotype (high vimentin and low E-cadherin expression) was associated with shorter survival and enhanced TGF- β pathway activity (increased TGF- β 1, phospho-SMAD-2, and phospho- β 1 integrin expression)[58].

Crosstalk and TGF- β coupling with other signalling pathways may be critically important for tumour

progression. As essential components of cell-to-matrix adhesion, integrins are involved in modulating the TGF- β response. In PDAC, α V β 6 integrin cooperates with TGF- β in its tumour-suppressor function whereas in HCC, α V β 1 integrins or α 3 β 1 integrins are stimulated by TGF- β to promote tumour invasion[16, 56, 59]. TGF- β can activate and cooperate with multiple other pathways involved in invasion and metastasis (e.g., MAPK, PI3K/AKT/mTOR, NF- κ B, Notch, Wnt, and CXCR4) through SMAD2/3-dependent and -independent signalling mechanisms[60]. For instance, in HCC, co-activation of the Wnt and TGF- β pathways define an HCC subclass with a more aggressive phenotype, and in PDAC SMAD4 was required to transduce Wnt signalling in a SMAD2/3-independent manner[61-63]. In contrast to early carcinogenesis, the presence of SMAD4 seems to be important for the metastatic potential of PDAC tumour cells *in vivo*[40]. Activation of the Ras-ERK and TGF- β -SMAD4 pathways was required for EMT induction and maintenance[52]. Mutated K-Ras and TGF- β cooperate to produce L/C forms of phospho-SMAD2 and phospho-SMAD3, which are translocated into the nucleus and activate transcription of pro-proliferative (c-Myc) and pro-invasive (MMPs) genes[49].

To summarize, TGF- β plays an important role in both PDAC and HCC in late-stage tumour progression by promoting EMT, invasion, and, as a result, cancer metastasis, in cooperation with other pathways.

3. ROLE OF TGF-B AT THE MICROENVIRONMENT LEVEL

3.1. PDAC: stromal avascular microenvironment

PDAC displays the most prominent desmoplastic stromal reaction of all epithelial tumours, often greater than the epithelial component of the tumour itself[1, 64, 65]. Fibrotic focus (evidence of intratumoural fibroblast proliferation following focal necrosis) and stromal abundance and activity (evaluated by collagen deposition and α -smooth actin immunostaining) correlated with poorer survival in resected PDAC patients, suggesting a prognostic role for desmoplasia in PDAC[66-69]. This desmoplastic stroma is a complex structure composed of ECM proteins and various cell types including pancreatic stellate cells (PSCs), endothelial cells and pericytes, nerve cells, immune cells, and bone marrow-derived stem cells[68].

Activated PSCs are responsible for excess ECM production in PDAC, and TGF- β 1 is a key signalling factor in this process[70]. Löhner et al.[71] demonstrated that TGF- β 1 overexpression induced up-regulation of ECM proteins *in vitro* in co-culture experiments in TGF-

β 1-transfected PDAC cells and fibroblasts, and also when fibroblasts were grown in conditioned medium from TGF- β 1-transfected PDAC cells. *In vivo*, TGF- β 1-transfected PDAC cells induced a rich stroma after orthotopic transplantation into nude mice pancreas. Consistent with this, Bachem et al.[72] showed that PDAC cell lines stimulated PSC proliferation. Using specific neutralizing antibodies, they demonstrated that the increase in ECM protein production was mediated by TGF- β 1 and FGF2, while PSC proliferation was likely mediated by PDGF. In addition, after subcutaneous injection of combined PDAC cells and PSCs into immunodeficient mice, tumours grew faster than with PDAC cells injected alone. On histologic examination, mixed PSCs-PDAC cells tumours displayed intense desmoplastic reaction, but also an increased number of cancer cells themselves[72]. Additional studies, using other physiological orthotopic and transgenic mice models, also demonstrated that human PSCs within the tumour stimulated fibrosis, local tumour growth, and importantly, promoted regional and distant metastasis[73-75]. Strikingly, PSCs were also detected in metastatic nodules in the liver in mice, suggesting that PSCs can migrate with PDAC cells to establish a potentially tumour-favourable microenvironment at distant sites[73, 76]. Taken together, these data highlight the crucial role of the interactions between cancer cells and PSCs in tumour progression in PDAC, via TGF- β 1 and desmoplasia[77, 78].

PSCs not only create a fibrotic microenvironment, but also contribute to make it hypoxic. Although activated PSCs produce pro-angiogenic factors such as VEGF, they are dominantly anti-angiogenic, through (1) enhancing anti-angiogenic endostatin production by PDAC cells, (2) compressing vessels by the dense and fibrotic stroma and (3) high interstitial pressure, all of which result in low vascularization and tumour hypoxia[64, 79]. In addition, hypoxia stimulates pro-fibrogenic functions of PSCs (production of ECM components and CTGF), thus perpetuating a hypoxia-fibrosis vicious cycle[80-82]. Avascular hypoxic microenvironments promote survival of anaerobic cancer cells that are intrinsically resistant to hypoxia-induced apoptosis[83]. This may explain the failure of anti-angiogenic treatment in PDAC[64, 84]. Moreover, hypoxia may select for tumour cells with a more aggressive phenotype[85], a well described phenomenon in other tumour types[86, 87]. This may be due to hypoxia-induced activation of EMT and invasion pathways such as HGF/c-Met or CXCR4/CXCL12, through HIF-1 α -dependent and independent mechanisms[88-93]. Hypoxia-induced modulations of tumour metabolism (glycolysis, glutaminolysis, lactate efflux) may additionally contribute to increased aggressiveness[93]. The TGF- β pathway cooperates with hypoxia in these processes[94]. In preclinical studies, TGF- β inhibition by various agents (LY2109761, SD-208,

and trabedersen) reduced PDAC cells invasion in vitro and metastasis in vivo[95-97]. Consequently, PSCs and TGF- β 1 in PDAC desmoplasia may contribute to create a hypoxic microenvironment exerting a selection pressure toward a more invasive cancer cell phenotype.

To summarize, TGF- β is a major pro-fibrotic factor in PDAC carcinogenesis. TGF- β therapeutic inhibition in PDAC might thus result in stromal depletion, vascularization enhancement with improved drug delivery, and an anti-metastatic effect. An anti-fibrogenic effect has yet to be well studied due to the lack of relevant preclinical models mimicking the stromal complexity of PDAC[64].

3.2. HCC: cellular highly vascularized microenvironment

The role of TGF- β signalling is quite different in the HCC microenvironment. In contrast to PDAC, HCCs are typically hypervascularized tumours with predominant arterial perfusion[98, 99]. Angiogenesis plays an important role in HCC development and growth as suggested by high circulating VEGF levels and pathological studies showing the development of unpaired arteries, increased histological microvessel density, and VEGF immunostaining on tissue biopsies[100-102]. Consistent with this, HCCs are responsive to intra-arterial embolization and anti-angiogenic agents such as sorafenib or sunitinib, suggesting angiogenic dependence[98].

TGF- β plays a pro-tumourigenic role in HCC mainly by promoting angiogenesis[103]. Ito et al.[104] showed that TGF- β plasma levels were positively correlated with tumour vascularity assessed by celiac angiography. TGF- β signalling can induce angiogenic factors such as VEGF and CTGF in epithelial cells and the fibroblasts that promote these epithelial cells[105]. Mazzocca et al.[106] demonstrated that the TGF- β RI inhibitor LY2109761 displayed anti-angiogenic activity by inhibiting VEGF secretion. Mechanistically, LY2109761 blocked paracrine crosstalk between HCC cells and endothelial cells involving SMAD2/3-mediated signalling, and consequently the formation of blood vessels. Interestingly, this anti-angiogenic effect was more effective than that of bevacizumab, a specific anti-VEGF monoclonal antibody.

In addition, TGF- β -mediated CTGF production is also involved in ECM deposition in HCC, despite that HCC stromal content is much less abundant than in PDAC. CTGF plays an important role in the crosstalk between HCC cells and HSCs (or cancer-associated fibroblasts, CAFs) to control stroma production[51]. TGF- β circulating levels increase in line with collagen deposition and the reduction of ECM degradation[107]. The Gianelli group[51] showed that HCC cell lines producing high levels of CTGF generated high stromogenic tumours, which was reversed by CTGF knock-down. Upon TGF- β 1 stimulation, low-CTGF HCC cells formed tumours with

a high stromal content and CTGF expression, which was inhibited by treatment with LY2109761. Blocking TGF- β signalling with LY2109761 inhibited CTGF synthesis and release from HCC cells (and CAFs) and reduced tumour stromal content by inhibiting CAF proliferation[51]. Inhibiting this pro-fibrogenic role of TGF- β may be of particular interest in forms of HCC exhibiting dense stroma such as fibrolamellar HCC, similar to PDAC.

Overall, TGF- β promotes HCC vascularization, with an important role of VEGF and CTGF as paracrine mediators. TGF- β inhibition may mainly have an anti-angiogenic role in HCC.

3.3. TGF- β in PDAC and HCC cancer microenvironment: immune system deregulation

Many lines of preclinical evidence suggest that TGF- β plays a crucial role in immune regulation[108, 109]. The immune system is responsible for the early detection and destruction of cancer cells. Some cancer cells become immunologically invisible by passive avoidance of immune surveillance (i.e., cancer cell “hiding”). Another mechanism for escaping immune surveillance is to actively secrete cytokines that “blind” the immune system to the presence of abnormal antigens at the cancer cell surface. TGF- β 1 is the most potent immunosuppressor and plays a crucial role in this process. TGF- β 1-null mice exhibit a phenotype of excessive inflammatory response and early death, with multifocal inflammatory disease in many tissues and massive tissue infiltration by lymphocytes and macrophages[110, 111]. Interestingly, marked immunosuppression is observed in patients with PDAC or HCC[112]. Tumour-associated TGF- β 1 downregulates the host immune response via several mechanisms: it (1) drives the T-helper (Th) balance toward the Th2 immune phenotype via IL-10 as an intermediate; (2) directly inhibits anti-tumoural Th1-type responses and M1-type macrophages; (3) suppresses cytotoxic CD8+ T-lymphocytes, natural killer lymphocytes and dendritic cells functions; (4) generates CD4+CD25+ T-regulatory cell (T-regs) that suppress activity of other lymphocyte populations; (5) promotes M2-type macrophages with pro-tumoural activity mediated by secretion of immunosuppressive cytokines (e.g., IL-10, TGF- β), pro-angiogenic factors (e.g., VEGF, MMP-9, CXC chemokines), pro-inflammatory cytokines (e.g., IL-6, TNF α , IL-1), and tumour growth factors, and generates reactive oxygen species with genotoxic activity[109, 113-115].

Together, these data suggest that TGF- β overexpression in PDAC and HCC generates a favourable immune microenvironment for tumour growth, and that TGF- β inhibition may contribute to restore anti-tumoural cytotoxic immune response. The effects of TGF- β signalling at the cellular and microenvironment levels

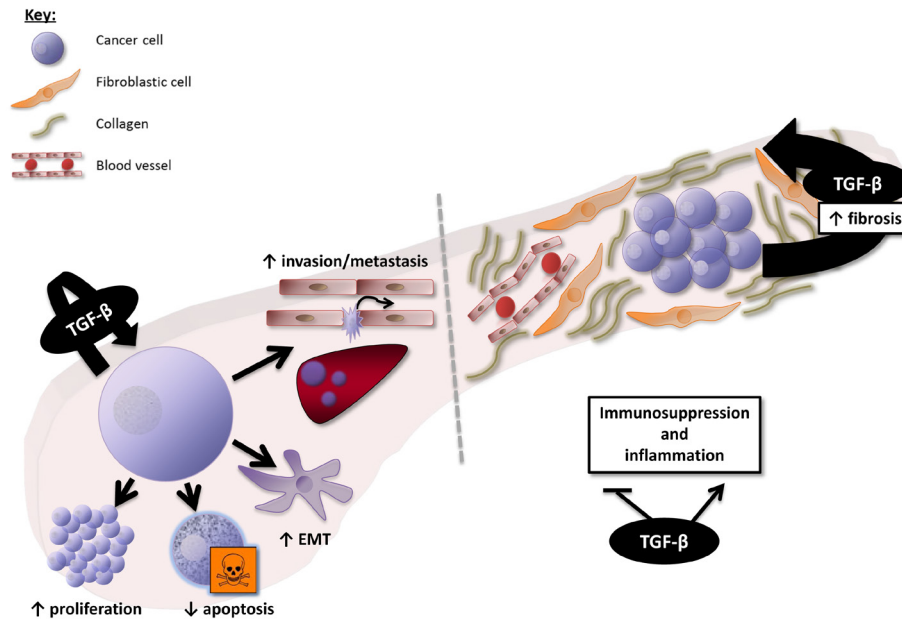


Figure 2: Overview of the effects of TGF- β signalling in PDAC. At the cellular level, TGF- β induces proliferation and survival of PDAC cells in the late phase of PDAC carcinogenesis (after SMAD4 inactivation), and promotes epithelial-to-mesenchymal transition (EMT), invasion, and metastasis. At the microenvironment level, TGF- β is a key mediator of the dialogue between cancer and stellate cells (fibrotic cells), involved in the production of a dense fibrotic stroma and the resulting low vascularization of PDAC. TGF- β also deregulates the immune microenvironment toward immunosuppression and inappropriate inflammation.

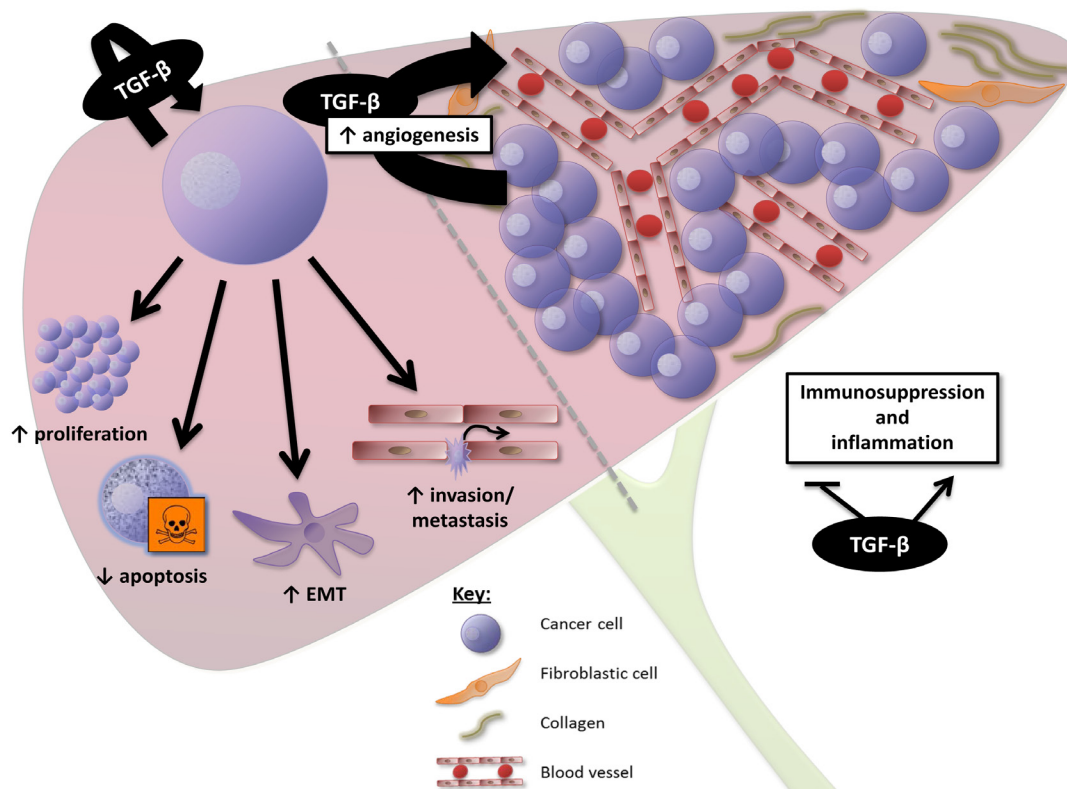


Figure 3: Overview of the effects of TGF- β signalling in HCC. At the cellular level, TGF- β induces proliferation and survival of HCC cells displaying a “late TGF- β signature”, promoting epithelial-to-mesenchymal transition (EMT), invasion, and metastasis. At the microenvironment level, TGF- β is a key mediator of angiogenesis in HCC, contributing to the high vascularization of these tumours. TGF- β also generates a favourable immune microenvironment for tumour growth.

Table 1: TGF- β pathway inhibitors in development in hepatocellular and pancreatic carcinomas

Name	Targets	Trial identifier	Current Status
TGF- β ligand inhibitors			
Lerdelimumab Genzyme®	TGF- β 2		Development stopped.
Metelimumab Genzyme®	TGF- β 1		Development stopped.
Fresolimumab Genzyme®/Aventis®	TGF- β 1, - β 2, - β 3		Not currently tested in PDAC or HCC. In progress in other cancer types.
LY2382770 Eli Lilly®	TGF- β 1		Not currently tested in PDAC or HCC. In progress outside oncology.
Trabedersen Antisens Pharma®	TGF- β 2	NCT00844064	Phase I/II completed. Phase II in progress. Results in a small PDAC cohort.
Lucanix NovaRx Corporation®	TGF- β 2		Not currently tested in PDAC or HCC. In progress in other cancer types.
Disitertide Digna Biotech®	TGF- β 1		Not currently tested in PDAC or HCC. In progress outside oncology.
TGF- β receptor inhibitors			
LY2157299 Eli Lilly®	TGF- β RI	NCT01246986 (HCC) NCT01373164 (PDAC)	Phase I completed. Phase II in progress in both PDAC and HCC. Early phase II results in HCC.
LY3022859 Eli Lilly®	TGF- β RII		Phase I in progress.

in PDAC and HCC are summarized in figures 2 and 3, respectively.

4. TGF-B AT THE PATIENT LEVEL: TGF-B PATHWAY INHIBITORS IN THE CLINIC

Many TGF- β pathway inhibitors have been investigated in the preclinical setting, some of which are now in clinical development targeting either TGF- β ligands (TGF- β 1, - β 2, - β 3) or receptors (TGF- β RI and TGF- β RII) (table 1). Nonetheless, very few of these inhibitors have been tested in the context of HCC or PDAC.

4.1. Inhibitors of TGF- β ligands

Four humanized monoclonal antibodies have been developed against TGF- β ligands, however data are not available in HCC and PDAC. Two antibodies, lerdelimumab directed against TGF- β 2 and metelimumab directed against TGF- β 1, were stopped for lack of efficacy before being evaluated in oncology. Fresolimumab, a pan-TGF- β antibody still under investigation, has completed phase II studies in glioma and relapsed malignant pleural mesothelioma, phase I in renal cell

carcinoma and malignant melanoma, and is currently in phase I in metastatic breast cancer in association with radiotherapy. LY2382770, targeting TGF- β 1, is currently being evaluated in diabetic kidney conditions but not in oncology.

Trabedersen (AP12009) is an antisense oligonucleotide targeting TGF- β 2. In preclinical models, it displayed potent anti-tumour efficacy in TGF- β 2-overexpressing PDAC cells, and drastically inhibited cell invasion, and also inhibited tumour growth, angiogenesis and lymph node metastasis in an orthotopic xenograft mouse model of metastatic PDAC[97]. It is currently in phase II development in glioblastoma. In a phase I/II study, a cohort of nine advanced PDAC patients received intravenous trabedersen (140 mg/m²/day, 4 days on/10 days off) as second-line therapy. Toxicities were limited and survival analysis showed a remarkable median overall survival of 13.4 months. One patient with liver metastasis had a complete response[116]. No clinical data are available in HCC to date.

Lucanix (belagenpumatucel-L) is a TGF- β 2 antisense gene-modified allogeneic tumour cell vaccine that has completed phase II evaluation and is currently in a phase III study against placebo in non-small cell lung cancer (NSCLC) patients as maintenance therapy after front-line treatment[117]. Phase II results showed good tolerance and a survival rate of 47% in stage IIIB and IV

NSCLC patients[117]. Lucanix has not yet been evaluated in PDAC or HCC.

Disitertide (P144) is a peptidic TGF- β 1 inhibitor specifically designed to block the interaction with its receptor. In a mouse model of metastatic colorectal carcinoma, P144 inhibited tumour growth, liver metastasis, EMT and angiogenesis[118]. Phase I studies of topical application for skin fibrosis are completed but it is yet to be evaluated in oncology.

4.2. Inhibitors of TGF- β receptors

LY3022859 (IMC-TR1) is a monoclonal antibody against TGF- β R2 that has just entered phase I clinical trial in patients with advanced solid tumors. No data are yet available in HCC or PDAAC

LY2157299 is a small molecule inhibitor of TGF- β R1. To date, it is the most advanced TGF- β signalling inhibitor under clinical development in HCC and PDAC. In preclinical models, LY2157299 has anti-tumour activity in NSCLC, breast and HCC models, affecting mainly tumour migration and invasion rather than proliferation[119]. In a triple-negative breast cancer xenograft model, TGF- β inhibition was synergistic with chemotherapy preventing the development of cancer stem cells[120]. Phase I evaluation showed a good safety profile and durable responses beyond one year in three patients. Several Phase II studies with LY2157299 are ongoing, including as second-line treatment in HCC after sorafenib (NCT01246986), in association with gemcitabine in advanced PDAC (NCT01373164), as well as in glioblastoma. Preliminary results of the HCC phase II were presented at the ASCO and ILCA 2013 meeting[121]; 106 patients have been randomized to receive LY2157299 at 160 or 300 mg/day. LY2157299 safety profile was suitable for patients with Child-Pugh A/B7 HCC. Median time to progression was 12 weeks. LY2157299 treatment was associated with AFP responses, reduction in TGF- β 1 and E-cadherin levels, and time to tumor progression was increased in patients with AFP and TGF- β 1 levels reduction from baseline. Further analysis is expected to confirm the signal to launch phase III clinical trials in patients with PDAC and/or HCC.

5. PERSPECTIVES AND CONCLUSIONS

The future of TGF- β -directed therapies is promising. However, many questions remain to be answered before optimal clinical use of these agents in PDAC and HCC is reached. At a fundamental level, there is a crucial need for pertinent and robust PDAC and HCC preclinical models to study the effects of the TGF- β pathway and its inhibition. Both these tumours have complex microenvironments in which cancer cells interact closely with ECM components and different cell types. Studying cancer cells in isolation

in vitro is a far from optimal representation of in vivo circumstances. Compared to an in vivo setting, cells grown on two-dimensional (2D) tissue culture substrates differ markedly in their morphology, differentiation, and cell-cell and cell-matrix interactions[122, 123].

TGF- β effects can also differ considerably according to the features of the culture system used, e.g. its rigidity, composition, and structure[10]. Dedifferentiation was seen in hepatocytes grown on monolayers of dried stiff collagen, caused by a specific signalling network triggered by the ECM, activating focal adhesion kinase (FAK) via Src, which in turn activated Akt, causing resistance to TGF- β -induced apoptosis by antagonizing p38[124]. In contrast, FAK was not activated when hepatocytes were grown on a softer collagen gel, keeping them sensitive to TGF- β -induced apoptosis. In the PDAC COLO-357 cell line, Sempere et al.[125] showed that TGF- β had anti-proliferative effects on PDAC cells cultured on standard plastic plates or in soft agar, while it promoted cell growth in a three-dimensional (3D) culture system. These limits of classic 2D in vitro culture models may explain some of the discrepancies between in vitro and in vivo models evaluating TGF- β functioning.

Moreover, TGF- β is a paracrine-signalling molecule mediating interactions between cancer cells and stromal cells, including stellate cells. In vitro co-culture systems of cancer and stromal cells grown within or on top of reconstituted ECM gels are able to model cancer more realistically than 2D systems. These “organotypic cultures” were first described for human skin cancer and have been exploited to investigate various tumour types, including ovarian, breast, prostate and oesophageal, providing important insights into the role of frequently altered genes in biological behaviour and mechanisms of tumour invasion[126]. Such models may provide a more accurate prediction of the in vivo situation and may be useful to study the effects of TGF- β and TGF- β inhibition.

There are many clinical challenges to developing TGF- β inhibitors, notably patient selection, timing of treatment and predictive biomarkers. Given the dual effects of TGF- β on proliferation, TGF- β inhibition may only be beneficial in tumours expressing the “late TGF- β signature” (i.e. promoting proliferation and invasiveness)[9, 46]. More so than tumour cell characteristics, predictive power of biomarkers in terms of TGF- β inhibitor efficacy may be affected by the tumour microenvironment or a patient’s overall blood biomarker profile. For example, patients with high intra-tumoural and/or circulating levels of TGF- β may be more likely to respond to specific TGF- β inhibitors. Thus, TGF- β inhibition (i.e., by inhibitors of TGF- β ligands) may be used to normalize tissue homeostasis by down-regulating excess TGF- β production of tumour and tumour-related tissues, with limited side effects on normal tissues. This raises the question of the timing and context in which TGF- β inhibition would be most beneficial. TGF- β

inhibition may be of particular interest as a preventive strategy in HCC, in which TGF- β overproduction, as a driver of the fibrotic process of cirrhosis, precedes tumour formation and create a favourable microenvironment for tumour cells[9]. This may be useful both in the primary prevention setting and as adjuvant treatment after complete HCC ablation in cirrhotic patients. In addition, although there may be a potential hazard of stimulating synchronous occult tumours through the inhibition of TGF- β -induced tumour suppression (particularly with inhibitors of TGF- β receptors), early clinical results of TGF- β inhibition in HCC patients do not show evidence of malignant transformation from underlying cirrhotic livers[9, 121]. Nonetheless, in future multidisciplinary strategies, TGF- β inhibitors should be considered with caution after extensive liver resection, as the TGF- β pathway plays a crucial role during liver regeneration.

In contrast, in PDAC, TGF- β overproduction is more a consequence of tumour development, i.e. microenvironment remodelling by tumour cells. Thus, TGF- β levels are expected to decrease after tumour resection and a preventive or adjuvant role of TGF- β inhibition may be limited to the small fraction of resectable PDAC emerging in chronic pancreatitis. In this case, TGF- β inhibitors should be preferentially used in the advanced PDAC setting. Moreover, radiotherapy and chemotherapy induce TGF- β activity, possibly promoting metastatic progression, and high levels of TGF- β are associated with resistance to anticancer treatments[10, 127]. Then, combined TGF- β inhibition may enhance tumour sensitivity to chemotherapy and radiotherapy[10]. TGF- β inhibition should thus be tested in association with conventional cytotoxic chemotherapy, both in advanced PDAC and HCC.

As TGF- β inhibitors are mainly anti-invasive agents and can display dual effects on proliferation, clinical development of these agents raises the question of whether they should be used as monotherapy or in combination. Gemcitabine is a reference chemotherapy in PDAC and can be combined with platinum salts in HCC, making it a potential cytotoxic partner with no expected overlapping toxicities. There is also a rationale for combination with targeted agents such as mTOR or MEK inhibitors, with preclinical data supporting a cooperative relationship between the Ras-ERK and TGF- β pathways[49, 52, 128]. Furthermore, EMT can be a predictive biomarker of response to MEK inhibitors; by attenuating the mesenchymal phenotype of tumour cells, TGF- β inhibition may sensitize them to MEK inhibition[129]. In addition, TGF- β cooperates with hypoxia to induce EMT and VEGF signalling through HIF-1 α induction[10, 94, 130]. This provides a rationale in HCC for combination with anti-angiogenic agents such as sorafenib (concomitant or sequential treatment after progression under sorafenib) or with hypoxia-inducing procedures such as arterial embolization. Finally, as TGF- β creates a vicious circle

of inflammation and immunosuppression, combination with immunotherapies may also be an option through the restoration of the immune response by TGF- β inhibitors[10, 109]. For example, by restoring lymphocyte cytotoxic activity, TGF- β inhibition may potentiate the effects of anti-CTLA4 or anti-PD1 antibodies.

In conclusion, despite being critical for development and tumour suppression in normal cells, in cancer, alterations of TGF- β pathway signalling do not suppress its signalling but rather change tumour cell fate through intrinsic (SMAD2/3-dependent and -independent pathways rewiring) and extrinsic (microenvironment remodelling) mechanisms. Microenvironment remodelling by TGF- β , in space and time, will generate a more hospitable environment for tumour growth and dissemination. Understanding the mechanisms mediating the dual role of the TGF- β signalling pathway is critical for the development of specific and efficient TGF- β -targeted therapies in PDAC and HCC.

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Conflicts of Interest:

Sandrine Faivre is a consultant for Merck, Pfizer, Novartis, Bayer, and Lilly; and Eric Raymond is a consultant for Pfizer, Novartis, Bayer, and Lilly. Other authors have no conflict of interest.

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