TGF-β signaling dynamics in epithelial-mesenchymal plasticity of cancer cells

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Chapter 1

General Introduction

1) TGF-β in Cancer Progression: From Tumor Suppressor to Tumor Promoter

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2) Long non-coding RNAs in TGF-β signaling and EMT

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1) TGF-β in Cancer Progression: From Tumor Suppressor to Tumor Promoter

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Introduction

Transforming growth factor (TGF)-β is a multifunctional secreted cytokine that exerts highly context dependent effects on many different cell types, including growth inhibition, extracellular matrix (ECM) production, apoptosis and differentiation[1, 2]. TGF-β1 is the prototype of a large family of evolutionarily conserved structurally and functionally related dimeric proteins that include TGF-βs, activins and bone morphogenetic proteins (BMPs). Signaling occurs via transmembrane serine/threonine kinase type I and type II receptors, that is TβRI and TβRII, respectively[3]. TGF-β induces the formation of a complex of TβRI and TβRII, upon which TβRII phosphorylates TβRI, thereby transmitting the signal across the cell membrane. Inside the cells, activated TβRI phosphorylates specific down-stream effector molecules, among which are canonical SMAD and non-SMAD signaling components. SMADs can act as transcription factors and thus relay the signal from the membrane into the nucleus[4]. Each step of the signaling pathway is intricately regulated to fine tune the cellular responses of TGF-β[5].

Misregulation of TGF-β signaling associates with many diseases, including cancer, fibrosis and cardiovascular diseases[6-8]. In this review, we focus on its dual role in cancer. Moreover, as TGF-β stimulates cancer cell invasion and metastasis, this pathway has been subject to therapeutic targeting by academic and industrial laboratories. We provide an update on the latest clinical developments of TGF-β targeting agents for the treatment of cancer[9, 10].

TGF-β Signaling

Ligands and Their Receptors

Shortly after the cDNA cloning of TGF-β1 in 1985[11], the structurally and functionally related TGF-β2 and TGF-β3 were characterized[12]. In this review, we indicate specific TGF-β isoforms when relevant, for example, when they have distinct functional properties; otherwise we refer to them as TGF-β. TGF-β is a conserved 12.5 kilodalton (kDa) polypeptide that forms a disulfide-linked dimer[13]. While predominantly present as homodimers, heterodimers between different TGF-β isoforms have been described[14]. Of note, TGF-β may exert diverse, sometimes even opposing, effects depending on cell types and development stages[1, 2]. The three TGF-β isoforms are differentially expressed. TGF-β1 is highly abundant in platelets and bone and is widely expressed and synthesized among diverse tissues. TGF-β is secreted in an inactive form in which the amino-terminal pro-peptide (also termed the latency-associated peptide) is non-covalently associated with the carboxy-terminal mature peptide[15]. Activation of TGF-β can be

mediated via specific proteases and cell surface-associated integrins that liberate the mature peptide, which can then bind to cell surface receptors[16]. This activation step is a pivotal control mechanism that regulates the local bioavailability of TGF-β.

Activated TGF-β initiates cellular responses by binding to cell surface single transmembrane TβRI and TβRII[17]. TGF-β induces the formation of a heterotetrameric complex containing two TβRIIs and two TβRIs[18]. Initially, TGF-β1 and TGF-β3 (but not TGF-β2) bind to TβRII, and thereafter, TβRI is recruited. TGF-β type III coreceptor (also termed betaglycan), which lacks intercellular enzymatic activity, can facilitate the interaction between TβRI and TβRII[19]. In particular, TGF-β2 requires TβRIII for efficient binding to signaling receptors[20]. Upon the ligand-induced TβRI/TβRII complex formation, TβRI is phosphorylated by TβRII on specific serine and threonine residues in the glycine/serine-rich (GS) domain. The extracellular ligand signal is thereby transduced across the membrane, and the activated TβR complex is ready to initiate intracellular responses by phosphorylating intracellular effector proteins[21-23] (**Fig. 1**).

TGF-β/SMAD and Non-SMAD Signaling

With the help of genetic approaches in worms and fruit flies, Sma- and Mad-related proteins, termed SMAD proteins, were identified in vertebrates as unique and pivotal intracellular effectors of TGF-β[24]. SMADs are classified into three groups: the receptor-regulated SMADs (R-SMADs), the common SMADs (Co-SMADs) and the inhibitory SMADs (I-SMADs)[25-27]. R- and Co-SMADs share two conserved domains, i.e, N-terminal Mad Homology 1 (MH1) and C-terminal Mad Homology 2 (MH2) domain. Both domains are separated by a proline-rich linker region. There is also an MH2 domain in I-SMADs.

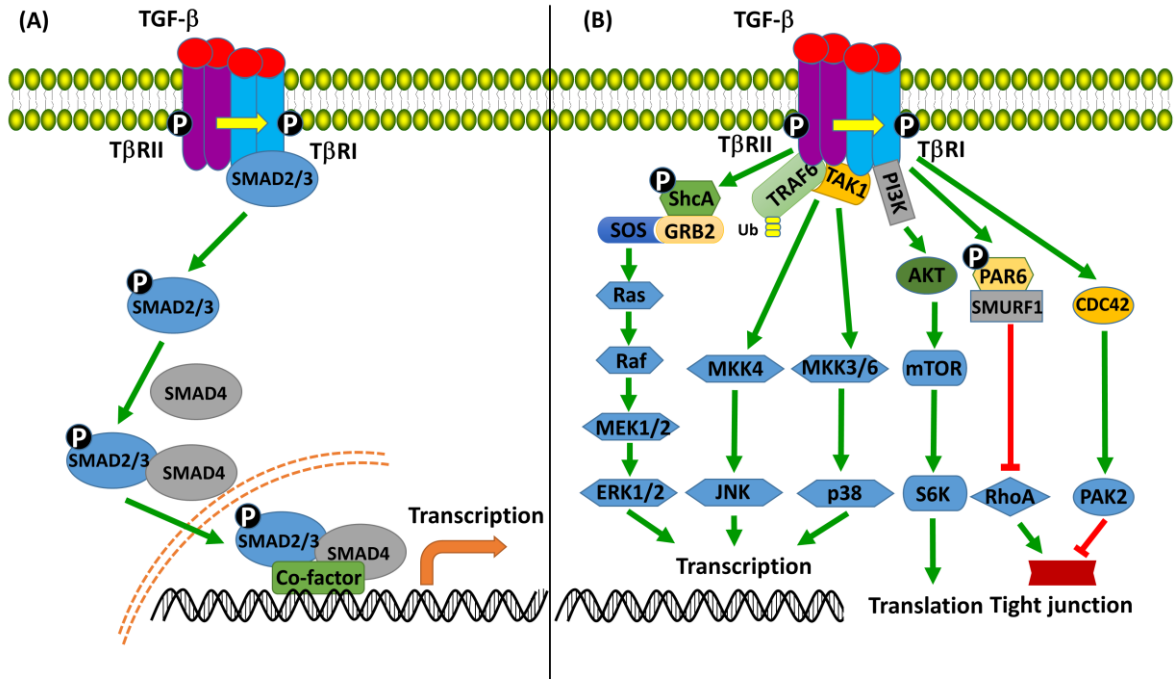

Fig. 1 TGF-β/SMAD and non-SMAD signaling. (A) In the SMAD-dependent pathway, binding of active TGF-β induces the assembly of TβRI and TβRII into a complex in which TβRI is phosphorylated by the TβRII kinase. Activated TβRI subsequently signals by recruiting and phosphorylating SMAD2/3, which form heteromeric complexes with SMAD4. The SMAD complexes then translocate into the nucleus and regulate target gene transcription by cooperating with other cofactors. (B) In the non-SMAD signaling pathways, TGF-β receptors activate other pathways including MAPKs (such as ERKs, p38 and JNK) and PI3K-AKT signaling to regulate transcriptional and translational events and modulate the Rho-like GTPase activity for tight junction dissolution. Abbreviations: ERK, extracellular regulated kinase; GRB2, growth factor receptor-bound protein 2; mTOR, mammalian target of rapamycin; PI3K, phosphatidyl inositol-3-kinase; S6K, S6 kinase; SMURF,

SMAD ubiquitin regulatory factor; SOS, son of sevenless; TAK1, TGF-β activated kinase; TβR, TGF-β receptor; TGF-β, transforming growth factor-β; TRAF, TNF associated factor; Ub, ubiquitin.

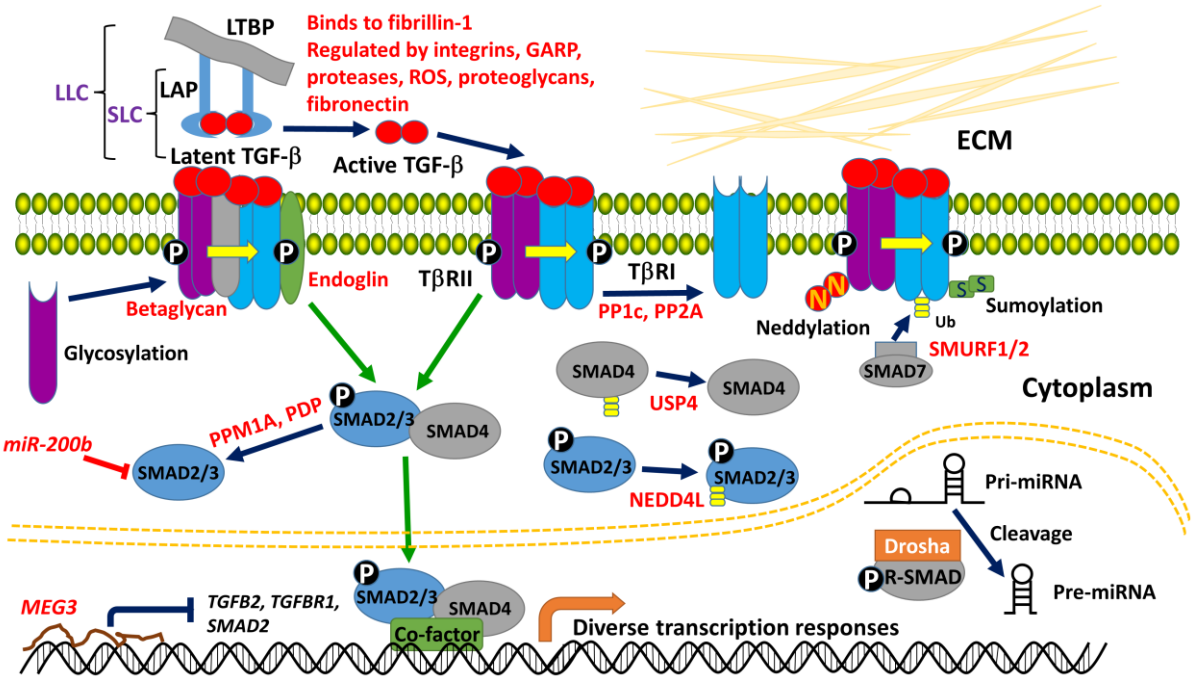

Fig. 2 Regulation of TGF-β/SMAD signaling. Fibrillin-1, proteases, ROS, GARP, integrin-mediated contractile forces and stromal-derived factors modulate the bioavailability of TGF-β ligands and accessibility to its receptors. At the cell membrane level, the activity of TβRs is modified by glycosylation, phosphorylation, ubiquitylation, deubiquitylation, sumoylation and neddylation, as well as the interactions with coreceptors and other accessory proteins. At the cytoplasmic level, SMAD proteins are under tight control by phosphatases, ubiquitylating enzymes, deubiquitylating enzymes and microRNAs (miRNAs). In the nucleus, the SMAD complex affects different transcriptional responses in combination with diverse cofactors. SMAD proteins are also required for the maturation process of miRNAs. Moreover, modulators such as long non-coding RNAs (lncRNAs) can regulate TGF-β pathway components at the transcription level.

Upon activation, TβRI recruits and phosphorylates R-SMAD family members, SMAD2 and SMAD3, at two serine residues in their C-terminal regions. Activated SMAD2/3 form heteromeric complexes with SMAD4 which subsequently translocate into the nucleus. Activated SMAD2/3/4 complexes can form transcriptional complexes in conjunction with a large variety of DNA binding cofactors and thereby gain high affinity and specificity to DNA. The intrinsic binding activity of SMAD3 and SMAD4 (via their MH1 domain) is weak, and their direct binding ability to DNA is lacking in the predominantly expressed splice variant of SMAD2. These SMAD-containing transcription factor complexes interact with coactivators, corepressors and chromatin remodeling factors to regulate the transcription of target genes in a cell type-dependent manner[22, 25, 28] (**Fig. 1A**).

In addition to the canonical SMAD-dependent pathway, non-SMAD signaling pathways can be initiated by activated TGF-β receptor complexes in specific cell types (**Fig. 1B**). These pathways can also modulate the SMAD pathway[29]. Via phosphorylation or direct interaction with signaling modules, TGF-β receptors can activate pathways such as the mitogen-activated protein kinase (MAPK) signaling cascade, which includes extracellular signal-regulated kinases (ERKs), p38 and c-Jun amino terminal kinase (JNK), phosphatidylinositol-3 kinase (PI3K)-AKT signaling and Rho-like GTPase activity[29-32]. TβRII is phosphorylated by non-receptor tyrosine kinase Src on Tyr284, which acts as a docking site for growth factor receptor-bound protein 2 (GRB2) and Src homology domain 2 containing (Shc), leading to the activation of ERK MAPK pathway[33]. Moreover, Shc is reported to be directly phosphorylated by TβRI,

which provides a docking site for GRB2 that interacts with the exchange factor SOS to activate the pro-oncogenic Ras-Raf-MEK1/2-ERK1/2 signaling[31]. Phosphorylated ERK1/2 translocate into the nucleus and regulate gene transcription by phosphorylating target transcription factors[34]. TGF-β activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK) that is recruited to the TGF-β receptor complex by polyubiquitylated TRAF6, phosphorylates specific MAP kinase kinases (MKKs), leading to the phosphorylation of JNK and p38[35]. In addition, TGF-β stimulation triggers the interaction between TβRI and the PI3K subunit p85, leading to AKT phosphorylation and the activation of downstream effectors (e.g., mTOR, P70S6K and 4EBP1)[36, 37]. PAR6 can also be phosphorylated by TβRI and recruit SMURF1 to degrade RhoA, which regulates cell–cell interactions via tight conjunctions[38]. CDC42, another GTPase, can be recruited to the TGF-β receptor complex and mediate the activation of p21-activated kinase 2 (PAK2), which stimulates tight conjunction disassociation[39, 40] (**Fig. 1B**).

Regulation of TGF-β/SMAD Signaling

As a pivotal cytokine in cell homeostasis, TGF-β signaling activity is under precise control, from ligand bioavailability to receptor and SMAD activation (**Fig. 2**). After synthesis and intracellular furin-mediated cleavage of the precursor protein (removal of the signal peptide), the bioactive growth-factor domain (mature TGF-β) and prodomain, also termed the latency-associated peptide (LAP), are secreted in a small latent complex (SLC) form. Binding of TGF-β ligand to its receptors is prevented by LAP. The large latent complex (LLC), a more commonly deposited complex, contains the SLC and the latent TGF binding protein (LTBP)[41-44]. LLC is bound to elastic microfibrils via the binding of LTBP to the extracellular protein fibrillin-1[45]. Stromal-derived molecules including proteases and reactive oxygen species (ROS) substantially contribute to the increase of active TGF-β levels by interacting with the latent TGF-β complex[43, 46-48]. Moreover, glycoprotein-A repetitions predominant protein (GARP) functions as a critical docking receptor on regulatory T cells to concentrate and activate latent TGF-β on the cell surface[49, 50]. In addition, contractile forces exerted by the integrins across the LLC play a vital role in the release of mature TGF-β[42, 51-53]. Fibronectin deposited in the ECM prior to LLC formation impairs TGF-β1 bioactivity by interacting with LTBP[54]. Decorin, a member of the proteoglycan family, also exerts a suppressive role in TGF-β activity via binding to all isoforms of soluble TGF-β[55].

Apart from the ECM level, TGF-β responsiveness is tightly controlled at the cell membrane. Glycosylation of the extracellular domain of TβRII inhibits its transportation to the cell membrane and lowers its TGF-β binding affinity[56, 57]. E3 ubiquitin ligases such as SMAD-specific E3 ubiquitin protein ligase 1/2 (SMURF1/2) cooperate with inhibitory SMAD7 to regulate the availability of TβRI receptor on the cell surface by polyubiquitylation and proteasomal degradation[58, 59]. In contrast, deubiquitinating enzymes ubiquitin-specific protease (USP) 4, 11 and 15 remove the polyubiquitin chains from TβRI[60]. Moreover, two phosphatases, i.e. protein phosphatase (PP)1c and PP2A, impair receptor activation by targeting TβRI for dephosphorylation[61, 62]. Akin to ubiquitylation, sumoylation and neddylation have also been implicated to regulate TGF-β receptor stability. The interaction between TGF-β receptors and the coreceptors located in the cell membrane is another determinant for the signaling strength[21, 63]. The coreceptor betaglycan stabilizes the receptor complex between TβRI and TβRII and propagates signaling transduction initiated by TGF-β2[64]. Endoglin, another accessory protein structurally related to betaglycan, inhibits TGF-β/ALK5-mediated SMAD2/3 signaling but promotes TGF-β/ALK1-induced SMAD1/5/8 signaling in endothelial cells[65, 66].

At the cytoplasmic level, phosphorylated SMAD proteins can be deactivated by phosphatases such as PPM1A and PDP, leading to signal termination[67-69]. Similar to the TGF-β receptors,

SMAD2/3 are destabilized by multiple ubiquitylating enzymes such as SMURF1/2 and NEDD4L[70, 71]. Conversely, USP4 promotes SMAD4 activity by removing the suppressive monoubiquitination triggered by SMURF2[72]. MicroRNAs (MiRNAs) also inhibit the expression of various signaling components[73]. *MiR-200b*, a miRNA whose expression is downregulated by TGF-β1, attenuates TGF-β signaling by targeting *SMAD2* mRNA at the post-transcriptional level, thereby forming a negative feedback loop[74].

Upon activation, the SMAD2/3/4 complex translocate into the nucleus and form a transcription complex with other cofactors. In combination with different sequence-specific transcription factors, the SMAD complex generate various transcriptional responses in a context and cell type-dependent manner[1, 75-77]. In addition, activated SMAD proteins participate in the maturation of miRNAs by recruiting the RNA helicase p68 (DDX5) to the Drosha complex[78]. *MEG3*, an intranuclear long noncoding RNA (lncRNA), can bind to the distal regulatory elements of genes encoding for TGF-β signaling components, including *TGFB2*, *TGFBR1* and *SMAD*, to inhibit their transcription[79].

TGF-β as a Tumor Suppressor

TGF-β-Induced Growth Inhibition

TGF-β induces growth inhibition (**Fig. 3**) and apoptosis (**Fig. 4**) in normal epithelial (and certain premalignant) cells; these properties are associated with its function as a tumor suppressor[80]. The molecular mechanisms by which TGF-β elicits these processes involve multiple intracellular pathways[81-83].

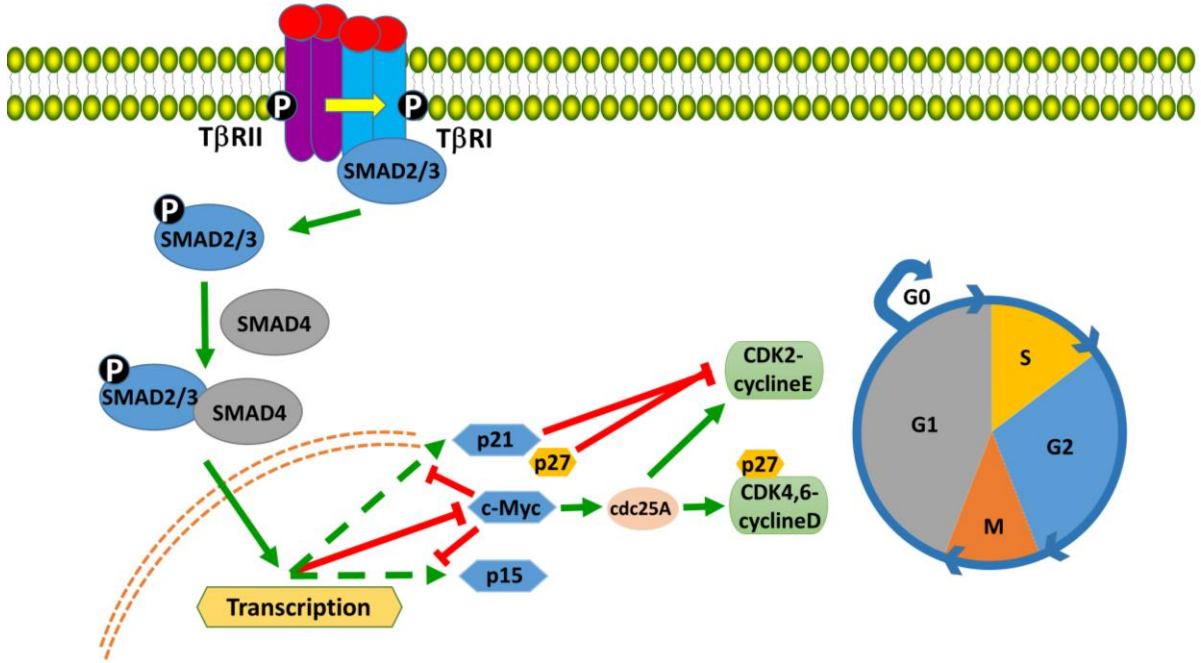

Fig. 3 Gene regulation in TGF-β-induced cell cycle arrest. TGF-β receptor activation leads to SMAD2/3 phosphorylation. Phosphorylated SMAD2/3 bind to SMAD4, and the SMAD2/3/4 complex translocate into the nucleus to modulate gene transcription. *C-Myc* and *cdc25A* gene expression is repressed, while *p15INK4b* and *p21CIP/WAF1* gene expression is induced by TGF-β, leading to the cell cycle arrest into the G1 phase. Abbreviations: CDK, cyclin dependent protein kinase; TβR, TGF-β receptor; TGF-β, transforming growth factor-β.

Numerous studies support the notion that TGF-β inhibits cell proliferation by arresting cells into the G1 phase of the cell cycle (**Fig. 3**). SMAD-containing protein and transcriptional coactivator complexes can activate the transcription of two major cell cycle inhibitors, CDK

inhibitors (CKIs), *p15* and *p21*[84, 85]. In keratinocytes, TGF-β/SMAD signaling induces the expression of cyclin-dependent kinase inhibitors $p15^{INK4b}$ and $p21^{CIP/WAF1}$, which inhibit the CDK4/6-cyclinD complex[86]. These cyclin-dependent kinase inhibitors suppress the CDK activities associated with the G1 to S phase progression, prevent cyclin-dependent kinases-mediated Rb phosphorylation, and arrest cells in the G1 phase[87]. The activated SMAD proteins target the promoters of *c-Myc* and *CDK* genes and repress their transcription in cooperation with nuclear corepressors[88]. TGF-β receptor-initiated non-SMAD signaling can also exert an anti-proliferative effect on some cell types[89].

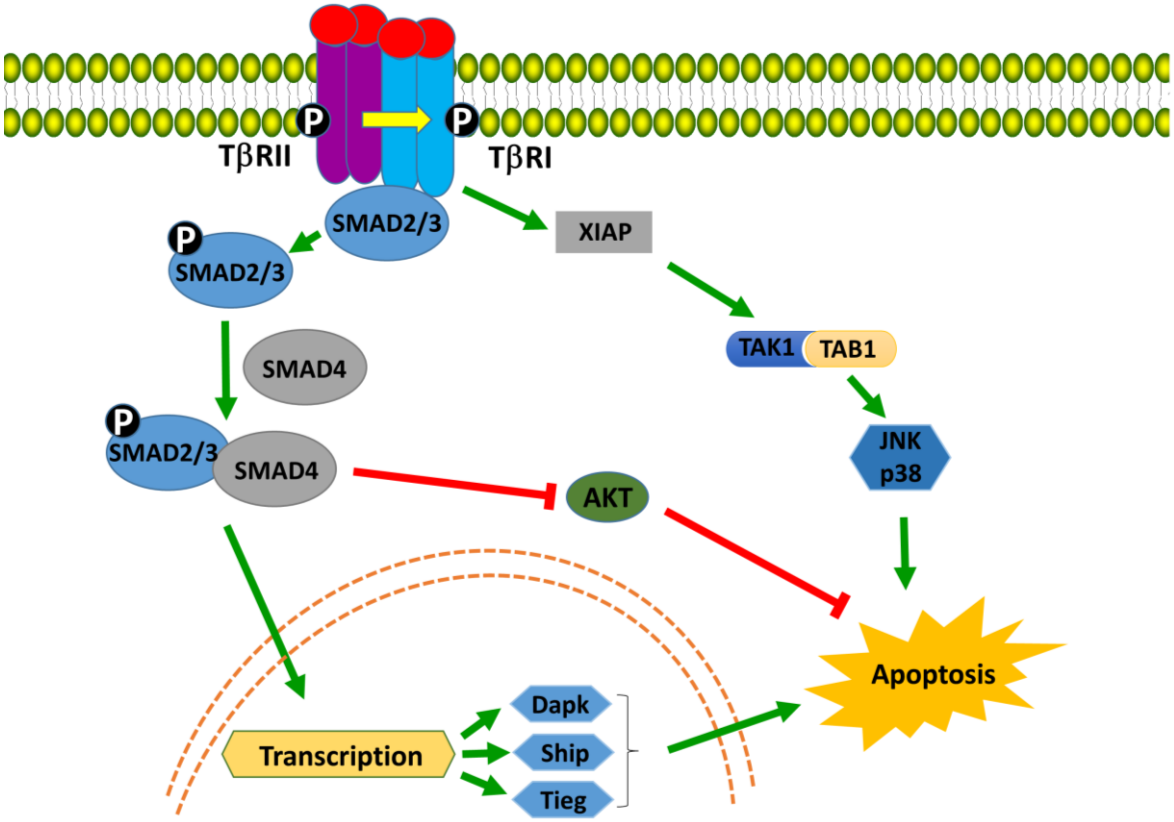

Fig. 4 TGF-β-induced cell apoptosis. TGF-β promotes the activation of SMADs and the expression of pro-apoptotic genes such as *Dapk*, *Ship* and *Tieg*. SMADs also bind and inactivate the survival kinase AKT, thereby inducing apoptosis. TGF-β-induced activation of the JNK and p38 pathways can also result in apoptosis. TGF-β can also induce, via the adaptor XIAP, the activation of the TAK1-TAB complex, leading to JNK or p38 activation, both of which can lead to apoptosis. Abbreviations: Dapk, death associated protein kinase; Ship, SH2-containing inositol phosphatase; TAB1, TAK1 binding protein; TAK1, TGF-β activating kinase; TGF-β, transforming growth factor-β; Tieg, TGF-β-inducible early-response gene; XIAP, X chromosome-linked inhibitor of apoptosis.

TGF-β-Induced Apoptosis

TGF-β can induce cell apoptosis in normal epithelial (and some premalignant) cells (**Fig. 4**). Several apoptotic regulators have been implicated as downstream targets of TGF-β signaling, often in a cell- or tissue-specific manner[90]. Induction of the pro-apoptotic genes such as *Ship* and *Tieg* have been shown in TGF-β-induced apoptosis[91]. In liver cancer cells, the Daxx adaptor protein couples TGF-β signaling to the cell death machinery through its interaction with TβRII[92]. In liver cancer cells, TGF-β can induce the expression of the death-associated protein kinase *DAPK*, which promotes cell death[93]. In addition, TGF-β-induced activation of TGF-β-activated kinase-1 (TAK-1), a protein of the MAPKKK family that activates p38 and JNK signaling, is involved in TGF-β-induced apoptosis[94]. TGF-β can also induce apoptosis

through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells[95] (**Fig. 4**).

Mutation in TGF-β Signaling Components in Cancer

Analysis from clinical tumor samples reveals that TGF-β-mediated signaling is indeed strongly implicated in the regulation of cancer[96]. Recent studies have shown that in various human tumor types, components of the TGF-β signaling pathway, namely, *TGFBR2*, *TGFBR1*, *SMAD2*, *SMAD3* and *SMAD4*, are commonly inactivated through mutation[81, 97]. Multiple genetic alterations in genes encoding central components in TGF-β signaling pathway are found in human cancers, in particular in pancreatic, esophagus, colorectal and head and neck cancer[98] (**Fig. 5**). Indeed, *TGFBR2*-inactivating mutations in its poly A gene tract are frequently found in cancers associated with microsatellite instability (MSI)[99]. SMAD point mutations associated with cancer are loss-of-function mutations that either target functional elements or affect the overall stability of the protein. Studies in cultured cells have shown that these inactivating mutations mediate an escape from TGF-β-induced growth arrest and apoptosis.

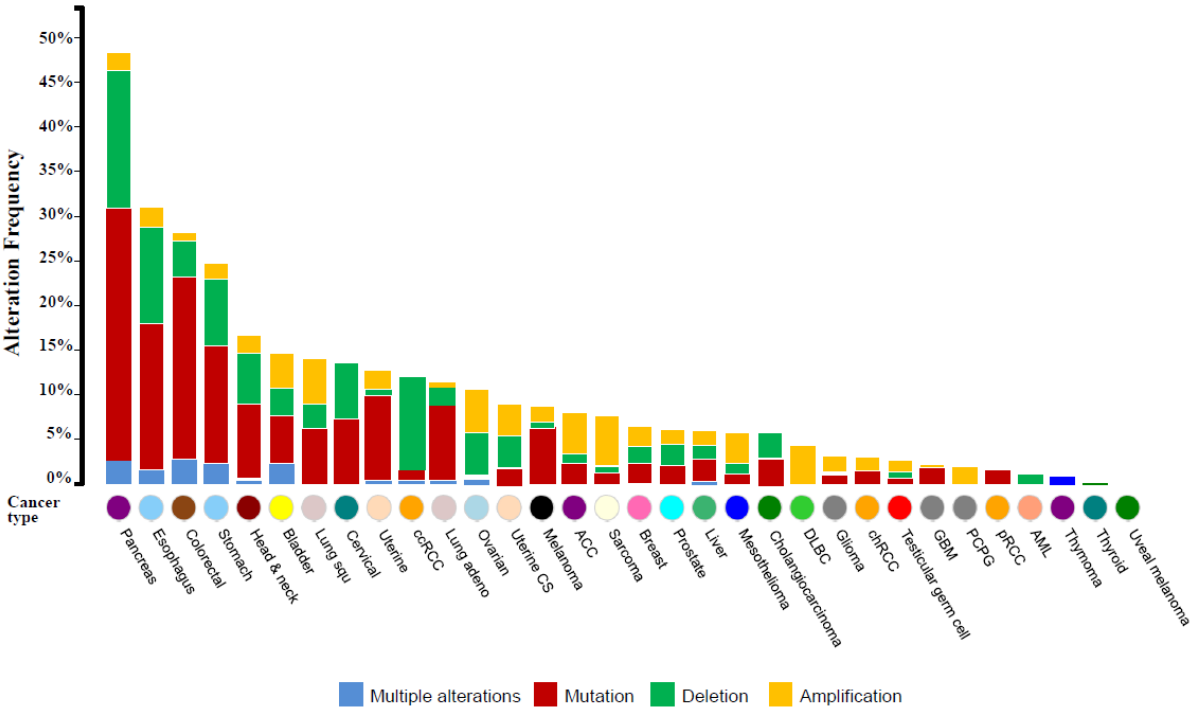

Fig. 5 Frequency of genetic alterations in *TGFBR1*, *TGFBR2*, *SMAD2*, *SMAD3* and *SMAD4* by cancer type. The graph displays the frequency of genetic alterations (point mutations, deletions, amplifications, or multiple alterations) in *TGFBR1*, *TGFBR2*, *SMAD2*, *SMAD3* and *SMAD4* in different types of cancer. Data were derived from TCGA datasets (The Cancer Genome Atlas, cancergenome.nih.gov/) at the time of this writing. Analysis was done using cBioPortal (www.cbioportal.org/).

In addition to the known mutations in the TGF-β receptors and SMAD pathway, other types of (epi)genetic alterations may also affect TGF-β signaling and tumor formation[89]. For example, oncogenic activation of the Ras-Raf-MAPK pathway and c-Jun NH2-terminal kinase in hepatocellular carcinoma has been reported to induce phosphorylation of the SMAD3 linker domain by MAPK, further preventing C-terminal phosphorylation of SMAD by the TβRI kinase domain and thereby inhibiting the TGF-β cytostatic effects[100].

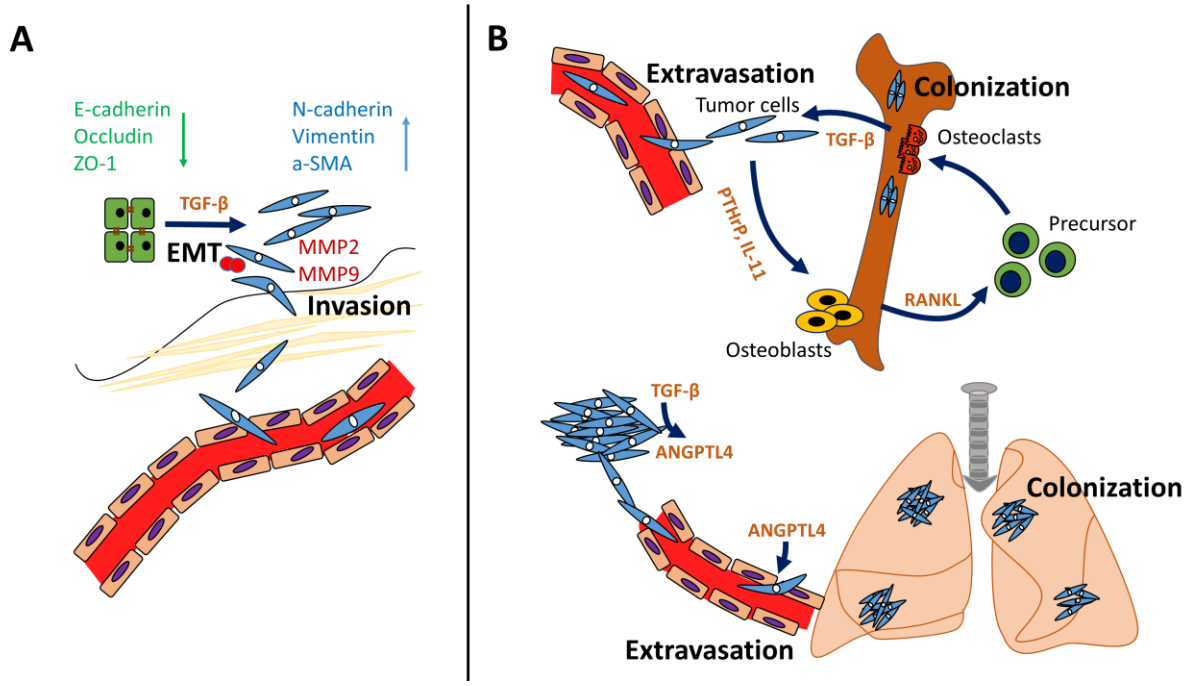

Fig. 6 TGF-β-induced EMT, invasion and metastasis. (A) TGF-β induces EMT by decreasing the expression of epithelial makers (in green) and increasing the expression of mesenchymal markers (in blue). TGF-β also promotes the secretion of MMP2 and MMP9, thereby conferring tumor cells highly invasive abilities. (B) Bone-derived TGF-β increases the secretion of PTHrP, which activates osteoclast activity through interacting with RANKL, thereby promoting osteolytic metastasis. IL-11 and CTGF are also key effectors induced by TGF-β in this process. Osteolysis leads to more local TGF-β release, causing the formation of a positive feedback loop. Moreover, TGF-β-induced ANGPTL4 plays a vital role in disrupting the junctions between pulmonary endothelial cells and contributes to lung metastasis formation. Abbreviations: ANGPTL4, angiopoietin-like 4; EMT, epithelial to mesenchymal transition; MMP, matrix metalloproteinase; SMA, smooth muscle actin; RANKL, receptor activator of nuclear factor κB ligand; TGF-β, transforming growth factor-β.

TGF-β as a Tumor Promoter

TGF-β-Induced EMT and Invasion

In the late stage of tumor progression, TGF-β switches from a tumor suppressor to a tumor promoter by inducing EMT, tumor invasion, distant dissemination, angiogenesis and immune evasion[101-104]. During EMT, tumor cells switch from an epithelial phenotype to a mesenchymal phenotype and gain highly migratory and invasive abilities. Moreover, they acquire cancer stem cell (CSC) properties and become more resistant to detachment-induced apoptosis[105]. During EMT, epithelial cells downregulate the expression of genes encoding epithelial markers, such as E-cadherin, Occludin and ZO-1, upregulate the expression of genes encoding mesenchymal markers, such as N-cadherin, Vimentin and α-smooth muscle actin (SMA), and dissolve the tight junctions. EMT greatly facilitate tumor cell invasion[106, 107] (**Fig. 6A**). In response to TGF-β, the SMAD complex directly increases the expression of multiple EMT-transcription factors including *ZEB*, *TWIST* and *SNAIL* family members by binding to their promoters. In addition, in combination with ZEB2 or SNAIL, the SMAD complex suppresses the transcription of genes encoding E-cadherin and Occludin, conferring the mesenchymal traits to cancer cells[108, 109]. In addition, SMAD4 binding enhances the promoter activity of *miR-155*. *MiR-155* dissolves the tight junctions by targeting *RhoA* mRNA and downregulates *CDH1* (mRNA encoding for E-cadherin) expression by inhibiting the expression of transcriptional activator *CEBPB* (mRNA encoding for C/EBPβ)[110, 111]. *LncRNA-ATB*, a long non-coding RNA activated by TGF-β, serves as a sponge for the *miR-200* family members that restrain ZEB1/2 protein expression, and thereby promotes EMT and hepatocellular carcinoma progression[112]. In combination with the SMAD-dependent pathway, SMAD-independent pathways also

potentiate TGF-β-induced EMT[29, 107, 113]. Activation of proto-oncogenes such as Ras and receptor tyrosine kinase pathways cooperate with TGF-β pathway to promote EMT[114, 115]. By directly modulating the activity of AP1 transcription factors that can cooperate with SMADs or phosphorylate R-SMADs, the ERK, p38 and JNK MAPK pathways play a key role in TGF-β-induced EMT and tumor invasion[116-119]. In addition, PI3K/AKT signaling participates in TGF-β-triggered EMT by activating the mTOR and EMT-related transcription factors such as SNAIL and TWIST1[37, 120-122]. Activation of the Rho family GTPases including RhoA, Rac1, and Cdc42 by TGF-β receptors contributes to cell–cell junction dissolution and cytoskeletal reorganization, which are important determinants for EMT[38, 123, 124].

Local invasion through the surrounding ECM and stromal cell layers is the first step of the invasion-metastasis cascade[125]. Results from human cancer specimens suggest that coexpression of SMAD3/4 and SNAIL is correlated with the loss of E-cadherin and coxsackie and adenovirus receptor (CAR), a tight junction-associated cell adhesion molecule, at the invasive front[109]. Apart from conferring EMT properties to cancer cells, TGF-β induces the expression and secretion of *matrix metalloproteinases 2/9* (*MMP2/9*) in tumor cells or/and stromal cells (e.g., myofibroblasts). These two proteinases promote ECM and collagen proteolysis, leading to the invasion of tumor cells into their stromal compartment[126, 127] (**Fig. 6A**). In addition, TGF-β employs *miR-181b* to inhibit the protein level of TIMP3, an inhibitor of metalloprotease. The latter promotes MMP2/9 activities and the invasion of hepatocellular carcinoma cells[128].

TGF-β-Induced Metastasis to Bone, Lung and Other Organs

Cancer metastasis contributes to the death of most cancer patients[129]. Bone metastasis is a common event in specific cancer types, including breast, lung and prostate cancers. The interaction between disseminated cancer cells and resident skeletal cells disrupts bone integrity, conferring a receptive microenvironment for the outgrowth of metastatic cancer cells[130, 131]. Bone-derived TGF-β promotes SMAD-dependent pathway activation in cancer cells, which increases the expression and secretion of *parathyroid hormone-related protein* (*PTHrP*), a major osteoclastogenic factor. PTHrP potentiates osteoclast activity by interacting with receptor activator of nuclear factor κB ligand (RANKL), thereby promoting bone metastasis[131-134] (**Fig. 6B**). By employing *in vivo* selection of highly metastatic cell lines and functional imaging, Kang *et al.* identified a bone metastasis gene signature that includes *C-X-C motif chemokine receptor 4* (*CXCR4*), *interleukin 11* (*IL-11*) and *connective tissue growth factor* (*CTGF*), which contribute to metastasis by directing the homing of breast cancer cells to bone, osteolysis and angiogenesis, respectively[135, 136]. *IL-11* and *CTGF* expression is induced by TGF-β. The degraded bone in turn secretes stored factors including TGF-β to form a positive feedback loop called “vicious cycle”[137].

TGF-β signaling also contributes to lung metastases formation. A TGF-β-induced gene expression signature in estrogen receptor (ER)-negative breast cancer cells was found to correlate with the potential to form lung metastases. Blockade of TGF-β signaling impairs the extravasation of ER-negative breast cancer cells in lung capillaries, while TGF-β pretreatment increases the metastatic abilities of tumor cells[138]. TGF-β-induced *adipokine angiopoietin-like 4* (*ANGPTL4*) plays a vital role in the disruption of junctions between pulmonary endothelial cells (**Fig. 6B**). However, bone metastasis is not affected by TGF-β preincubation or *ANGPTL4* knockdown, which can be explained by the microvasculature difference in these two organs[96].

TGF-β also participates in the metastatic growth of tumor cells in liver[139, 140]. Upon extravasating into liver parenchyma, TGF-β released by colorectal cancer cells promotes the

transformation of surrounding hepatic satellite cells (HSCs) into myofibroblasts. Tumor-associated myofibroblasts in turn increase the expression of *C-X-C motif chemokine ligand 12* (*CXCL12*) and *hepatic growth factor* (*HGF*), which trigger the metastatic growth of cancer cells[141].

Stimulation of Angiogenesis and Immune Evasion by TGF-β

Angiogenesis is indispensable for solid tumors larger than 2–3 mm^3 to obtain oxygen and nutrients, remove waste products and spread through the circulatory system[142]. An elevated level of TGF-β in plasma correlates with an increase of tumor angiogenesis and poor clinical outcomes in many cancer types[143-146]. TGF-β can directly activate endothelial cells by promoting TGF-β/ALK1 signaling[147]. The coreceptor endoglin, which is highly expressed in activated endothelial cells, can potentiate this signaling response[148]. Moreover, in the tumor niche with low oxygen, hypoxia and TGF-β signaling can cooperate to initiate an angiogenic program in cancer cells. Mechanistically, hypoxia-induced HIF-1, in cooperation with SMAD3, enhances the transcription of *vascular endothelial growth factor* (*VEGF*), which is of importance in capillary formation and endothelial cell migration, thereby promoting tumor angiogenesis[149, 150].

Table 1 Overview of clinical trials with TGF-β targeting agents

Stage	Drug	Type	Target	Disease	Clinical trial identifier
Phase 1	AP 12009	AON	TGF-β2	Multiple cancers	NCT00844064
	TAG Vaccine	Vaccine therapy	TGF-β2 & immune	Carcinoma/Advanced Metastatic	NCT00684294
	TEW-7197	Kinase inhibitor	TβRI	Advanced Stage Solid Tumors	NCT02160106
	NIS793	Antibody	TGF-β	Multiple cancers	NCT02947165
	Paclitaxel/Carboplatin + Galunisertib	Kinase inhibitor	TβRI	Carcinosarcoma, Ovarian	NCT03206177
	TEW-7197	Kinase inhibitor	TβRI	Multiple Myeloma	NCT03143985
	LY2157299	Kinase inhibitor	TβRI	Multiple myeloma	NCT00689507
	LY573636	Kinase inhibitor	TβRI	Hematopoietic malignancies	NCT00718159
	Fresolimumab (CG1008)	Antibody	TGF-β2	Multiple cancers	NCT00356460
Phase 1/Phase 2	Fresolimumab (CG1008)	Antibody	TGF-β2	Multiple cancers	NCT02581787
	LY2157299	Kinase inhibitor	TβRI	Multiple cancers	NCT02423343
	LY2157299	Kinase inhibitor	TβRI	Glioma	NCT01220271
Phase 2	LY2157299	Kinase inhibitor	TβRI	Prostate Cancer	NCT02452008
	Lucanix (belagen-pumatucel)	Vaccine therapy	TGF-β2 & immune	Multiple cancers	NCT01058785
	PF03446962	Antibody	ALK1	Transitional Cell Carcinoma of Bladder	NCT01620970
	Fresolimumab (CG1008)	Antibody	TGF-β2	Primary Brain Tumors	NCT01472731
	LY2157299	Kinase inhibitor	TβRI	Metastatic Breast Cancer	NCT02538471
	LY2157299	Kinase inhibitor	TβRI	Rectal Adenocarcinoma	NCT02688712
	AP 12009	AON	TGF-β2	Glioblastoma/Anaplastic Astrocytoma	NCT00431561
	Fresolimumab (CG1008)	Antibody	TGF-β2	Pleural Malignant Mesothelioma	NCT01112293
	Fresolimumab (CG1008)	Antibody	TGF-β2	Renal Cell Carcinoma	NCT00923169
	LY2157299	Kinase inhibitor	TβRI	Hepatocellular Carcinoma	NCT02178358
	Fresolimumab(CG1008)	Antibody	TGF-β2	Metastatic Breast Cancer	NCT01401062

	LY2157299	Kinase inhibitor	TβRI	Hepatocellular carcinoma	NCT01246986
	LY573636	Kinase inhibitor	TβRI	Melanoma	NCT00383292
	LY573636	Kinase inhibitor	TβRI	Non-small cell lung carcinoma	NCT00363766
	Fresolimumab(CG1008)	Antibody	TGF-β2 & immune response	Kidney cancer	NCT00899444
Phase 3	AP 12009	AON	TGF-β2	Anaplastic Astrocytoma/Glioblastoma	NCT00761280
	Lucanix	Vaccine therapy	TGF-β2 & immune response	Non-small Cell Lung Cancer	NCT00676507
Phase 4	Vitamin D3		TGF-β1	Multiple cancers	NCT02460380
Preclinical	LY2109761	Kinase inhibitor	TβRI/TβRII	Pancreatic cancer	
	SD208	Kinase inhibitor	TβRI	Melanoma	
	SM16	Kinase inhibitor	TβRI	Multiple cancers	
	TβRII Antibody	Antibody	TβRII	Multiple cancers	
	sTβRII (Fc)	Ligand Trap	TβRII	Multiple cancers	
	sBetaglycan	Ligand Trap	Betaglycan	Multiple cancers	
	1D11	Antibody	TGF-β1/2/3	Multiple cancers	
	2G7	Antibody	TGF-β1/2/3	Multiple cancers	

Data from www.clinicaltrials.gov

Silencing *SMAD2* (in contrast to *SMAD3* depletion) in breast cancer MDA-MB-231 cells enhances TGF-β-induced VEGF secretion *in vitro* and promotes the formation of bone metastases *in vivo*[151]. TGF-β also enhances the transcription of *CTGF*, another key angiogenic factor, in breast cancer cells with high bone metastatic potential[135].

In addition to supporting EMT, invasion, metastasis and tumor angiogenesis, TGF-β also contributes to tumor progression by stimulating tumor evasion from immune surveillance. $CD8^+$ cytotoxic T cells are a cell population that can induce cancer cell apoptosis. TGF-β represses the transcription of *granzyme*, *perforin* and *interferon-γ* through SMAD and ATF1 in $CD8^+$ T cells, thereby inhibiting the cytotoxic activity of $CD8^+$ cytotoxic T cells[152, 153]. TGF-β can also induce the differentiation of regulatory T-cells (Tregs), which suppress the proliferation and activation of $CD8^+$ cytotoxic T cells, resulting in immunosuppression and a decrease in immunosurveillance[154-156]. The activation of natural killer (NK) cells, another cytotoxic cell type, is attenuated by TGF-β-induced downregulation of *IL-15* and *NKG2D*, an activating receptor of NK cells[157, 158]. In addition, TGF-β-triggered *miR-183* expression represses DAP12 protein expression, leading to the destabilization of the NK receptor and inhibition of cytotoxicity[159]. In addition, TGF-β is a driver of the tumor-suppressive M1 macrophage phenotype transition into the tumor-promoting M2 phenotype, thereby promoting the production of tumor-promoting factors and inhibiting the activity of T cells[160, 161].

Targeting TGF-β Signaling in Cancer

Due to the strong pro-oncogenic effects of TGF-β, inhibitory agents targeting TGF-β have been developed, including antisense oligonucleotides (AONs), small molecule receptor kinase inhibitors and neutralizing antibodies. The mechanisms of these inhibitors involve the inhibition of TGF-β and receptor expression, the interference of receptor kinase signaling, and the blockade of TGF-β ligand and receptor binding (**Fig. 7**, **Table 1**). These agents have been tested in preclinical and clinical stages. While inhibiting tumor progression by blocking TGF-β signaling is a promising approach, the biphasic action of TGF-β in cancer progression and its multifunctionality make it a challenging target.

Antisense Oligonucleotides

The binding of ligands to receptors is the first step of TGF-β signaling activation; AONs have been developed to degrade *TGFB* mRNA[162] (**Fig. 7**). The antisense RNA drugs AP12009 and AP11014 targeting *TGFB2* and *TGFB1*, respectively, have been used in (pre)clinical cancer treatment studies. AP12009 has been reported to inhibit neovascularization and tumor invasion and has been used to treat high-grade glioma and anaplastic astrocytoma patients[163-165]. In addition, AP11014 has been reported to display an anti-tumor effect in animal models of colon cancer, prostate cancer and lung cancer and is being studied in preclinical research[166, 167].

TGF-β Receptor Kinase Inhibitors

Small ATP-mimetic compounds have been synthesized to selectively inhibit TβRI (and TβRII) kinase activity (**Fig. 7**). These compounds have been tested in preclinical and clinical studies of multiple cancer types. Systemic administration of the TβRI kinase inhibitor SD208 can increase the median survival of mice with malignant glioma inoculation[168] and reduce tumor metastasis in pancreatic and breast cancer[169, 170]. LY2157299 is the first TβRI kinase inhibitor that has been reported to inhibit primary tumor growth in breast and lung cancer cell lines[171, 172]. To optimize the applicability of LY2157299 to cancer therapy, a first-in-human dose evaluation found that LY2157299 administration at 300 mg per day is safe[173]. Another kinase inhibitor, LY2109761, inhibits both the activity of TβRI and TβRII. A large number of studies have indicated that LY2109761 exhibits great potential in the prevention of cancer metastasis in multiple cancer types including colon[174] and pancreatic cancer[175], glioblastoma[176] and ovarian cancer[177, 178]. TEW-7197 is an orally administered small molecule that targets TβRI kinase activity. It stimulates apoptosis and suppresses TGF-β-induced activation of SMAD2/3 in human and murine myeloma cells *in vitro*, leading to the inhibition of myeloma cell growth and viability[179]. While the preclinical results of these studies are promising, the clinical translation has been difficult. On-target side effects on the cardiovascular system have halted clinical advancement. By using an intermittent dosing strategy, these adverse side effects may be overcome[172, 180].

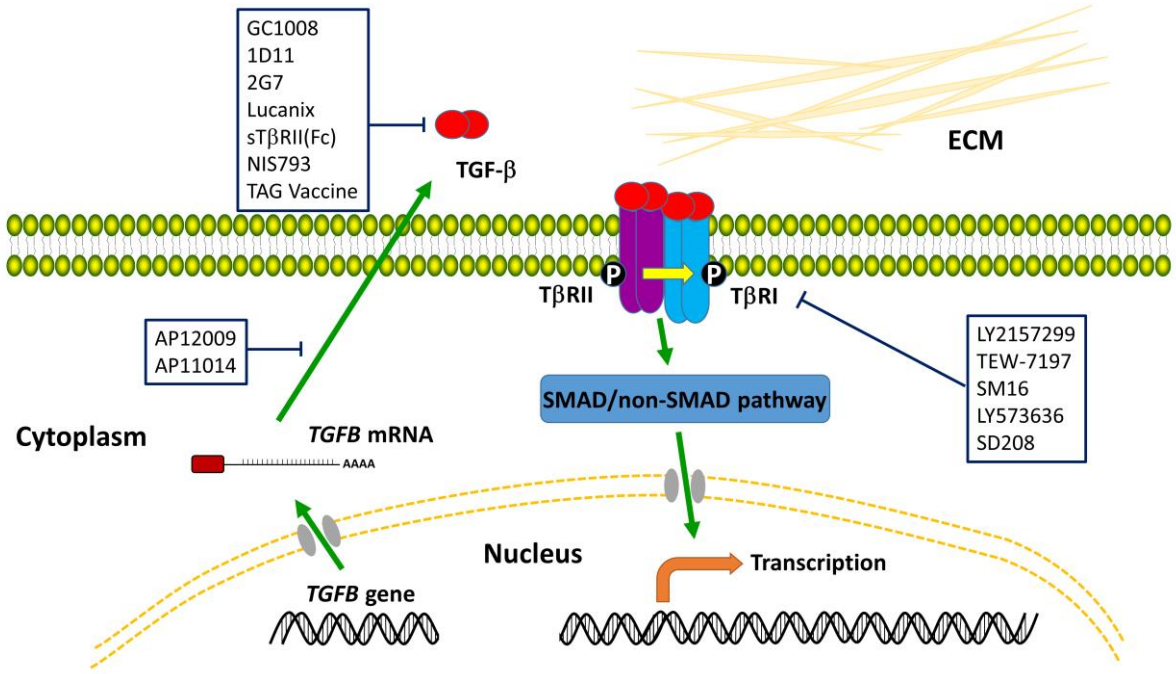

Fig. 7 Targeting TGF-β in cancer. TGF-β signaling has an important effect on tumor progression and provides a new approach for tumor targeting therapy. Many inhibitors of the TGF-β pathway (including kinase inhibitors, AONs, and antibodies) have already been applied in preclinical and clinical trials (see **Table 1**). Abbreviations: ECM, extracellular matrix; TβR, TGF-β receptor; TGF-β, transforming growth factor-β.

Antibodies Against TGF-β Ligands and Extracellular Domains of TGF-β Receptors

1D11, an antibody that recognizes all three TGF-β isoforms, interferes with TGF-β and TGF-β receptor binding and, thus, neutralizes TGF-β activity (**Fig. 7**). This antibody has been used in (pre)clinical studies. 1D11 significantly increases NK cell and nuclear T cell invasion, as well as *NKG2D* expression and cytotoxic perforin and granzyme B release in breast cancer cells, thereby enhancing the anti-tumor effect of $CD8^+$ T cells and NK cells[181]. Additionally, 1D11 has also been found to suppress bone metastasis in prostate cancer[182]. Like 1D11, 2G7 also inhibits MDA-MB-231 cell invasion. Additionally, the combination of dendritic cell (DC)-based vaccines and 2G7 potently inhibits the development of the established murine mammary tumors[183, 184]. A clinical trial of another monoclonal antibody, fresolimumab (CG1008), which inhibits all three TGF-β isoforms, demonstrated its safety and efficacy in suppressing metastatic melanoma and renal cell carcinoma[185]. Fresolimumab may help to stabilize the condition of patients during malignant pleural mesothelioma therapy. Importantly, adverse effects, such as skin toxicity (including formation of cutaneous squamous-cell carcinomas and basal cell carcinoma), have been reported in cancer patients after fresolimumab treatment[185].

Similar to neutralizing antibodies, soluble TβRII and TβRIII ligand traps are also used to block TGF-β signaling. These molecules are expressed in the extracellular domain of the receptor, which prevent ligands from binding to TGF-β receptors[103]. The ligand trap TβRII:Fc (a fusion of the extracellular TGF-β-binding domain of TβRII with IgG1 Fc domain) shows anti-tumor effects on multiple cancers, including inhibition of mesothelioma growth and suppression of breast cancer cell viability and migration[186, 187]. The expression of soluble TβRIII (sBetaglycan) effectively suppresses tumor growth in MDA-MB-231 xenograft-bearing athymic nude mice[188] and inhibits glioma and non-small cell lung cancer progression in other mouse models[189, 190]. Due to the risk of tumor development caused by the TGF-β soluble receptors[191, 192], these receptors have not yet entered the clinical research phase.

Targeting TGF-β signaling provides a new approach and opportunity for cancer therapy. Since TGF-β pathway is also involved in many normal biological functions, the exact mechanism of action in the patients and the adverse reactions caused by systemic inhibition of TGF-β are still not clear. A further understanding of the dual roles of TGF-β will be beneficial to the development of therapeutics specifically targeting TGF-β in tumor progression. Sole treatment with TGF-β-targeting agents will likely not be successful in curing cancer patients, and a combination of TGF-β targeting therapies with chemo- and radiotherapy or other forms of targeted therapy should be explored.

Concluding Remarks

TGF-β has a dual action in cancer by acting as a tumor suppressor in the early stages and a tumor promoter in the late phases of tumor progression. Cancer cells are insensitive to the cytostatic effects of TGF-β through the activation of proto-oncogenes and inactivation of tumor suppressor genes. The latter (epi)genetic changes also cooperate with TGF-β to mediate EMT, thereby facilitating invasion and metastasis. Moreover, TGF-β promotes tumorigenesis by stimulating immune evasion and promoting angiogenesis. The biphasic role in cancer and its multifunctional properties in the maintenance of tissue homeostasis make TGF-β a challenging pathway to target for treatment of cancer patients. A more detailed understanding of the mechanism of action in cancer patients, careful dosing and the selection of patients who will most benefit from the TGF-β targeting agents will be important for their clinical implementation.

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2) Long non-coding RNAs in TGF-β signaling and EMT

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Abstract
Transforming growth factor-β (TGF-β) signaling can have a dual role during cancer progression and suppress tumorigenesis at initial stages of cancer but promote cancer progression at advanced stages. The latter is achieved, in part, by acting directly on cancer cells by inducing a transition from epithelial to a highly invasive mesenchymal state (EMT). Ligand-induced activation of transmembrane TGF-β receptor triggers EMT through activation of intracellular SMAD transcription factors. TGF-β signaling is regulated by modulators at multiple levels during EMT. Although the importance of protein coding genes that are modulated in response to TGF-β/SMAD signaling have been well studied, an important role of long non-coding RNAs (lncRNAs) in TGF-β/SMAD signaling action is emerging. This mini-review focusses on the mechanisms by which lncRNAs interplay with TGF-β signaling.

Molecular Basis of LncRNAs
Although more than 70% of human genome can be actively transcribed, only around 2% of it is transcribed into protein coding messenger RNAs (mRNAs)[1, 2]. However, a large amount of lncRNAs, which had been recognized as “transcription noise” for a long time, is extensively transcribed within the human genome[3, 4]. A recent study that collected the sequencing results from various publicly available databases revealed 95,243 human lncRNA genes and 323,950 human lncRNA transcripts[5]. LncRNAs are arbitrarily defined by that their transcript length is longer than 200 nucleotides (nt). Similar to mRNAs, most lncRNAs are transcribed by RNA polymerase II (Pol II), and a large proportion of lncRNAs undergo alternative splicing and polyadenylation[6, 7]. Unlike mRNAs, lncRNA primary sequences are less conserved among species and lncRNA expression generally exhibits high tissue specificity[2, 8-10].

Mechanisms of LncRNAs
LncRNAs can be divided into nuclear and cytoplasmic lncRNAs depending on their subcellular localization (Fig. 1). By interacting with chromatin modifiers or transcription (co)factors, nuclear lncRNAs can alter the epigenetic landscape or the transcription process, and thereby change target gene expression[11-15]. Nuclear lncRNAs can influence RNA splicing by interacting with the serine and arginine-rich (SR) protein[16, 17]. Moreover, a subgroup of lncRNAs called enhancer RNAs, which are transcribed from active enhancers, can modulate chromatin looping *in cis* or *in trans*, leading to the activation of target gene transcription[18-21]. Cytoplasmic lncRNAs can regulate mRNA stability or translation through directly binding to mRNAs or RNA binding proteins (RBPs). LncRNAs localized in the cytoplasm can also act as sponges

for microRNAs (miRNAs)[22, 23]. Recent studies have shown that functional small peptides can be encoded by cytoplasmic lncRNAs that associate with ribosomes[24, 25]. Additionally, both cytoplasmic and nuclear lncRNAs can regulate protein post-translational modifications (PTMs) or molecular complex formation by functioning as scaffolds or decoys[26-29].

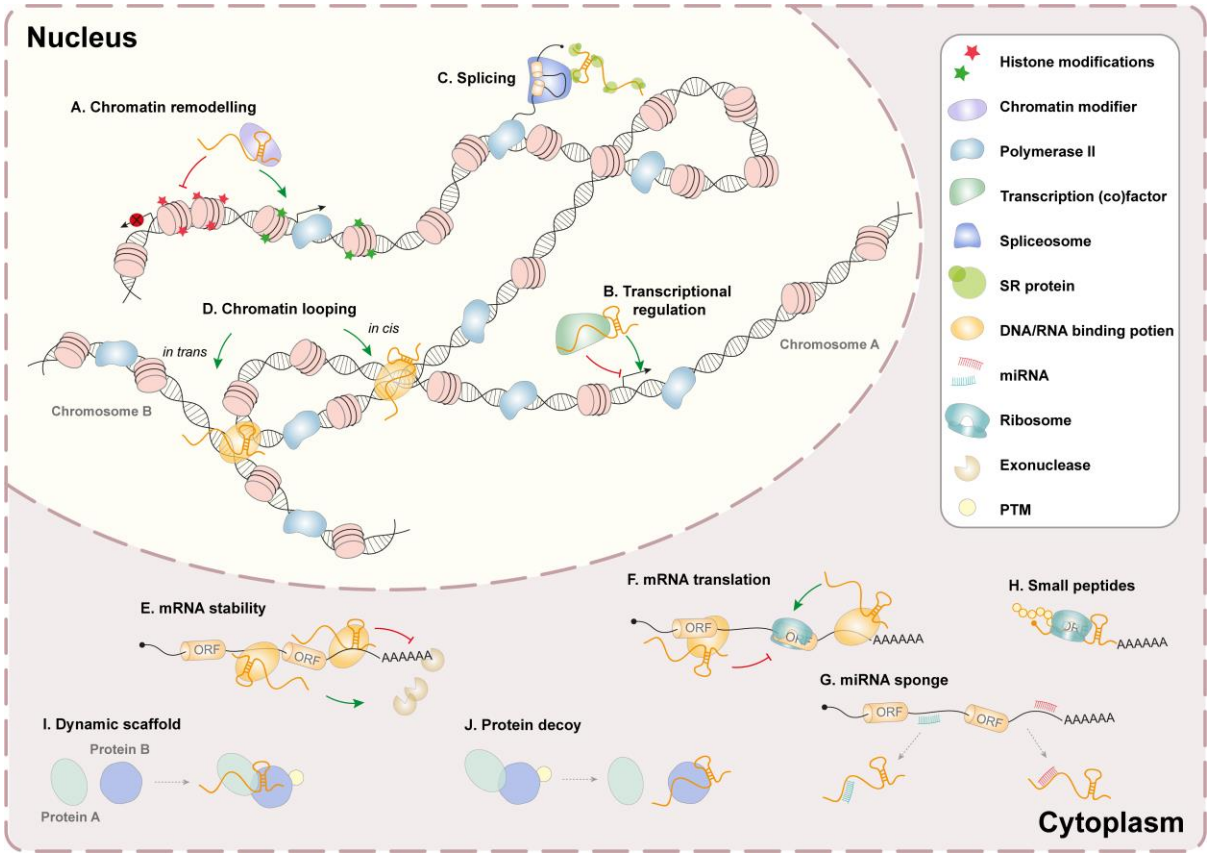

Fig.1 Mechanisms of lncRNAs function. Nuclear lncRNAs can interact with chromatin modifiers (A) or transcription (co)factors (B) to regulate chromatin landscape or gene transcription. They can also mediate alternative splicing (C) and chromatin looping (D). Cytoplasmic lncRNAs can affect mRNA stability (E) or translation (F), sponge miRNAs (G), encode small peptides (H), and modulate protein interactions and post-translational modification (I, J). ORF: open reading frame; SR: serine and arginine-rich; PTM: post-translational modification.

LncRNAs Function as Effectors of TGF-β Signaling

TGF-β-induced gene products frequently function as effectors of TGF-β-induced responses, for example EMT[30, 31]. Consistent with this scenario, TGF-β-induced lncRNAs can drive TGF-β-induced EMT in cancer. ***Lnc****RNA-****H****OXA transcript* ***i****nduced by* ***T****GF-β* (*LncRNA-HIT*) promotes TGF-β-induced EMT and migration by specifically mitigating E-cadherin expression in mouse mammary NMuMG cells[32]. TGF-β promotes the expression of ***lnc****RNA-****a****ctivated by* ***TGF-β*** (*lncRNA-ATB*), which stabilizes *interleukin-11* (*IL-11*) mRNA, resulting in the promotion of hepatocellular carcinoma (HCC) cell colonization in secondary tissues[33]. In addition, *lncRNA-ATB* drives EMT by serving as a sponge for *miR-200*, leading to the upregulation of EMT transcription factor ZEB1/2[33]. Moreover, expression of other TGF-β downstream EMT transcription factors including SNAIL[34, 35], SLUG[34-36] and TWIST[37, 38] can be activated by TGF-β-induced lncRNAs.

TGF-β can induce lncRNA expression to influence the transcriptional output by altering epigenetic modifications. TGF-β-induced ***M****etastasis* ***A****ssociated* ***L****ung* ***A****denocarcinoma* ***T****ranscript* ***1*** (*MALAT1*) interacts with H3K27 methyltransferase suppressor of zeste 12 (suz12),

a component of the polycomb repressive complex 2 (PRC2), to promote H3K27me3 abundance at the promoter of *CDH1* (the gene that encodes E-cadherin) and to potentiate EMT in bladder cancer cells[39]. *TGFB2-antisense RNA1* (*TGFB2-AS1*) associates with PRC2 adaptor protein EED to facilitate H3K27me3 modification at the promoter of TGF-β target genes[40].

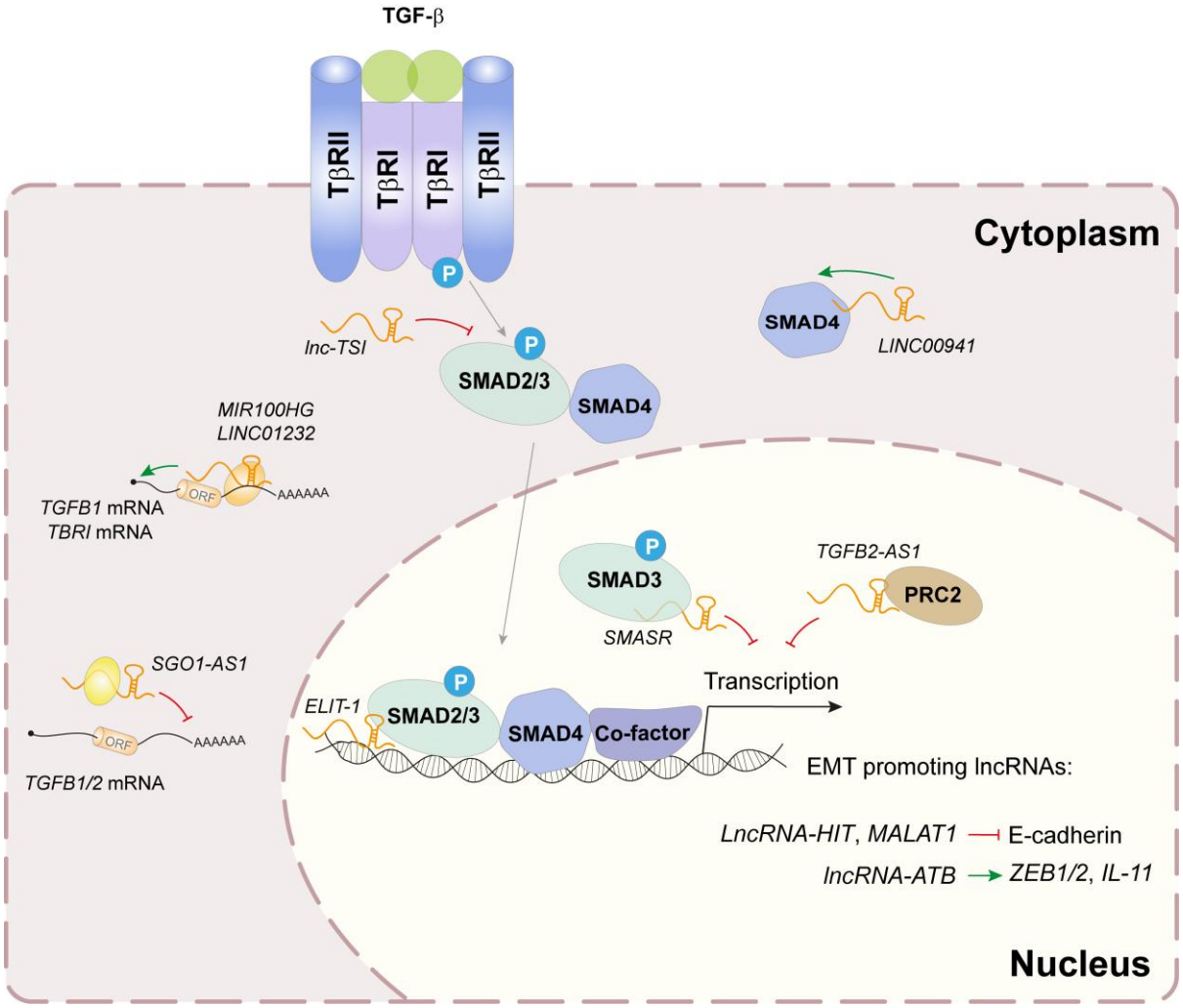

Fig.2 Interplay between lncRNAs and TGF-β signaling. TGF-β signaling induces lncRNAs to regulate EMT in cancer. LncRNAs can also modulate TGF-β signaling transduction at different levels, from ligand production to transcriptional output. (For description see text)

LncRNAs Function as Modulators of TGF-β Signaling

LncRNAs can act as modulators to fine-tune TGF-β signaling transduction in a negative or positive feedback manner[41, 42]. TGF-β induced ***mir-100****-let-7a-2-mir-125b-1 cluster* ***h****ost* ***g****ene* (*MIR100HG*) enhances *TGFB1* mRNA stability by promoting the binding of RBP HuR to *TGFB1* mRNA in multiple cancer cells[43]. *MIR100HG* enhances TGF-β1 autocrine to potentiate TGF-β signaling[43]. *SGO1-AS1* facilitates *TGFB1/2* mRNA decay by competing their binding to PTBP1, an RBP that stabilizes *TGFB1/2* mRNA[44]. TGF-β1/2 production is therefore decreased by *SGO1-AS1*, leading to the attenuation of EMT and cancer metastasis[44].

Expression of TGF-β signaling receptors is regulated by lncRNAs. ***SM****AD3-****as****sociated long non-coding* ***R****NA* (*SMASR*) expression is suppressed by TGF-β/SMAD signaling in lung adenocarcinoma cells[45]. *SMASR* interacts with SMAD3 to attenuate *TBRI* mRNA transcription, thus leading to inactivation of TGF-β/SMAD signaling[45]. *LINC01232* recruits the RBP insulin

like growth factor 2 mRNA binding protein 2 (IGF2BP2) to protect *TBRI* mRNA from degradation[46]. As a consequence, TGF-β signaling and cell stemness are potentiated by *LINC01232* in lung adenocarcinoma cells[46].

R-SMADs (i.e. SMAD2/3) and the co-SMAD SMAD4 are reported to be modulated by lncRNAs. TGF-β/SMAD-induced ***T**GF-β/**S**MAD3-**i**nteracting long noncoding RNA* (*lnc-TSI*) binds to the MH2 domain of SMAD3 to diminish its interaction with TβRI in human tubular epithelial cells[47]. ***E**MT-associated **l**ncRNA **i**nduced by **T**GFβ**1*** (*ELIT-1*) selectively binds to SMAD3, but not SMAD2, and recruits SMAD3 to target gene promoter in multiple cancer cell lines[48]. *ELIT-1* depletion greatly abrogates TGF-β-induced EMT and migration[48]. *LINC00941* functions as a molecular decoy to bind SMAD4 MH2 domain and to protect SMAD4 from being degraded by the E3 ligase β-TrCP in colorectal cancer cell[49].

Perspectives

The interplay between lncRNAs and TGF-β signaling reveals the important effector role of lncRNAs in TGF-β-induced biological responses and also the intricate and multi-level regulation of TGF-β signaling by lncRNAs to fine-tune its strength and duration. Manipulating critical lncRNA expression in cancer cells may provide a new strategy to target TGF-β-triggered EMT in cancer progression. The tissue-specific expression of lncRNAs can be exploited to selectively target TGF-β signaling in highly-malignant mesenchymal cancer cells to circumvent the on-target effects caused by systemic TGF-β signaling intervention. However, considering the dichotomous role of TGF-β signaling in early and late phases of cancer progression, it is key to understand the mechanisms by which lncRNAs modulate TGF-β signaling in cancer cells in different stages or with difference genetic mutations. Differentially expressed lncRNAs that functionally correlate with TGF-β-induced pro-tumorigenic responses may serve as biomarkers to select cancer patients who can benefit from TGF-β targeted therapies.

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3) Scope of this thesis

In **Chapter 1**, we review the transduction of TGF-β signaling and the intricated regulation of TGF-β signaling at multiple layers. The biphasic role of TGF-β signaling in cancer progression is discussed. We also review the interplay between long non-coding RNAs (lncRNAs) and TGF-β signaling in EMT. In **Chapter 2**, we identified a lncRNA *LITATS1* that functions as a protector of TGF-β-induced EMT in breast and non-small cell lung cancer. *LITATS1* enhances the polyubiquitination and proteasomal degradation of TβRI by strengthening the interaction between TβRI and the E3 ligase SMURF2. *LITATS1* maintains the cytoplasmic localization of SMURF2. In **Chapter 3**, we uncovered an unannotated lncRNA *LETS1* as a novel enforcer of TGF-β signaling and TGF-β-induced EMT in breast and non-small cell lung cancer cells. Mechanistic study revealed that *LETS1* cooperates with NFAT5 to bind *NR4A1* promoter and induces the expression of *NR4A1*, a critical determinant of a destruction complex for inhibitory SMAD7. In **Chapter 4**, we found that a transcriptional repressor Ovo like transcriptional repressor 1 (OVOL1) inhibits TGF-β-induced EMT by facilitating TβRI degradation. We uncovered that OVOL1 interacts with and prevents SMAD7 polyubiquitination and degradation. A small molecule compound 6-formylindolo(3,2-b)carbazole (FICZ) was identified to activate OVOL1 expression and thereby antagonizes (at least in part) TGF-β-mediated EMT and migration in breast cancer cells.