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REVIEW

Intracellular and extracellular TGF-β signaling in cancer: some recent topics

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Abstract **Transforming growth factor (TGF)-β regulates a wide variety of cellular responses, including cell growth arrest, apoptosis, cell differentiation, motility, invasion, extracellular matrix production, tissue fibrosis, angiogenesis, and immune function. Although tumor-suppressive roles of TGF-β have been extensively studied and well-characterized in many cancers, especially at early stages, accumulating evidence has revealed the critical roles of TGF-β as a pro-tumorigenic factor in various types of cancer. This review will focus on recent findings regarding epithelial-mesenchymal transition (EMT) induced by TGF-β, in relation to crosstalk with some other signaling pathways, and the roles of TGF-β in lung and pancreatic cancers, in which TGF-β has been shown to be involved in cancer progression. Recent findings also strongly suggested that targeting TGF-β signaling using specific inhibitors may be useful for the treatment of some cancers. TGF-β plays a pivotal role in the differentiation and function of regulatory T cells (Tregs). TGF-β is produced as latent high molecular weight complexes, and the latent TGF-β complex expressed on the surface of Tregs contains glycoprotein A repetitions predominant (GARP, also known as leucine-rich repeat containing 32 or LRRC32). Inhibition of the TGF-β activities through regulation of the latent TGF-β complex activation will be discussed.**

Keywords TGF-β; EMT; lung cancer; pancreatic cancer; latent form; immune function; GARP

Introduction

Transforming growth factor-β (TGF-β) is a prototype of a large family of structurally related growth regulatory factors known as the TGF-β family (or TGF-β superfamily). The TGF-β family includes more than 30 members in mammals, including TGF-β1, -β2, and -β3, activins and inhibins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [1, 2]. TGF-β was originally isolated in 1981 as a factor that induces transformation of some fibroblast cell lines, and allows these cells to grow in an anchorage-independent manner [3–5]. However, TGF-β was then found in 1984 to act as a potent growth inhibitor of epithelial cells, and further studies revealed that TGF-β inhibits growth of various types of cells, including endothelial cells and lymphocytes. TGF-β was also found to induce the accumulation of extracellular matrix (ECM) proteins and tissue fibrosis. In 1994, TGF-β was discovered to induce trans-differentiation of mammary epithelial cells to mesenchymal cells, which is now widely known as epithelial-mesenchymal transition (EMT) [6]. TGF-β accelerates metastasis of various types of cancer, and inhibition of TGF-β signaling results in prevention of cancer metastasis in various animal models. TGF-β thus regulates a variety of biological events, and abnormalities in TGF-β signaling are critically involved in the pathogenesis of various diseases, including cancer [7–10].

Although the molecular mechanisms of the growth inhibitory activity of TGF-β have been extensively studied [11] and loss of the TGF-β signaling activity is linked to pathogenesis in various cancers, TGF-β is now known to be also involved in the progression of cancer, particularly at advanced stages [12]. Roles of TGF-β signaling in cancer have been reviewed by others [7–10]. In addition, roles of other members of the TGF-β family in cancer have been discussed in other review articles [13, 14]. Thus, we focus this review on some recent topics on TGF-β. We first describe the biological activities of TGF-β, including EMT. We then discuss the roles of TGF-β in progression of lung and pancreatic cancers, because some intriguing findings have been reported in these cancers. Finally, we

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describe recent findings on latent forms of TGF-β and its activation *in vivo* [15]. Latent TGF-β complexes contain either latent TGF-β-binding proteins (LTBPs) or glycoprotein A repetitions predominant (GARP, also known as leucine-rich repeat containing 32 or LRRC32) [16, 17]. Because the latent TGF-β complex containing GARP is expressed in regulatory T cells (Tregs) and suppresses immune function in cancer, targeting latent TGF-β complexes is a potentially interesting way to specifically regulate the activity of TGF-β in some cancers.

Intracellular TGF-β signaling

TGF-β receptors contain protein kinase domains with dual kinase activities, i.e., serine/threonine and tyrosine kinase activities, which transduce unique intracellular signals. In this section, we describe the mechanisms of intracellular signaling of TGF-β, abnormalities of which play critical roles in the development of cancer [18–20].

Activation of the intracellular TGF-β signaling pathway

TGF-β ligands bind to specific type II (TβRII) and type I receptors (TβRI, also known as activin receptor-like kinase 5 or ALK-5), which, by forming a hetero-tetrameric complex, activate downstream signaling pathways (Fig. 1). Betaglycan, also known as the TGF-β type III receptor, facilitates binding of TGF-β ligands, particularly TGF-β2, to TβRII, and in the absence of betaglycan, TGF-β2 is less active than TGF-β1 or TGF-β3. The TβRII kinase transphosphorylates the Gly-Ser-rich (GS) domain of TβRI, and induces the activation of the TβRI kinase. The TβRI kinase then transduces intracellular signals by phosphorylating the C-terminal two serine residues of the receptor-regulated class of Smads (R-Smads). TGF-βs and activins induce phosphorylation of Smad2 and Smad3 (activin/TGF-β-specific R-Smads), whereas BMPs induce phosphorylation of Smad1, Smad5, and Smad8 (BMP-specific R-Smads). The activated R-Smads form oligo-

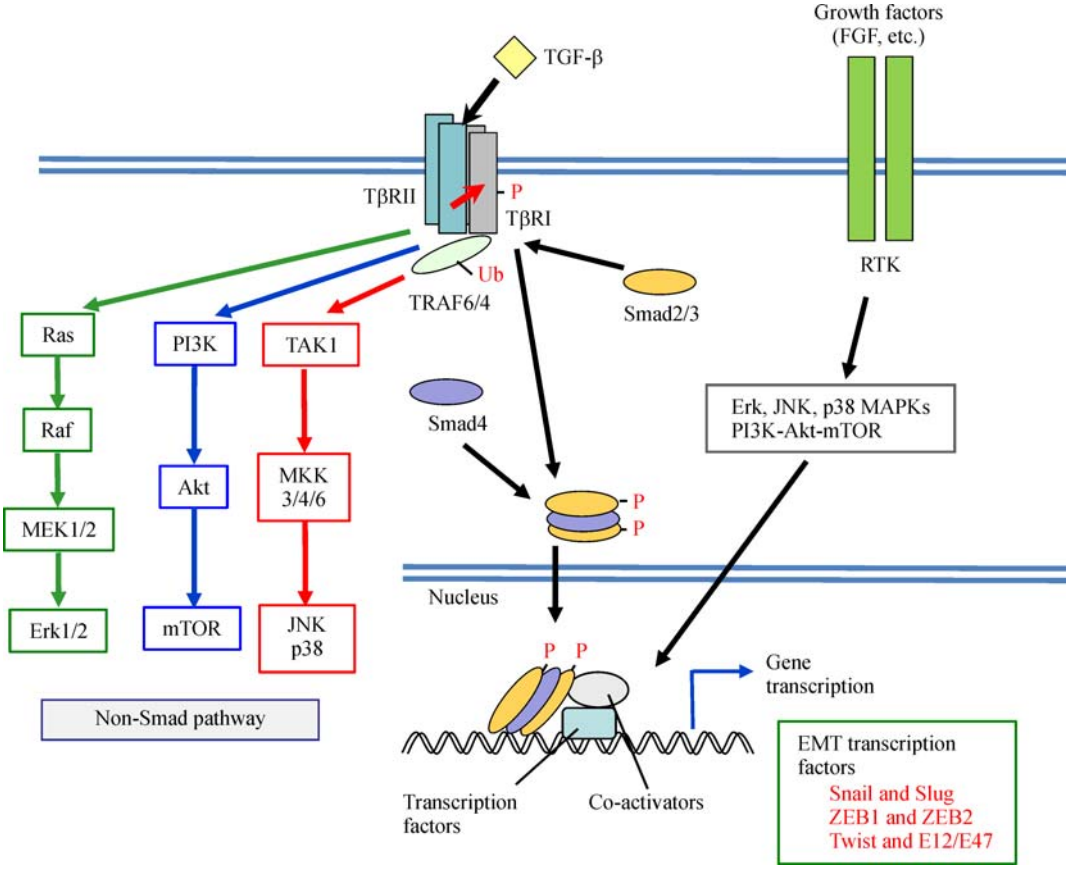

Fig. 1 Intracellular signal transduction by TGF-β. Upon binding of TGF-β ligands to the receptors, the Smad pathway involving Smad2 and/or 3 (Smad2/3) and Smad4 is activated (middle). The TGF-β-Smad pathway regulates the expression of various target genes, and EMT transcription factors induced by TGF-β signaling are shown. TGF-β also activates non-Smad pathways, including the TRAF6 and/or 4 (TRAF6/4)-TAK1-JNK and/or p38 pathway, PI3K-Akt-mTOR pathway, and Ras-Erk1 and/or 2 (Erk1/2) pathway (left). In addition, the growth factor-RTK pathway modulates the TGF-β signaling pathway (right). Ub, ubiquitin; P, phosphorylation.

meric complexes with the common-partner Smad (co-Smad), Smad4. The Smad complexes then move into the nucleus and regulate the expression of various target genes, such as those encoding inhibitory Smads (I-Smads), i.e., Smad6 and Smad7. Smad7 inhibits TGF-β signaling through multiple mechanisms, including inhibition of R-Smad activation through competition for binding to the TGF-β receptors [21].

In addition to the Smad pathway, TGF-β activates non-Smad pathways, including extracellular signal-regulated kinase (Erk) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways, phosphoinositide 3'-kinase (PI3K)-Akt pathway, Src tyrosine kinase pathway, and Rho GTPase pathway (Fig. 1) [22]. Notably, some of the non-Smad pathways play critical roles in tumorigenesis: Erk activation is initiated by recruitment of the adaptor protein Shc to phospho-Tyr residues in TβRI. Shc then recruits Grb2-Sos1, and activates Ras and the downstream Erk MAP kinase pathway [23]. TβRI also contains a binding motif for the E3 ubiquitin ligase tumor necrosis factor (TNF) receptor activated factor 6 (TRAF6) and TRAF4 in its juxtamembrane region. Upon formation of the ligand-induced TβRII and TβRI complexes, TRAF6 and/or 4 are recruited to TβRI, and auto-ubiquitination of these molecules is induced. TRAF6 and 4 subsequently cause the poly-ubiquitination of TGF-β activated kinase 1 (TAK1), leading to activation of its kinase activity. Activated TAK1 then phosphorylates and activates MAP kinase kinases, such as MKK3, 4 and 6, which in turn activate the downstream p38 MAP kinase and JNK pathways, leading to promotion of cell migration and apoptosis [24–26].

In addition to the Smad and non-Smad pathways, ligand binding induces cleavage of the TβRI protein, resulting in liberation of its intracellular domain (ICD) in the cytoplasm. After translocation into the nucleus, the ICD of TβRI regulates gene transcription and activates some cellular programs, such as cell invasion [27, 28].

Regulation of gene expression

Smads directly bind regulatory gene sequences and activate or repress gene expression in cooperation with other DNA-binding transcription factors, such as AP-1 and 2, ETS, and hepatocyte nuclear factor (HNF)-4α, and transcriptional co-activators (p300 and CBP) or co-repressors (Ski and SnoN). c-Ski and the related SnoN protein (Ski-like) directly interact with Smad2 and 3 and Smad4 and repress transcription by recruiting histone deacetylases. c-Ski also interferes with the formation of the R-Smad-co-Smad complex to repress TGF-β signaling [29]. The functions of Smads are regulated by other signaling pathways, including non-Smad pathways activated by the TGF-β receptors. They are also regulated by post-transcriptional regulation, such as phosphorylation, ubiquitination, sumoylation, and acetylation [30]. Analyses using next-generation sequencers, such as chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) demonstrate genome-wide DNA-binding landscapes of Smad proteins in various types of cells under different conditions [31]. TGF-β also regulates the expression of noncoding RNAs, such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) [32, 33], and regulates various cell responses, including EMT.

Multiple functions of TGF-β

Regulation of cell proliferation and apoptosis

TGF-β exhibits potent growth inhibitory activity in various types of cells [34, 35]. The cytostatic effects by TGF-β are mediated mainly through induction of cyclin-dependent kinase inhibitors, including $p15^{INK4B}$ and $p21^{CIP1/WAF1}$, and inhibition of the expression of proliferation factors, such as c-Myc, Cdc25A, and Id proteins. Cancer cells often acquire resistance to the growth inhibitory activity of TGF-β. In contrast, TGF-β can also induce cell proliferation in some cell types, including fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, and mesenchymal stem cells. The growth promoting effects of TGF-β are mediated by induction of some growth factors, such as platelet-derived growth factors (PDGFs) and fibroblast growth factors (FGFs).

In addition to induction of growth arrest, TGF-β also induces apoptosis [1, 35, 36]. TGF-β increases the expression of death-associated protein kinase (DAPK) and growth arrest and DNA damage-inducible 45β (GADD45β) in hepatocytes or hepatoma cells, and activates the Bcl-2 family pro-apoptotic effector Bim in some epithelial cells, and B lymphocytes. Moreover, TGF-β inhibits the expression of the pro-survival protein survivin, leading to apoptosis in colon cancer and prostate epithelial cells. TGF-β also induces apoptosis by activation of JNK and/or p38 MAP kinases through the TRAF6-TAK1-MKK3/4/6 pathway in some cell types. Thus, TGF-β induces apoptosis of various epithelial cells and lymphocytes; however, mechanisms of the TGF-β-induced apoptosis appear to be dependent on cell type and culture conditions. In contrast to pro-apoptotic effects, TGF-β also stimulates survival of certain types of cells in a context-dependent manner through activation of the PI3K-Akt signaling pathway or induction of certain pro-survival proteins, such as Bim.

Cell differentiation, EMT, and maintenance of stemness by TGF-β

TGF-β regulates cell differentiation to a variety of cell

lineages [1, 2], e.g., immune, blood, and neural cells. TGF-β inhibits differentiation of mesenchymal cells towards adipocytes and skeletal myocytes, while it stimulates their differentiation toward chondrocytes.

EMT is a crucial step in which epithelial cells differentiate into mesenchymal cells, and TGF-β induces EMT in various epithelial cells [37–39]. EMT is important in embryonic development and tissue morphogenesis, wound healing, and cancer. During the process of EMT, reduced expression of epithelial markers, including E-cadherin and epithelial splicing regulatory proteins (ESRPs), and increased expression of mesenchymal markers, including N-cadherin, fibronectin, vimentin, and α-smooth muscle actin (α-SMA), are observed. Cells that have undergone EMT display disruption of tight junctions connecting epithelial cells, loss of cell polarity, increased cell motility, and induction of a spindle-shaped morphology with actin stress fiber formation. E-cadherin is critical for cell-cell attachment of epithelial cells at the adherens junction, and loss of E-cadherin is an essential event for EMT. The roles of EMT induced by TGF-β in cancer will be further discussed below.

TGF-β plays essential roles in the acquisition and maintenance of stem cell-like properties of some cancer cells [40, 41], e.g., glioma-initiating cells, breast cancer stem cells, pancreatic cancer-initiating cells, and leukemia-initiating cells in chronic myeloid leukemia. On the other hand, TGF-β has also been shown to reduce the cancer-initiating cell (CIC) populations in certain cancers, including breast cancer, pancreatic cancer, and diffuse-type gastric cancer. These findings suggest that the effects of TGF-β on CICs may be regulated in context-dependent manners [42, 43], possibly reflecting the properties of the original tissue stem cells. EMT mediated by TGF-β can induce a stem cell-like phenotype in cancer cells. Inhibition of TGF-β signaling thus decreases the expression of stemness markers and induces the differentiation of cells to less aggressive phenotypes [44].

Tissue fibrosis and angiogenesis

Increased expression of TGF-β, especially TGF-β1 and -β2, are observed in tumor tissues compared to normal surrounding tissues, and high expression of TGF-βs correlates with poorer prognosis of cancer patients. Roles of TGF-β in tumor microenvironment have been discussed by others [7, 45, 46].

TGF-β promotes tissue fibrosis through induction of the migration of fibroblasts and monocytes at the sites of injury [45–47]. TGF-β is also a potent inducer of the production of ECM proteins, such as fibronectin and collagens. Fibrotic response to TGF-β is relevant to its roles in cancer progression, because desmoplastic response is observed in some types of cancer, especially in pancreatic cancer and diffuse-type gastric cancer.

TGF-β potently inhibits the growth of vascular and lymphatic endothelial cells *in vitro*; however, it functions as a pro-angiogenic factor and stimulates angiogenesis *in vivo* under certain conditions [48]. High expression of TGF-β is correlated with increased vascularity in some types of tumors. For induction of angiogenesis, TGF-β induces the expression of angiogenic factors, such as vascular endothelial growth factors (VEGFs). Moreover, TGF-β has been reported to stimulate the production of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, and repress that of tissue inhibitor of metalloproteinases (TIMPs) *in vivo*. Migration and invasion of vascular endothelial cells are induced by increased MMP activity, leading to induction of angiogenesis. It should be noted that TGF-β suppresses angiogenesis in a context-dependent manner through regulation of the expression of some angiogenic factors and inhibitors. In diffuse-type gastric carcinoma, TGF-β induces the synthesis of thrombospondin-1 and suppresses angiogenesis *in vivo* [49].

In addition to regulation of cell growth, TGF-β disrupts cell-cell junctions of vascular endothelial cells through repression of the expression of claudin-5 [50]. TGF-β also induces differentiation of some endothelial cells into mesenchymal cells, known as endothelial-mesenchymal transition (EndMT) [51]. Furthermore, TGF-β disrupts endothelial cell-cell junctions by inducing the expression of angiopoietin-like 4 (Angptl4), and stimulates the trans-endothelial movement of cancer cells. TGF-β may thus accelerate the colonization of tumor cells to establish metastatic foci [52].

Immune responses

TGF-β functions as a potent immunosuppressive cytokine [53–56] and therefore, inhibition of TGF-β signaling in the immune system may lead to an enhancement of tumor immunity. TGF-β suppresses the proliferation of T and B cells and the functions of cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells. TGF-β1-deficient mice show rapid development of lethal inflammation after birth [57, 58]. Moreover, T cell-specific deletion of TβRII or TβRI results in neonatal lethal inflammatory disease. In contrast, TGF-β induces the differentiation of Tregs in the presence of interleukin-2 (IL-2) [59, 60] and stimulates the generation of IL-17-positive pro-inflammatory helper T cells (Th17) in the presence of IL-6 or IL-21 [61, 62]. TGF-β can thus induce both regulatory and pro-inflammatory T lymphocytes, depending on the presence of pro-inflammatory cytokines. In addition, TGF-β suppresses the generation of natural killer (NK) cells in the presence of interferon-γ [63]. TGF-β also acts on macrophages and neutrophils and polarizes them towards immunosuppressive phenotypes [55]. The roles of TGF-β in immune responses will be further discussed below.

TGF-β-induced EMT in cancer progression

Carcinoma cells activate the EMT program to drive cancer progression. EMT is a reversible process in which epithelial cells acquire a mesenchymal phenotype and enhanced motility and invasion. EMT contributes to initiation and progression of cancer through induction of cell dissemination, stromal formation, cancer stem cell generation, and chemoresistance [37–39, 64, 65].

Smad pathway and non-Smad pathways in the regulation of EMT

Epithelial plasticity of cancer cells is controlled by signals from the tumor microenvironment. Multiple signaling pathways need to be activated and coordinated to induce EMT. TGF-β in the tumor microenvironment regulates the plasticity of cancer cells and stromal cells in cooperation with other signaling pathways [37]. TGF-β signaling induces EMT through both Smad and non-Smad signaling pathways. The TGF-β-Smad signaling pathway directly activates the expression of the EMT transcription factors, including the zinc-finger transcription factors Snail and Slug, two-handed zinc-finger factors ZEB1 (zinc finger E-box binding homeobox 1, also known as δEF1) and ZEB2 (also known as Smad-interacting protein 1 or SIP1), and the basic helix-loop-helix (bHLH) factors Twist and E12/E47 (Fig. 1). The expression of EMT transcription factors and signal components of the TGF-β signaling pathways is controlled by miRNAs [66, 67]. During TGF-β-induced EMT, ZEB1 and ZEB2 repress the expression of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429), which inhibit the expression of ZEB proteins and TGF-β2 [68–71]. Snail binds to the promoter regions of the miR-34 family and represses their expression. Snail is targeted by the miR-34 family, and the expression of the gene encoding Snail is repressed by miR-34 [72]. Thus, double-negative feedback loops between transcription factors and miRNAs regulate TGF-β-induced EMT. Mathematical predictions and experimental confirmation suggest that feedback loops between transcription factors and miRNAs function as reversible switches to promote TGF-β-induced EMT in a stepwise manner [73–75]. lncRNAs also contribute to TGF-β-induced EMT [76–78]. TGF-β-induced lncRNA-ATB (activated by TGF-β) enhances ZEB1 and ZEB2 expression by binding to the miR-200 family in hepatocellular carcinoma [76]. TGF-β-induced EndMT is also regulated by multiple miRNAs. Activation of some miRNAs, such as miR-31, is required for TGF-β-induced EndMT in MS-1 mouse pancreatic microvascular endothelial cells [79].

TGF-β also promotes EMT through activation of non-Smad pathways. Activation of the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway by TGF-β is required for the transition to the mesenchymal phenotype and the induction of cell motility and invasion (Fig. 1) [80–83]. TGF-β-induced Erk and p38 MAP kinase signaling activation also promotes EMT, and inhibition of the Erk MAP kinase pathway prevents EMT induced by TGF-β [84–86]. TGF-β also influences junctional integrity and epithelial cell polarization through direct interaction between the TGF-β receptors and the tight junction proteins. TGF-β-activated TβRI phosphorylates the polarity protein Par6 at tight junctions leading to RhoA degradation and dissolution of the junction [87, 88]. On the other hand, during the course of EndMT in MS-1 cells, TGF-β activates the Rho signal and myocardin-related transcription factor (MRTF)-A in a Smad4-dependent manner, resulting in the induction of a mesenchymal marker, α-SMA [89].

Cooperation with diverse signaling pathways in cancer-related EMT

Cooperation of TGF-β signaling and other signaling pathways regulates epithelial plasticity. Receptor tyrosine kinase (RTK) signaling pathways activated by growth factors such as hepatocyte growth factor (HGF), FGF, PDGF and epidermal growth factor (EGF) collaborate with TGF-β signaling to control the process of EMT in cancer cells. These ligands activate the Erk, p38, and JNK MAP kinase pathways as well as the PI3K-Akt-mTOR pathway, which enhance TGF-β-induced non-Smad pathways and also affect Smad-mediated transcription. Increased activation of RTK signaling, which is observed in many cancers, enhances TGF-β-induced EMT and cell invasion. For example, Ras-mediated cell transformation activates the Erk MAP kinase pathway, and cooperates with TGF-β in the induction of EMT transcription factors [90]. In pancreatic cancer cells, activation of the oncogenic K-Ras signals is required for the induction of Snail by TGF-β. Silencing of K-Ras attenuates the Snail induction by TGF-β and TGF-β fails to induce EMT in the absence of Ras signaling [91]. Ras and TGF-β signaling activation also induces a p63-dependent transcriptional program, which leads to cell migration, invasion, and metastasis [92].

In addition to coordinately controlling the EMT transcriptional program, Ras-induced transformation and TGF-β signaling have been found to induce more global alterations in chromatin accessibility during the process of EMT. In mouse mammary epithelial EpH4 cells and Ras-transformed EpRas cells, TGF-β and Ras alter chromatin accessibility either cooperatively or independently, and AP1, ETS, and RUNX binding motifs are enriched in the accessible chromatin regions. Oncogenic ETS family transcription factors Etv4 and Etv5, which are strongly induced by Ras signaling and bind to accessible chromatin regions in EpRas cells, may regulate transcriptional regulation during Ras- and TGF-β-induced EMT [93].

Cooperation of RTK signaling with TGF-β signaling

also regulates epithelial plasticity in cancer stromal cells. EMT of epithelial cells adjacent to cancer cells plays important roles in the generation of cancer-associated fibroblasts (CAFs) [94–96]. FGF-2 (also known as basic FGF) collaborates with TGF-β in inducing differentiation of normal epithelial cells to fibroblastic cells, which may promote the invasion of adjacent cancer cells [97]. In the absence of FGF-2, prolonged treatment of normal mammary epithelial cells with TGF-β induces differentiation to α-SMA-expressing myofibroblastic cells. Addition of FGF-2 inhibits TGF-β-induced expression of α-SMA, and thus myofibroblastic differentiation. Instead, combined FGF-2 and TGF-β treatment drives the differentiation of the cells towards more migratory and invasive α-SMA-negative fibroblastic cells.

This crosstalk between FGF signaling and TGF-β signaling is controlled by alternative splicing of mRNAs. The RNA-binding proteins ESRP1 and ESRP2 are expressed in epithelial cells, and activate the epithelial-specific splicing program [98]. TGF-β-induced ZEB1 and ZEB2 repress the expression of ESRP1 and ESRP2 during EMT [99]. Downregulation of ESRPs alters the splicing patterns of many mRNAs to generate mesenchymal forms of the proteins. ESRPs induce alternative splicing of FGF receptors (FGFRs), resulting in the expression of the IIIb isoforms of FGFRs in epithelial cells. In contrast, ESRP expression is decreased during EMT, leading to the increased expression of the IIIc isoforms of FGFRs [98, 99]. Epithelial cells that express the FGFR IIIb isoforms respond to FGF-7 (also known as keratinocyte growth factor). TGF-β-induced transition to the mesenchymal state results in isoform switching, and mesenchymal cells that express the FGFR IIIc isoform become responsive to FGF-2, promoting the generation of fibroblastic cells [97].

Crosstalk between FGF and TGF-β signaling also regulates EndMT, which contributes to generation of CAFs. Similar to the roles of FGF-2 in preventing myofibroblast differentiation from epithelial cells, FGF-2 inhibits TGF-β-induced α-SMA expression in endothelial cells. FGF-2 prevents TGF-β-induced EndMT through the induction of miRNAs that target the TGF-β signal components, and through the activation of the MEK-Erk pathway that inhibits Smad2 phosphorylation [100–102].

Fundamental roles of TGF-β-induced EMT in the progression of cancer

EMT and its reversibility play important roles in multiple aspects of cancer initiation and progression. Using *in vivo* models, TGF-β-induced EMT is shown to be required for cancer cell invasion and dissemination. Targeted inactivation of TGF-β receptor expression in cancer cells or pharmacological inhibition of TGF-β signal activation inhibits the invasive phenotype and cancer cell dissemination [8, 103]. TGF-β signaling also contributes to generation of cancer stem cells through the induction of EMT [104, 105]. Autocrine TGF-β signaling is required for maintenance of the mesenchymal phenotype and tumorigenicity in breast cancer cells [106]. TGF-β-induced EMT is also linked to increased resistance to anti-cancer drugs [107, 108]. In addition to the effect on cancer cells, increased TGF-β expression and enhanced signaling activation in many cancers regulate epithelial plasticity in stromal cells, and TGF-β signaling in fibroblast-like stromal cells contributes to cancer progression [109, 110]. TGF-β promotes the generation of CAFs from epithelial cells and endothelial cells in tumor stroma through EMT and EndMT, respectively [46, 51, 111].

The roles and molecular mechanisms of TGF-β signaling and crosstalk with other signals in EMT have been well studied in cell culture. Studies using animal models and patient tumor samples provide support for the importance of TGF-β-induced EMT in cancer progression [8, 43]. The development of novel techniques, including intravital imaging, has helped demonstrate the roles of the epithelial plasticity program in cancer. More recently, tissue-clearing based 3D imaging strategies have been applied to cancer models, and these techniques have enabled the visualization of cancer micrometastases throughout the body [112, 113]. One of the tissue-clearing protocols, clear unobstructed brain/body imaging cocktails (CUBIC)-based cancer analysis, allowed spatiotemporal visualization and quantification of the metastatic cancer cells at single cell resolution [113]. This approach provides tools to visualize EMT at better resolution in mouse models at the whole organ level, and promotes understanding of the dynamics of the EMT program in cancer progression. Indeed, CUBIC-cancer analysis suggests that the TGF-β-induced EMT promotes cell survival at metastatic sites as well as extravasation of cancer cells (Fig. 2) [113]. These technical advances in visualization of EMT in animal models, together with molecular mechanistic studies, advanced bioinformatics, and mathematical modeling, will provide a better understanding of the roles of TGF-β signaling and crosstalk with other signaling pathways in the progression of cancers.

TGF-β signaling in lung cancer

Comprehensive analyses of the genomic alterations in lung cancer suggested that genes encoding core components of the TGF-β signaling pathway are largely not common sites of somatic mutations. However, accumulating evidence indicates dysregulated TGF-β signaling and its pathogenic roles in lung cancers. Lung adenocarcinoma cells, which may arise from lung epithelial stem cells [114], undergo EMT in response to TGF-β and acquire tumor-progressive phenotypes (Fig. 3). TGF-β also regulates tumor progression through the expression of its various target genes. In

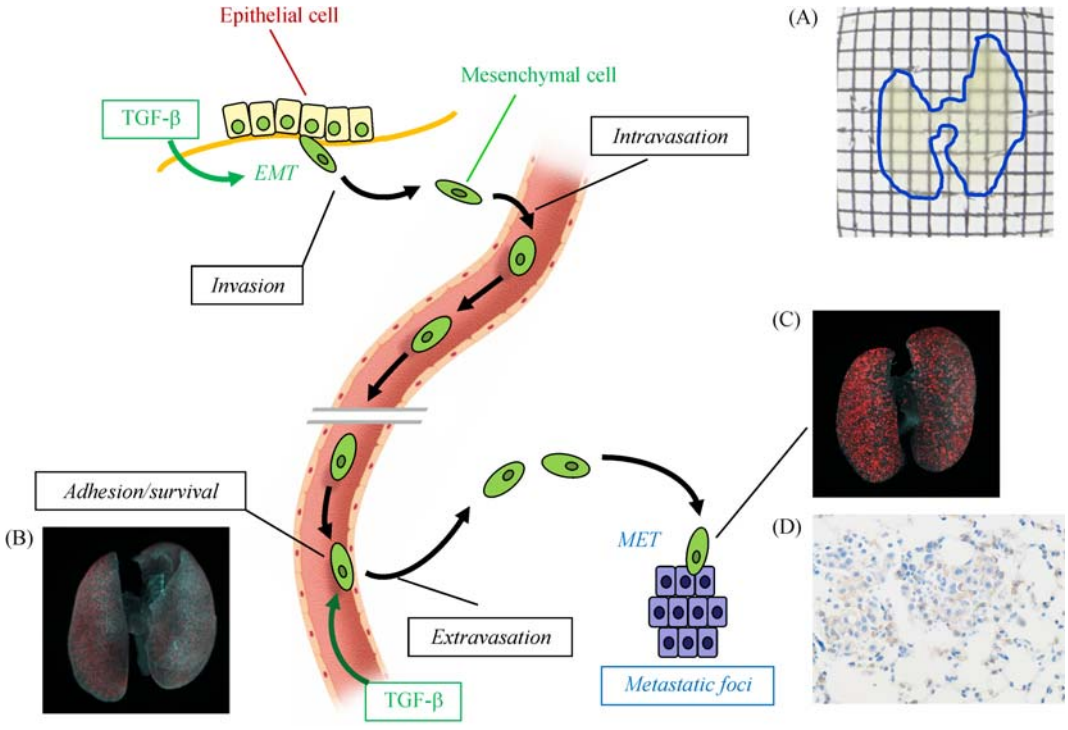

Fig. 2 Regulation of cancer metastasis by TGF-β and analyses by whole-body tissue-clearing. TGF-β acts on epithelial cells and accelerates the invasion of cells through induction of EMT. After intravasation, TGF-β stimulates cell adhesion and survival at distant organs, and facilitates extravasation. Then, cancer cells may undergo mesenchymal-epithelial transition (MET), a reverse process of EMT, and form metastatic foci, where the cancer cells often express an epithelial cell marker E-cadherin. (A) Whole lung of mice treated with the CUBIC tissue-clearing reagents. Blue dotted line indicates the outline of the lung. (B, C) Mice were injected with A549 lung adenocarcinoma cells pretreated with TGF-β through tail vein. Cancer cells expressing mCherry (shown in red) were visualized after 1 hour (B) and 14 days (C) after injection of cells into mice. Cell nuclei were visualized by RedDot2 (shown in blue). (D) Immunostaining of lung tissue of the mouse injected with the TGF-β-treated A549 cells. Cancer cells in the metastatic foci are positively stained by anti-E-cadherin antibody. (Courtesy of Drs. Shimpei I. Kubota, Kei Takahashi, and Hiroki R. Ueda.) See Kubota *et al.* [113].

contrast, disrupted TGF-β signaling in small cell lung carcinoma (SCLC) cells represses apoptosis and induces cell survival. Importantly, molecular mechanisms involved in these processes are related to the interaction of the TGF-β signaling pathway with the lineage-specific transcription factors NK2 homeobox 1 (NKX2-1, also known as thyroid transcription factor-1, TTF-1) or achaete-scute family bHLH transcription factor 1 (ASCL1, also known as achaete-scute homolog 1 or ASH1). This section focuses on the recent advances in our understanding of the roles of TGF-β signaling in lung cancers in relation to the recent development of targeted drugs.

TGF-β-induced target gene expression and EMT in lung adenocarcinoma cells

Non-small cell lung carcinoma (NSCLC) constitutes approximately 80% of lung cancer, of which lung adenocarcinoma is the most frequent histological subtype. Experimentally, TGF-β is a well-known inducer of EMT in A549 lung adenocarcinoma cells. Similar to other cancers of different organs, lung adenocarcinoma cells frequently acquire constitutive Ras activation through its mutations or *EGFR* (EGF receptor) mutations [115]. This allows the cells to be prone to TGF-β-induced EMT. The regulatory process of EMT induced by TGF-β in lung adenocarcinoma cells follows the common mechanisms with other types of cancers, which include the contribution of EMT-related transcription factors, such as Snail and ZEB1 [116, 117]. Likewise, TGF-β-induced EMT in lung adenocarcinoma cells is enhanced by co-stimulation with TNF-α or IL-1β secreted by other cells in the tumor microenvironment [118].

NKX2-1 plays a central role in tissue-specific regulation of EMT. NKX2-1 is a transcription factor essential for the development of thyroid, lung, and a part of the brain [119]. NKX2-1 is expressed in the adult lung epithelium and is important for the expression of genes related to lung epithelium-specific functions. In lung cancer, NKX2-1 is frequently expressed in both adenocarcinoma cells and SCLC cells. As described in detail previously, NKX2-1 works as both a tumor suppressor and oncogenic factor in

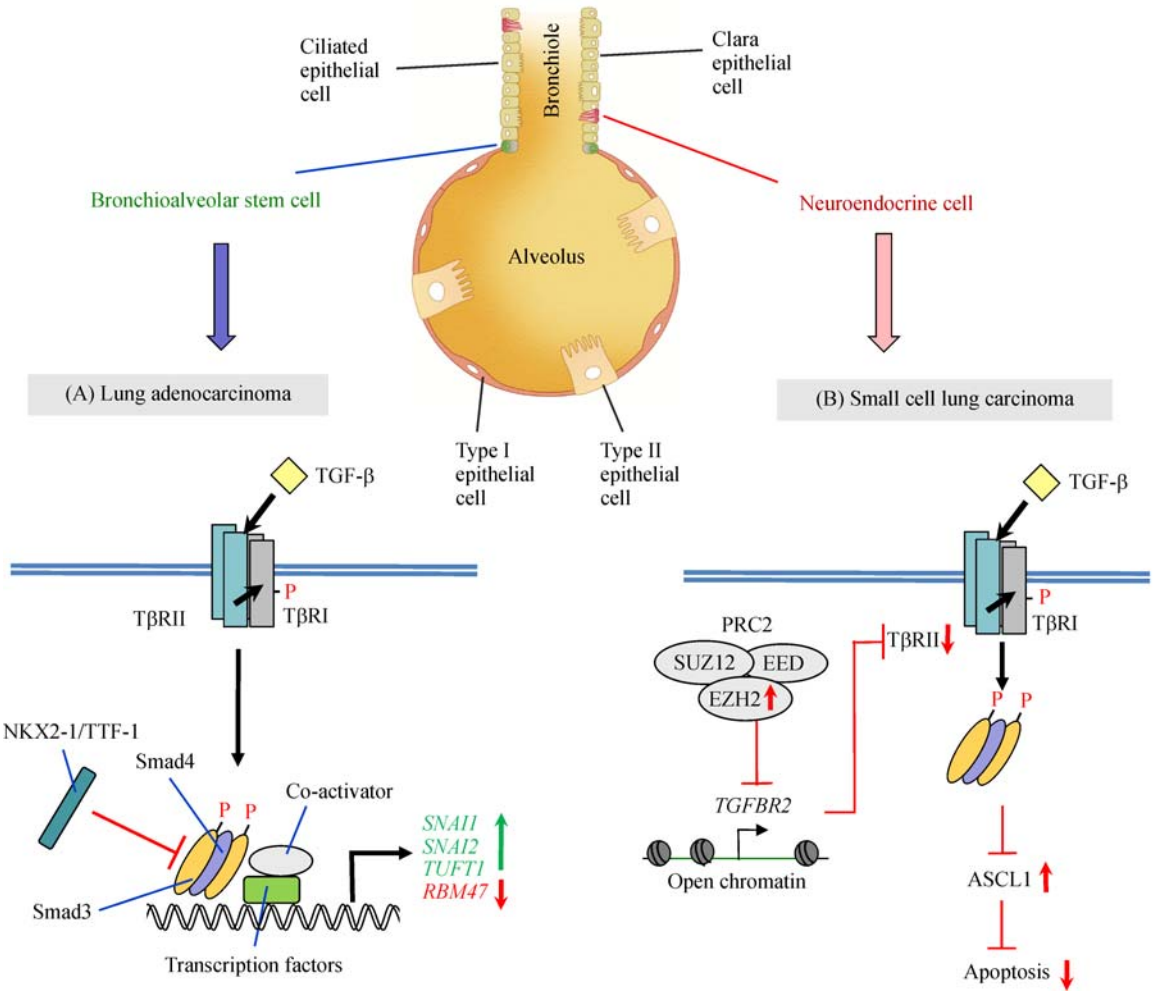

Fig. 3 Roles of TGF-β signaling in lung adenocarcinoma and small cell lung carcinoma. (A) TGF-β signaling in lung adenocarcinoma. TGF-β signaling induces the expression of *SNAI1* and *SNAI2* genes, encoding Snail and Slug, respectively, and regulates the expression of other target genes involved in progression of cancer. NKX2-1/TTF-1 antagonizes the effects of TGF-β-Smad signaling. (B) TGF-β signaling in small cell lung carcinoma (SCLC). TGF-β inhibits the expression of ASCL1/ASH1 through the Smad signaling pathway. Because ASCL1 induces cell survival, TGF-β signaling attenuates the induction of cell survival by ASCL1. In SCLC cells, expression of TβRII (encoded by the *TGFBR2* gene) is downregulated through an epigenetic mechanism by an increase in the EZH2 expression. Thus, TGF-β signaling is suppressed in SCLC cells, leading to enhanced cell survival in SCLC cells.

lung adenocarcinoma [120, 121]. NKX2-1 is highly expressed in some lung adenocarcinoma cell lines, including NCI-H441 cells, but not in A549 cells [116]. Accordingly, E-cadherin expression is high in NCI-H441 cells, whereas it is low in A549 cells. Overexpression of NKX2-1 in A549 cells reverses TGF-β–induced EMT, decreases MMP-2 activity, and suppresses cell migration and invasion. Furthermore, exogenous NKX2-1 stimulates the expression of E-cadherin and induces the appearance of an epithelial phenotype. Conversely, TGF-β induces the expression of EMT transcription factors, i.e., Snail and Slug, in A549 cells, and knockdown of NKX2-1 in NCI-H441 cells accelerates the TGF-β-induced EMT program. On the other hand, Smad3 inhibits binding of NKX2-1 to the *SFTPB* (encoding surfactant protein B) promoter [122], suggesting a close relationship between Smads and NKX2-1 in the transcriptional regulation.

Transcription factor binding sites are determined by genomic sequences, epigenetic status, and the repertoire of binding molecules expressed in the cells. Indeed, chromatin accessibility and epigenetic modifications are altered during the EMT process [93, 123], and emerging roles of lncRNAs in the regulation of histone modification have been revealed. A lncRNA MEG3 is induced by TGF-β and involved in the process of EMT in lung adenocarcinoma cells through recruitment of polycomb repressive complex 2 (PRC2) and its accessory component jumonji and AT-rich interaction domain containing 2 (JARID2) to the promoter regions of *CDH1* (encoding E-cadherin) and *MIR200* family genes (encoding the miR-200 family) to

induce tri-methylation of lysine 27 of histone H3 (H3K27) [78]. In Smad signaling, the binding regions of the Smad family in the genome are strikingly different depending on the cellular context [124, 125]. A genome-wide analysis of Smad3 binding regions in NCI-H441 cells demonstrates that most of the Smad3 binding regions are shared with NKX2-1. Further investigation revealed that NKX2-1 disrupts the Smad3-Smad4 complex in the nucleus and dramatically alters both the binding strength and distribution of Smad3 throughout the genome (Fig. 3A) [126]. In addition, NKX2-1 forms a complex with Smad3, but without Smad4, and the Smad3-NKX2-1 complex regulates the expression of target genes related to other processes of cancer, such as LMO3 [126].

Analyses of lung adenocarcinoma cells by next-generation sequencers allowed the identification of new target molecules of TGF-β. An RNA binding motif protein RBM47 is induced by NKX2-1 and suppressed by TGF-β, and RBM47 functions as a tumor suppressor by inhibiting the activity of NF-E2-related factor 2 (Nrf2), a master regulator of various cytoprotective genes [127]. RBM47 binds to kelch-like ECH-associated protein 1 (Keap1) and Cullin 3 mRNAs and increases their protein expression [127]. Because Keap1 is a component of the Cullin 3-based E3 ubiquitin ligase complex and decreases the stability of Nrf2 protein [128], a decrease in the expression of RBM47 results in the activation of Nrf2 in lung adenocarcinoma cells. In contrast, a cytoplasmic protein tuftelin 1 (TUFT1) is induced by TGF-β and functions as a pro-tumorigenic factor. TUFT1 enhances mTOR complex 1 (mTORC1) signaling by modulating the Rab GTPase-regulated processes through interaction with RABGAP1 [129].

Inhibition of the Smad2 activity by chaperonin containing TCP1 subunit 6A (CCT6A), with the function of Smad3 intact, was shown to be associated with metastasis of NSCLC cells [130]. In contrast, Smad3 activates a transcriptional program that promotes cell survival and cancer metastasis in NSCLC cells. ChIP-seq analysis using A549 cells revealed differential binding of Smad2 and Smad3 to the genome and regulation of distinct target genes for Smad2 and Smad3, which explains their opposite functions. Mechanistically, CCT6A directly binds to Smad2 and inhibits the interaction of Smad2 with Smad4. This regulatory process appears not to be restricted to lung adenocarcinoma cells because of the expression of CCT6A in several types of cancers.

Inactivated TGF-β signaling pathway in SCLC cells

SCLC constitutes a smaller subset of primary lung cancer. Somatic mutations are found in *TP53* and *RB1* genes in most cases [131]. Mice carrying mutant alleles for both *Trp53* and *Rb1* exclusively develop SCLC by intratracheal administration of a Cre-expressing adenoviral vector [132]. Importantly, a lineage-specific transcription factor, ASCL1/ASH1, specifically regulates neuronal and oncogenic gene expression and provides tumor-initiating capacity in SCLC cells [133, 134]. Tumors are not formed in the absence of ASCL1 in the SCLC mouse model.

Somatic mutations in the genes involved in the TGF-β signal transduction pathway are rare in SCLC. Thus, SCLC mouse models related to TGF-β signaling have not been reported. However, most SCLC cells have low expression of *TGFBR2,* and downstream TGF-β signaling is suppressed (Fig. 3B) [135–137]. Mechanistically, altered epigenetic regulation of gene expression is a characteristic of SCLC, and elevated expression of enhancer of zeste 2 (EZH2) and other PRC2 proteins are found in SCLC cells compared with NSCLC cells [138–141]. Indeed, the elevated expression of EZH2 contributes to the down-regulation of *TGFBR2* expression in SCLC [137]. Both EZH2 shRNAs and an EZH2 inhibitor restore *TGFBR2* expression, which in turn suppress the expression of lineage-specific gene *ASCL1* and its anti-apoptotic function via activation of Smad2 and/or 3. By using patient-derived xenograft (PDX) samples, SCLC was classified based on the patterns of CpG methylation. Elevated expression of EZH2 was also observed and PDX tumor growth was suppressed by EZH2 inhibitors [142]. EZH2 not only induces histone H3K27 tri-methylation but also recruits DNA methyltransferases (DNMTs) by direct interaction [143]. The expression of *EZH2* correlates with high promoter CpG methylation in the development of SCLC among various cancers in The Cancer Genome Atlas (TCGA) data [142]. These findings suggest central roles of EZH2 in SCLC, partly through the suppression of TGF-β signaling.

Heterogeneity in SCLC is related to chemoresistance of the cancer cells, and differential expression of *ASCL1* and *NEUROD1* and amplification of the *MYC* gene define molecular subgroups of SCLC [134, 144]. Future analysis of TGF-β signaling in SCLC, therefore, should focus on this aspect with evaluation of the therapeutic efficacy of EZH2 inhibitors.

Pancreatic cancer and TGF-β signaling

Pancreatic cancer is one of the leading causes of cancer death, with a five-year survival of less than 5% due to its high recurrence rate [145, 146]. The prognosis has not improved for more than half a century [145, 147], despite extensive research and novel insights in the field of cancer biology. Although surgical resection provides a chance of cure, median survival of patients in Stage IA (T1N0M0, the size of primary tumor is less than or equal to 2 cm without any lymph node involvement or distant metastasis) is still approximately 24 months [148]. The major histological subtype is pancreatic ductal adenocarcinoma

(PDAC), which accounts for over 90% of pancreatic cancer. PDACs originate from pancreatic ductal epithelium and evolve from premalignant lesions to fully invasive cancer with successive accumulation of gene mutations [149, 150]. Since *SMAD4* is identical to a putative tumor suppressor gene "*deleted in pancreatic cancer locus 4, DPC4*" [151], there have been extensive efforts to clarify the impact of this pathway on the development of PDACs.

Multistep progression of PDACs

Similar to colorectal cancers, in which the multistep progression of cancer is well described and is supported by organoid models [152–155], PDACs are thought to develop through a particular sequence of genetic alterations: *KRAS* activation followed by loss of function of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and then mutations in *TP53* and *SMAD4*. The most common precursor lesions of PDACs are known as pancreatic intraepithelial neoplasia (PanIN), which is graded from 1 to 3 depending on the extent of dysplasia and the risk of malignant transformation (Fig. 4A) [149]. Genomic sequencing confirmed that the most common genetic alterations in low-grade dysplasia PanIN-1 lesions are mutations in *KRAS*, whereas *KRAS* wild-type PDACs are rare (less than 7%) [156, 157]. The mutant *KRAS* gene produces a constitutively active form of Ras, which results in aberrant activation of proliferative and survival signaling pathways. During tumorigenesis, inactivation of tumor suppressor genes is required to further drive clonal expansion. Inactivating mutations in *CDKN2A*, encoding the cell cycle regulator $p16^{INK4A}$ protein, can be detected as early as PanIN-1/2 lesions [157, 158]. In addition, mutations in *TP53* and *SMAD4* genes can be detected in the PanIN-3 stage, or severe dysplasia/carcinoma *in situ* [157, 159]. *TP53* is abnormal in approximately 50%–75% of tumors with most changes as mutations rather than deletions. *SMAD4* is inactivated in approximately 20%–50% of tumors. These four genes are major genes with alterations found in PDACs, while recent exome and whole-genome sequencing data revealed recurrent somatic mutations in genes such as *ARID1A*, *RNF43*, and *RREB1*, but to lesser extents [160–163]. It is of note that examination of the genomes of 107 PDAC patients showed that two or more somatic alterations occurred simultaneously rather than sequentially [164]. Although the sequential stepwise-progression model has been well-established, it is also possible that a few catastrophic events

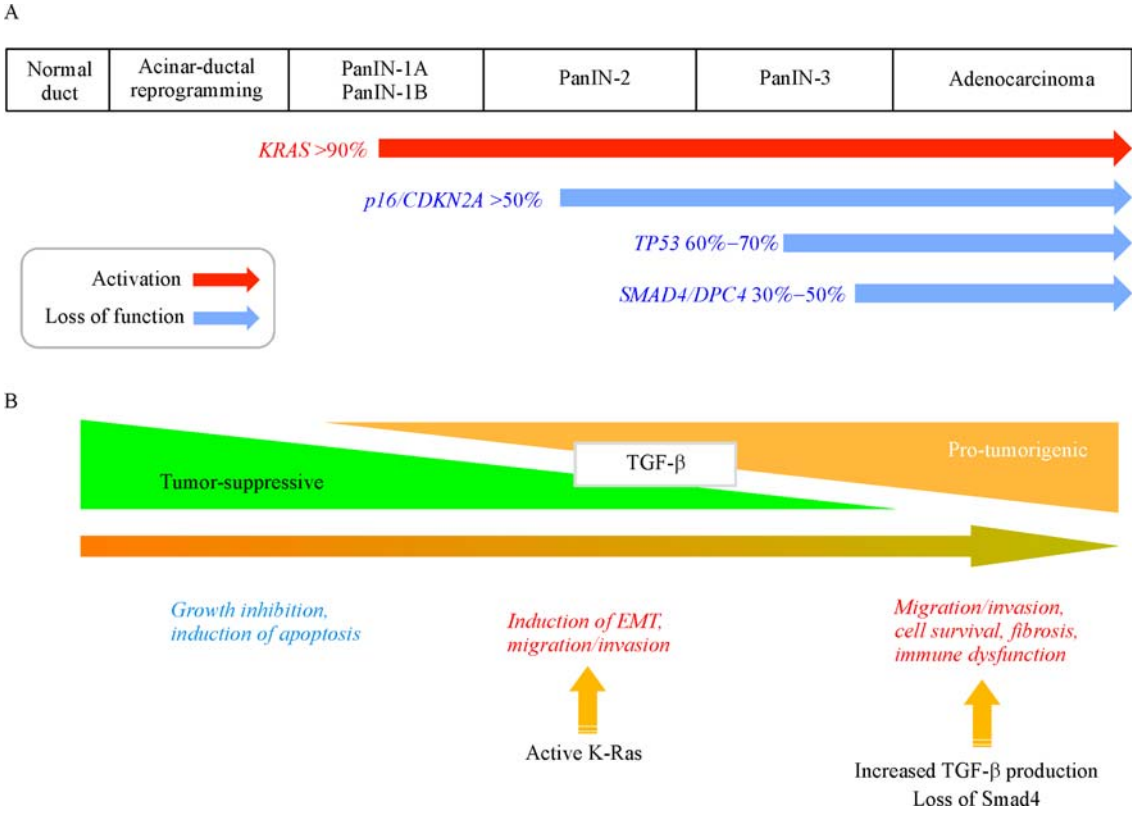

Fig. 4 Roles of TGF-β signaling in pancreatic carcinoma. (A) Multistep progression of pancreatic carcinoma. Genes involved in progression of pancreatic carcinoma and the frequencies of abnormalities of these genes are shown [239]. Red, oncogene; blue, tumor-suppressive genes. (B) Tumor-suppressive and pro-tumorigenic activities of TGF-β during the development of pancreatic carcinoma.

like chromothripsis strongly promote the evolution of PDACs.

Tumor-suppressive roles of TGF-β in PDACs

TGF-β is a bifunctional regulator during tumorigenesis, which functions as a tumor suppressor in early stages and as a tumor promoter in later stages of cancer [8, 41]. A growing body of evidence demonstrates that the bifunctional nature of TGF-β involves both cell-intrinsic and environment-mediated mechanisms. In several cancers, for example, inactivation of *SMAD4* gene serves as the cell-intrinsic switch, causing the escape from the cytostatic effects of TGF-β. However, Smad4-intact cancer cells, such as breast cancer cells, have a different cell-intrinsic switch of TGF-β signaling: accumulation of pro-oncogenic stimuli switches and stabilizes the TGF-β-induced migratory and invasive phenotype of Ras-transformed mammary epithelial cells [165].

During the course of PDAC development, TGF-β signaling functions as a tumor suppressor by inducing cell cycle arrest and apoptosis of epithelial cells, thereby preventing clonal expansion caused by Ras activation [34]. Indeed, several components of the TGF-β signaling pathway, not only *SMAD4 (DPC4)* [151], but also *TGFBR2*, *TGFBR1* (ALK-5) [166], and *ACVR1B* (also known as ALK-4) [167], become genetically inactivated in PDACs. Recent exome and whole-genome sequencing data confirmed these findings and showed that the frequency of the latter three mutations is approximately 5% or less [160–163]. Intriguingly, *SMAD4* gene inactivation is associated with a poorer prognosis [168, 169], indicating that the TGF-β-Smad4 signaling functions as a tumor suppressor in PDACs (Fig. 4B).

The tumor-suppressive role of TGF-β is well recapitulated in PDAC mouse models, which use the pancreatic and duodenal homeobox gene 1 *(Pdx1)* or pancreatic transcription factor 1a *(Ptf1a,* or *p48*) promoter system [170]. Endogenous expression of oncogenic $Kras^{G12D}$ serves to initiate PanIN, which can spontaneously progress to fully invasive and metastatic disease at a low frequency [170]. Pancreas-selective *Smad4* knockout on top of the *Kras* activation results in the rapid development of pancreatic cystic neoplasms, i.e., intraductal papillary mucinous neoplasms (IPMNs) [171] and mucinous cystic neoplasms (MCNs) [172], although *Smad4* inactivation alone causes no pancreatic neoplasm formation. On the other hand, *in vivo* disruption of TGF-β signaling in the pancreas by Smad7 overexpression induces premalignant ductal lesions through promoting proliferation of ductal and acinar cells [173]. Interestingly, inactivation of *Tgfbr2* has stronger effects than inactivation of *Smad4* [174]. It suggests that Smad4-independent cellular signaling activated by TβRII also hinders clonal expansion. It is of note that tripartite motif containing 33 (TRIM33, also known as transcriptional intermediary factor-1γ or TIF1γ, or ectodermin), which forms a complex with Smad2 and/or 3 without Smad4 [175], functions as a tumor suppressor during the course of PDAC development [176].

Pro-tumorigenic functions of TGF-β in pancreatic cancer

In the later stage of pancreatic cancer development, TGF-β functions as a tumor-promoting factor. Cancer cells secrete larger amounts of TGF-β1 than their normal cell counterparts do, and this overexpression is strong in the later stages of pancreatic cancers and other malignancies (Fig. 4B) [177]. Consistently, inhibitors for the TGF-β signaling components possess therapeutic potential against pancreatic cancers, at least those in orthotopic mouse models, e.g., a soluble TβRII protein [178], TβRI kinase inhibitor SD-208 [179], TβRI inhibitor SB431542 [180], dual inhibitor of TβRI/II kinase LY2109761 [181], and neutralizing antibody against TβRII [182]. In addition, TβRI kinase inhibitor Galunisertib (LY2157299) is now in phase I/IB clinical trials in Japanese patients with metastatic or locally advanced pancreatic cancer, with promising preliminary results [183, 184]. Because the Smad4-dependent signaling pathway may function as a tumor suppressor, Smad4-independent intracellular signaling in cancer cells or environment-mediated mechanisms can explain the tumor-promoting effects of TGF-β, which may be blocked by these inhibitors.

The molecular mechanisms regarding how TGF-β promotes tumor progression in the later stage are still elusive. In general, TGF-β enhances migration, invasion, and survival of tumor cells through stimulating ECM deposition and tissue fibrosis, perturbing immune and inflammatory function, stimulating angiogenesis, maintaining the stem cell-like properties of CICs, and promoting EMT [12, 53, 64, 185]. However, some of these characteristics are regulated through the Smad pathway, and Smad4 loss attenuates them. For example, activin and Nodal drive self-renewal and tumorigenicity of pancreatic cancer stem cells in a Smad4-dependent manner [186], whereas TGF-β impairs the activity of pancreatic CICs [187]. Since *SMAD4* mutations predict a poor prognosis in patients with PDACs [168, 169], the latter case may apply to patients with PDACs.

In addition, pancreatic cancer cell lines with Ras-activation have been widely used to analyze TGF-β-induced EMT. As discussed in the EMT section, the gene induction of transcription factors associated with EMT such as Snail, Slug, ZEB1, and ZEB2 are usually Smad4-dependent. Since Smad4 is a tumor suppressor, the induction of EMT through TGF-β-Smad4 is not necessarily associated with aggressiveness of the disease. Indeed,

Smad4-deleted cancer cells were reportedly resistant to induction of EMT by TGF-β *in vitro*, although these cells were highly proliferative *in vivo* [188]. It was also claimed that complete loss of *Smad4* in mice was associated with elevated levels of Runx3, which increased the migratory and metastatic potential of PDACs. On the other hand, another report proposed a detailed role for Smad4 in switching TGF-β signaling [189]. They suggest that in PDAC cells with Ras activation, TGF-β induces the expression of SOX4 in a Smad4-independent manner, which cooperates with the Krüppel-like factor 5 (KLF5) and functions as a pro-oncogenic factor. In the Smad4 intact cells, KLF5 is repressed in a Smad4-dependent manner, resulting in the switches of the role of SOX4 to a tumor suppressor, which induces apoptosis [189]. Interestingly, in PDACs with chromosomal rearrangement, *SMAD4* loss is accompanied by a gain in a region of chromosome 18 that harbors *GATA6* [164]. Thus, several transcription factors are induced in *SMAD4*-deleted PDACs, some of which empower the cells to acquire more migratory and more metastatic characteristics.

Induction of desmoplasia in pancreatic cancer

Abundant stroma, commonly referred to as desmoplasia, is one of the characteristics of PDACs. The stroma is composed of excessive deposition of ECM and cellular components, such as fibroblasts, myofibroblasts, pancreatic stellate cells (PSCs), and vascular and immune cells. Although it is still under debate whether depletion of stroma is protective or pathogenic [190], the stroma plays essential roles in promoting pancreatic cancer cell progression and determining response to therapy. In addition, several stroma-targeted therapies are already in clinical trials [191]. TGF-β is established as a key profibrotic cytokine, and the effects of TGF-β on stromal cells have been reviewed elsewhere [46]. In mouse orthotopic models, the TβRI inhibitor SB431542 was shown to selectively target stromal cells, not cancer cells, within the pancreatic tumor [180]. Moreover, it was recently shown that the application of TβRII neutralizing antibody mainly targets stromal cells that participate directly in the tumor cell phenotype and pancreatic cancer progression [182]. The tumor miocroenvironment plays critical roles in the induction of highly malignant pancreatic cancer cells and confers a mesenchymal phenotype to these cells [192]. Intriguingly, several members of the nuclear receptor superfamily, such as the vitamin D receptor (VDR), have been reported to repress fibroblast activation induced by TGF-β [193]; activation of stromal VDR antagonizes TGF-β-Smad3 and overcomes chemotherapeutic drug resistance [194]. Moreover, VDR ligand plus gemcitabine enhances survival in a PDAC mouse model [194]. Thus, the enhanced TGF-β signaling in stroma may explain the tumor-promoting effects of TGF-β in PDACs.

Extracellular regulation of TGF-β signaling

TGF-β is overexpressed in advanced cancers, and accumulating evidence demonstrates that TGF-β drives the progression of most solid tumors. Elevated TGF-β expression correlates with tumor progression and poorer prognosis. Thus,various inhibitors for TGF-β signaling, e.g., ligand trap using the extracellular domain of TβRII, neutralizing antibodies against TGF-βs or TβRII, and inhibitors for TβRI and/or TβRII kinases, have been developed, and some of them are in clinical trials [103, 195].

Platelets store large amounts of TGF-β1 [196], and serum levels of TGF-β1 are affected by the number of platelets. TGF-βs are produced as latent forms, and active forms of TGF-βs are below the detectable levels under physiological conditions; therefore, total levels of TGF-β, after transient acidification of samples, are usually assessed using enzyme-linked immunosorbent assay (ELISA) and other methods. Circulating plasma levels of TGF-β1 are increased in patients with breast and colorectal cancer and decreased following surgical resection of tumors [197, 198]. However, TGF-β in platelets may also play critical roles in cancer progression. During the process of cancer metastasis, platelets adhere to tumor cells in blood circulation, and function as a source of TGF-β; thus, platelet contact induces a mesenchymal phenotype in cancer cells and enhances cancer metastasis [199].

Latent TGF-βs

TGF-βs are 25-kDa disulfide-linked dimeric proteins. The mature proteins of TGF-β1, -β2, and -β3 are highly conserved in their amino acid sequences, including nine conserved cysteine residues [1, 5]. TGF-βs are produced as large precursor polypeptides composed of three segments: N-terminal signal peptides that are involved in secretion of the TGF-β precursors, large precursor segments known as latency-associated peptides (LAPs), and the C-terminal TGF-β monomer peptides that form the mature dimeric TGF-β proteins (112 amino acid residues for TGF-β1, -β2, and -β3) (Fig. 5A). Only some of the TGF-β family members, including TGF-β1, -β2, and -β3 and myostatin/GDF-8, are produced as latent forms [200].

The amino acid sequences of LAPs are not highly conserved among TGF-β1, -β2, and -β3 (249, 281, and 279 amino acid residues for β1-, β2-, and β3-LAP, respectively) compared to the mature TGF-β monomer peptides. The LAPs are cleaved from the mature TGF-β peptides by a furin protein convertase. However, the LAPs remain non-covalently associated with the mature TGF-β dimer and form the small latent complexes (SLCs) (Fig. 5B). The SLC is thus unable to bind and activate the TGF-β receptors. The SLC binds to other proteins by disulfide bonding through one of the cysteine residues in the LAP

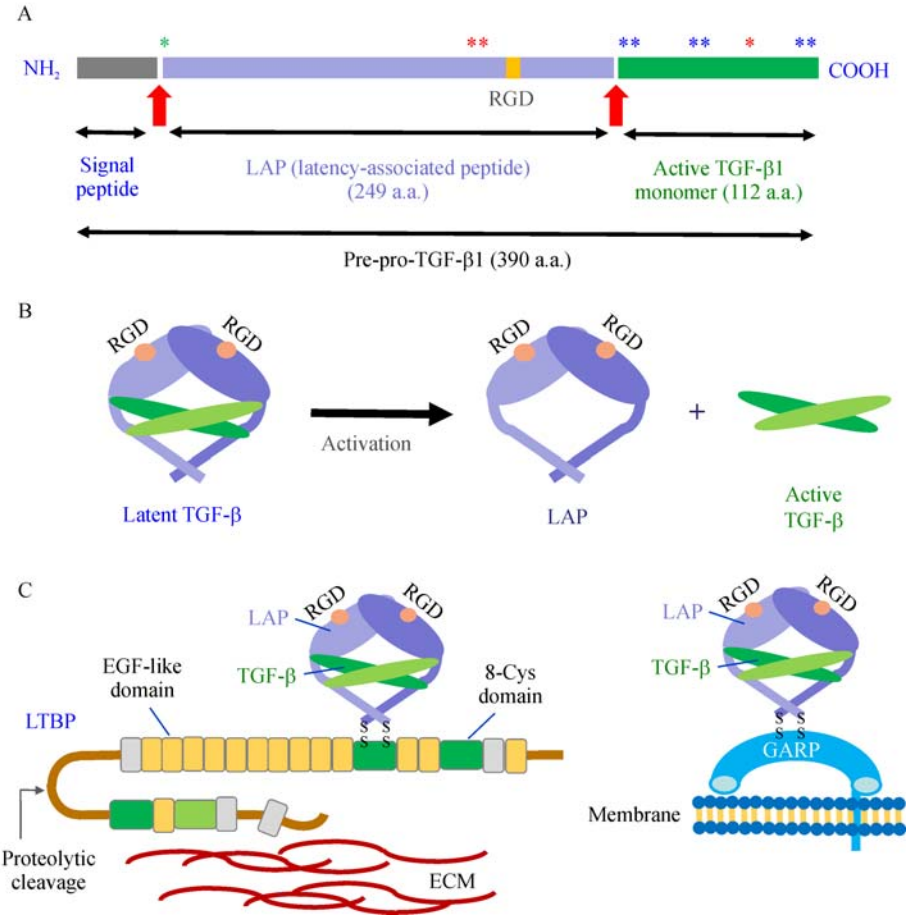

Fig. 5 Structure of pre-pro-TGF-β1 and latent forms of TGF-β. (A) Structure of pre-pro-TGF-β1. Red arrows, proteolytic processing sites; blue asterisks, cysteine residues, which form intramolecular disulfide bridges; red asterisks, cysteine residues, which form intermolecular disulfide bridges; green asterisk, cysteine residue, which forms a disulfide bridge with LTBPs or GARP; RGD, integrin recognition sequence. (B) Small latent TGF-β complex (SLC). TGF-β is produced as a latent form, consisting of the dimeric LAP proteins, which are non-covalently associated with the dimeric mature TGF-β. The RGD integrin recognition sequence is present in TGF-β1 and β3, but not in β2. Latent TGF-β is activated by various mechanisms; among those, mechanisms of activation by integrins have been best-characterized (see text). (C) Structures of the large latent TGF-β complexes (LLCs). SLCs are bound to LTBPs (LTBP-1, 3, and 4) or GARP. LTBPs are comprised of multiple EGF-like domains and 8-cysteine domains. The latent TGF-β complexes with LTBPs are released from the producer cells. LTBPs are associated with ECM proteins, which are involved in activation of the latent TGF-β. GARP is a transmembrane protein with a horseshoe-like structure. The latent TGF-β complex with GARP is thus anchored to the cell surface. The extracellular domain of GARP is comprised of multiple leucine-rich repeats.

(Cys33) and forms the large latent complex (LLC). At least two different groups of proteins have been reported to bind to the LAPs, i.e., LTBPs (LTBP-1, -3, and -4) and GARP/LRRC32 (Fig. 5C).

LTBPs are involved in TGF-β functions, such as incorporation into the ECM and storage for future activation [15, 201]. LTBPs are broadly expressed in various cells and tissues. LTBPs are structurally related to fibrillin-1, which is a component of extracellular microfibrils. Fibrillins interact with LTBPs and keep the latent TGF-β complexes bound to elastic microfibrils [202]. Abnormalities in the *FBN1* gene (encoding fibrillin-1) are responsible for Marfan syndrome [1].

Activation of latent TGF-β1

Activation mechanisms of latent TGF-β have been extensively studied for TGF-β1. β1-LAP contains a proline-rich loop termed the latency lasso, which encapsulates the TGF-β1 monomer peptide, thereby keeping TGF-β1 in an inactive form [203]. β1-LAP and β3-LAP contain an Arg-Gly-Asp (RGD) sequence, the recognition motif for integrins, and bind to cell surface integrins, particularly αvβ1, αvβ6, and αvβ8, while LTBPs anchor the latent TGF-β complex in the ECM [204]. Biological and structural studies demonstrate that the molecular tension mediated by the physical stretch between the β-LAP-

integrin interaction at the cell surface and LTBP anchorage in the ECM is responsible for the release of active TGF-β1 from the latent TGF-β1 complex [203–205]. It should be noted that the RGD sequence is not present in β2-LAP, and therefore, the latent TGF-β2 complex may not be activated by the interaction with integrins. Latent TGF-β complexes are also activated by other mechanisms, such as proteolytic cleavage of the LAPs, action of reactive oxygen species (ROS), ionizing radiation, and thrombospondin-1 [15].

GARP anchors the latent TGF-β complex on the cell surface

The *GARP/LRRC32* gene encodes an 80-kDa transmembrane protein, comprised of an extracellular domain with a horseshoe-like structure, almost entirely made of leucine-rich repeat sequences, followed by a transmembrane domain, and a short intracellular domain [206, 207]. GARP is expressed in various tissues and cells, including megakaryocytes/platelets, endothelial cells, lymphocytes, and mesenchymal stromal cells [207]. Notably, GARP is co-expressed with latent TGF-β on the surface of activated Tregs but not on helper T cells, and is thus regarded as a specific marker of activated Tregs [16, 17, 208]. miRNAs that target a short region of the 3' UTR of *GARP* have been identified in stimulated human Tregs, including miR-142-3p, which represses the expression of GARP [209, 210].

Garp-deficient mice do not exhibit apparent abnormalities in major organs; however, they show defective palatogenesis and die within 24 hours after birth [211]. Interestingly, the failure to develop the secondary palate and reduction of Smad2 phosphorylation without other defects in *Garp*-deficient mice are similar to the phenotype of *Tgfb3*-null mice. Although GARP forms a complex with the SLC containing TGF-β1 in human Tregs [212], GARP is co-localized with TGF-β3 in the medial edge epithelial cells in mouse embryos, and it directly interacts with latent TGF-β3, suggesting that GARP plays a crucial role in regulation of TGF-β3 signaling during mouse development.

Immunosuppressive roles of GARP through the regulation of TGF-β

As described in the earlier section, TGF-β regulates the differentiation and function of multiple types of immune cells, which in turn inhibits immune responses [56]. Among them, TGF-β plays a central role in conversion of naïve T cells into Tregs. TGF-β induces and maintains the expression of the master transcription factor of Tregs, forkhead box P3 (Foxp3) [213]. TGF-β induces the expression of Foxp3 through a Smad2 and Smad3-dependent manner [214]. Analyses of the TCGA-skin cutaneous melanoma dataset and TCGA-breast cancer dataset revealed correlation between TGF-β signaling and *FOXP3* expression [215]. IL-2 signaling through STAT5 is essential for the development of $Foxp3^+$ Tregs [216]. Foxp3 inhibits secretion of pro-inflammatory cytokines and enhances the expression of anti-inflammatory cytokines as well as immune checkpoint molecules, including cytotoxic T lymphocyte-associated molecule-4 (CTLA-4). In addition, TGF-β released from Tregs acts on effector T cells (Teffs) in a paracrine manner, which inhibits the proliferation and differentiation of Teffs.

GARP is expressed on the surface of Tregs and tethers latent TGF-β1 on the cell surface. Similar to LTBPs, GARP directly binds to the SLC of TGF-β by disulfide linkages through Cys192 and Cys331 of GARP and Cys33 of pro-TGF-β1 as well as by noncovalent association, which prevents the secretion of TGF-β1 [217]. Integrin αvβ8 dimers are present on stimulated Tregs, recognize the RGD motif in β1-LAP, and release active TGF-β1 from the latent TGF-β1-GARP complex [212]. GARP overexpression in T cells induces expression of Foxp3 and enhances their immunosuppressive functions, while silencing of GARP in Tregs attenuates their suppressive activity [218, 219].

GARP and human diseases

In accordance with these findings, immunosuppressive roles of GARP are implicated in various inflammatory diseases. GARP is expressed on megakaryocytes/platelets, and may be important for platelet-endothelium interactions [220]. Impaired immunosuppressive functions of $GARP^+$ Tregs have also been reported to be involved in acute coronary syndrome [221, 222].

Increasing evidence has revealed the roles of GARP in cancer progression. Amplification of the *GARP* gene has been found in colorectal cancer, head and neck cancer, and breast cancer [223]. Aberrant expression of GARP is observed in human breast, lung, and colon cancers and it promotes immune tolerance by activating latent TGF-β in the tumor microenvironment [224]. Moreover, the frequency of $GARP^+Foxp3^+$ Tregs is significantly higher in patients with advanced hepatocellular carcinoma than in controls, and the levels of GARP expression are elevated on the $Foxp3^+$ Tregs of these patients [225], suggesting that increased expression of GARP promotes cancer progression through activation of immunosuppressive functions of Tregs.

Application of GARP for cancer immunotherapy

Targeting immune checkpoints, such as CTLA-4 or programmed death-1 (PD-1)/PD-1 ligand (PD-L1) has introduced a paradigm shift in recent basic and clinical cancer research [226]. Since only some patients respond to the immune checkpoint therapies, an important strategy to improve their efficacies may be combination with other

immune therapies. Inhibition of TGF-β signaling is potentially a very interesting way to treat cancers, and many preclinical and clinical trials are ongoing [195]. Thus, combination of the immune checkpoint therapy with inhibition of TGF-β signaling may be an attractive strategy to treat cancers, especially those resistant to current immune checkpoint inhibitors. Recent studies revealed that co-administration of anti-PD-L1 antibody and TGF-β inhibitor (Galunisertib or anti-TGF-β antibody) showed high anti-tumor immunity and tumor regression [227, 228]. Moreover, enhanced anti-tumor activities could be observed by using bifunctional fusion proteins containing the PD-L1 (or CTLA-4) antibody and the TβRII extracellular domain [215, 229].

Since TGF-β exhibits a wide variety of biological activities, methods to selectively inhibit certain activities of TGF-β are desirable to provide an opportunity to inhibit tumor progression without severe side effects. Indeed, the TGF-β receptor kinase inhibitor Galunisertib has side effects on the heart [230]. Considering its immunoregulatory functions, GARP is thus expected to be a potential target for cancer treatment. When Tregs are transfected with an siRNA targeting GARP, inhibition of helper T cell proliferation by human Tregs is attenuated [17]. Platelet-specific deletion of the *Garp* gene in mice diminishes TGF-β activity within the tumor and enhances immunity against both melanoma and colon cancer [231], suggesting that the inhibition of GARP-TGF-β axis through a combination of immunotherapy and platelet inhibitors may be a new therapeutic strategy for cancer. Anti-GARP monoclonal antibodies that recognize a critical epitope for the function of the GARP-pro-TGF-β1 complex (including amino acids 137 – 139 of GARP) were generated and these antibodies inhibit the production of active TGF-β1 by human Tregs and their immunosuppressive activity *in vivo* [232]. In addition, the therapeutic efficacy of GARP-specific monoclonal antibodies was evaluated using immunocompetent mice [224]. GARP overexpression promotes Treg activity and cancer progression in breast cancer-bearing mice, whereas administration of GARP-specific monoclonal antibodies attenuates the progression of cancer metastasis. Most recently, antibodies against the integrin β8 subunit as well as antibodies against GARP have been shown to inhibit the immunosuppression induced by human Tregs in a model of xenogeneic graft-versus-host disease [212]. Administration of these antibodies alone or in combination with immune checkpoint inhibitors may improve the efficiency of cancer immunotherapy.

Conclusion and perspectives

TGF-β is a multifunctional cytokine that regulates various cellular responses. Recent findings reveal that TGF-β functions as a pro-tumorigenic factor in various types of cancers. Thus, it is expected that inhibition of TGF-β signaling may lead to prevention of the cancer progression. In this review, we have discussed the roles of TGF-β in lung and pancreatic carcinomas, but TGF-β also acts in other cancers, including breast cancer (reviewed in [233–235]), colorectal cancer, melanoma, leukemia/myelodysplastic syndromes, and glioblastoma [9, 236]. TGF-β activities occur in a context-dependent manner and some tissue-specific molecules may regulate TGF-β activities. Therefore, further studies are required to understand the functional regulation of TGF-β in each type of cancer.

In the present study, we have shown intriguing mechanisms of the activation of latent TGF-β complexes. Although activation mechanisms of the latent TGF-β complexes with LTBPs, and more recently with GARP, have been elucidated, latent TGF-β complexes may be associated with other molecules. TGF-β2 has been reported to be increased in some cancers [237, 238]; however, the mechanisms of activation for the latent TGF-β2 complex have been poorly investigated. In addition, although the functions of GARP in Tregs have recently been elucidated [212], its function in platelets and other cells need clarification. It is thus intriguing to characterize the latent TGF-β complexes and determine how these complexes are activated under physiological and pathological conditions.

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Compliance with ethics guidelines

Kohei Miyazono, Yoko Katsuno, Daizo Koinuma, Shogo Ehata, and Masato Morikawa declare no competing or financial interests. This manuscript does not involve any research protocols requiring approval by the relevant ethical committee or institutional review board.

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