Transforming growth factor-β (TGF-β) elicits both tumor-suppressive and tumor-promoting functions during cancer progression. Here, we describe the tumor-promoting functions of TGF-β and how these functions play a role in cancer progression. Normal epithelial cells undergo epithelial-mesenchymal transition (EMT) through the action of TGF-β, while treatment with TGF-β and fibroblast growth factor (FGF)-2 results in transdifferentiation into activated fibroblastic cells that are highly migratory, thereby facilitating cancer invasion and metastasis. TGF-β also induces EMT in tumor cells, which can be regulated by oncogenic and anti-oncogenic signals. In addition to EMT promotion, invasion and metastasis of cancer are facilitated by TGF-β through other mechanisms, such as regulation of cell survival, angiogenesis, and vascular integrity, and interaction with the tumor microenvironment. TGF-β also plays a critical role in regulating the cancer-initiating properties of certain types of cells, including glioma-initiating cells. These findings thus may be useful for establishing treatment strategies for advanced cancer by inhibiting TGF-β signaling.

**Key words:** Angiogenesis, cancer-initiating cell, EMT, invasion, metastasis, TGF-β

**Introduction**

Transforming growth factor-β (TGF-β) is a multifunctional regulator of cell growth, apoptosis, differentiation, and migration. TGF-β1 was originally discovered as a secreted protein that induces anchorage-independent growth in normal rat kidney NRK49F fibroblasts in the presence of epidermal growth factor (EGF) (1). TGF-β was shown to potently inhibit the proliferation of most cell types, including epithelial cells, endothelial cells, hematopoietic cells, and lymphocytes, and is widely known as a tumor suppressor. Studies investigating TGF-β signaling have revealed that perturbations of the TGF-β signaling pathway, such as mutations of TGF-β receptors or Smad proteins, lead to cancer progression and are related to poor prognosis of certain types of cancer. However, recent findings have shown that cancer cells become resistant to the growth inhibitory activity of TGF-β and that TGF-β facilitates invasion and metastasis of these cells both in vitro and in vivo.

Accumulating evidence has revealed that TGF-β plays a bidirectional role in cancer progression (2,3). TGF-β acts as a tumor suppressor by inhibiting cell growth through suppressing c-Myc expression and stimulating certain cyclin-dependent kinase inhibitors, including p21WAF1 and p15Ink4b, and by inducing cellular apoptosis through inducing DAP kinase, GADD45β, and Bim (4). Conversely, TGF-β functions as a tumor-promoting factor by stimulating extracellular matrix deposition and tissue fibrosis, perturbing immune and inflammatory function, stimulating angiogenesis, and promoting epithelial-mesenchymal transition (EMT).

In this review article, we discuss the tumor-promoting functions of TGF-β, particularly on EMT, on the basis of recent findings in our
laboratory. We also describe the function of TGF-β in some cancer-initiating cells and discuss how inhibition of TGF-β signaling can be used for treating different types of cancer.

**TGF-β family signaling**

TGF-β binds to two different serine/threonine kinase receptors, TβRII and TβRI (5). Betaglycan, also known as the TGF-β type III receptor, facilitates binding of TGF-β (particularly TGF-β2 among the three isoforms of TGF-β) to TβRII. TβRII activates TβRI through phosphorylation of the Gly-Ser-rich (GS) domain of TβRI, which in turn phosphorylates and activates Smad2 and Smad3, receptor-regulated Smads (R-Smads) specific for TGF-β and activin signaling (Figure 1). Bone morphogenetic proteins (BMPs) activate another set of R-Smads, including Smad1, Smad5, and Smad8 (6). Activated Smad2 and Smad3 form complexes with Smad4, common partner Smad (co-Smad), and translocate into the nucleus. R-Smad/co-Smad complexes associate with various transcription factors (AP-2, Ets, and HNF-4α (7–9)) and transcriptional co-activators (p300, CBP, and GCN5) or co-repressors (p107, Ski, and SnoN) in the nucleus and regulate transcription of a wide spectrum of TGF-β target genes. Smad7, an inhibitory Smad (I-Smad), represses TGF-β signaling through multiple mechanisms; among these mechanisms, binding to activated type I receptors and competition with R-Smads for receptor binding play a major role in regulation of TGF-β signaling (10). c-Ski (also known as SKI) and the related SnoN (also known as SKIL) bind directly to Smad2/3 and Smad4 and function as transcriptional co-repressors by recruiting histone deacetylases and competing for binding with p300/CBP. C-Ski also disrupts formation of the R-Smads and Smad7 complexes.

Figure 1. Schematic representation of TGF-β signal transduction pathways. TGF-β transduces signals through two different types of serine/threonine (and tyrosine) kinase receptors, termed TβRI and TβRII. Upon TGF-β binding, TβRI and TβRII form heterotetrameric complexes, and TβRII kinase transphosphorylates the juxtamembrane portion (GS domain) of the cytoplasmic region of TβRI. Phosphorylated TβRI transmits intracellular signaling through R-Smad phosphorylation. Smad2 and Smad3 are R-Smads phosphorylated by TβRI kinase and form heteromeric complexes with Smad4 (co-Smad). Smad complexes translocate into the nucleus and act as transcriptional regulators of target genes by interacting with other transcription factors and transcriptional regulators. Smad7 (I-Smad), which lacks the typical MH1 domain, interferes with the activation of R-Smads by interacting with TβRI and competitively prevents R-Smads from being phosphorylated by TβRI. TGF-β activates other intracellular signaling pathways in addition to Smads in order to regulate a wide array of cellular functions. These non-Smad pathways are activated by TGF-β receptors through phosphorylation or direct interaction.
EMT induction. Additionally, non-Smad signaling pathways activated by TGF-β and cross-talk with other signaling pathways, including fibroblast growth factor (FGF) and tumor necrosis factor-α (TNF-α) signaling, play important roles in EMT promotion.

**Induction of EMT in tumor stromal cells by TGF-β**

Epithelial cells in the tumor stroma undergo EMT (type 2 EMT) and play a critical role in cancer progression. We cocultured NMuMG cells with mouse mammary tumor JygMC(A) cells and found that NMuMG cells that have undergone EMT express α-SMA (18). The effect of the JygMC(A) cells was abolished by treatment with the TβRI inhibitor SB431542. Interestingly, when NMuMG cells were cocultured with the mouse mammary tumor cell line 4T1, NMuMG cells underwent EMT and produced mesenchymal cells with an activated fibroblastic phenotype, which lacked α-SMA expression. 4T1 cells produced TGF-β1 at a level comparable to that produced by JygMC(A) cells. When 4T1 cells were treated with FGF receptor 1 (FGFR1) inhibitor SU5402, α-SMA-positive NMuMG cells were detected, indicating that the loss of α-SMA expression is due to FGF(s) secreted from 4T1 cells. We have shown that treatment of NMuMG cells with TGF-β and FGF-2 prevents the production of mesenchymal cells expressing α-SMA and calponin by activating the MEK-ERK pathway. Interestingly, NMuMG cells that have undergone EMT following treatment with TGF-β and FGF-2 exhibit drastic morphological changes with marked actin reorganization, enhanced cell migration, and increased production of matrix metalloproteinases (MMPs), including MMP-9. Moreover, NMuMG cells treated with TGF-β and FGF-2 enhanced the invasion of cocultured breast cancer cells into collagen gels *in vitro*. Thus, TGF-β and FGF-2 co-operate with each other to produce ‘activated’ fibroblasts in the tumor microenvironment, and activated fibroblasts may in turn secrete substances such as MMPs to induce invasion and metastasis of adjacent cancer cells (Figure 2).

During EMT progression, TGF-β induces isoform switching of FGFRs. Of the 22 FGFRs (19), epithelial cells respond to specific FGFRs, including FGFR-7 (also known as keratinocyte growth factor (KGF)), but not to FGFR-2 (basic FGF) or FGFR-4. However, cells that have undergone EMT become responsive to FGF-2 and FGF-4, but not to FGF-7 (18). We have shown that TGF-β-mediated EMT induces isoform switching of FGFRs through alternative splicing, following which expression of the IIIb isoform of FGFR decreased and that of the IIIc isoform increased.
Exon array analysis showed that TGF-β alters a broad spectrum of splicing patterns by reducing the expression of epithelial splicing regulatory proteins (ESRPs) 1 and 2 (20). Warzecha et al. (21) recently reported that the ESRP-regulated splicing pathway is abrogated during EMT. We found that repression of the expression of ESRPs by TGF-β is mediated by up-regulation of the δEF1 family proteins δEF1 and SIP1, which suppress the transcription of ESRP(s) by binding to the ESRP promoter(s). Interestingly, the expression profiles of ESRPs were reciprocally correlated with those of δEF1 and SIP1 in human breast cancer cell lines as well as in tumor specimens. In addition to FGFRs, TGF-β induces alternative splicing of CD44, Mena, and CTNND1 (also known as δ-catenin or p120 catenin), which are reportedly involved in cancer progression. We have also shown that over-expression of ESRPs attenuates TGF-β-induced EMT and restores the expression of E-cadherin and some other epithelial phenotypes. Thus, ESRPs are downstream targets of TGF-β and serve as antagonists to EMT by regulating alternative splicing of specific genes involved in TGF-β-induced EMT.

**Induction of EMT in cancer cells**

EMT is observed in some transformed epithelial cells (type 3 EMT) to facilitate their invasive and metastatic properties. Type 3 EMT can be regulated by specific oncogenic and anti-oncogenic signals. We have shown that a zinc-finger transcription factor Snail is induced by TGF-β in pancreatic cancer Panc1 cells and plays a key role in EMT progression (22). Panc1 cells express active K-ras, and we found that induction of Snail by TGF-β is dependent on oncogenic Ras signals. Snail was strongly induced by TGF-β in Panc1 cells, but knock-down of Ras in Panc1 cells abolished Snail induction by TGF-β. Consequently, TGF-β failed to efficiently induce EMT in Panc1 cells in the absence of active Ras signaling. Exogenous expression of constitutively active Ras into HeLa cells resulted in marked induction of Snail by TGF-β, while induction of other direct targets of TGF-β, including Smad7 and PAI-1, was not enhanced by Ras signaling. MAP kinases have been reported to phosphorylate the linker region of Smad2 and Smad3, which both positively and negatively regulates TGF-β signaling.
we have also shown that TGF-
with Smad3 (28) and may inhibit Smad3 function.

EMT. TTF-1 has been reported to interact physically
TTF-1 in H441 cells enhances TGF-
Snail and Slug in A549 cells, and silencing of
ion. Conversely, TGF-
thelial phenotype through high E-cadherin expres-
sion. Additionally, TGF-
activates K-Ras and inactive p53. Interestingly, we found
that TTF-1 functions as a tumor-suppressor during
EMT induction. TTF-1 is highly expressed in certain
types of lung adenocarcinoma cell lines, including
H441 cells and LC-2/ad cells, but not in A549 cells
(27). A549 cells show a spindle-like phenotype and
grow rapidly, while H441 cells show tight cell–cell
junctions with cobblestone-like morphology and grow
much more slowly than A549 cells. A549 cells express
low levels of TTF-1 and E-cadherin, while H441 cells
express high levels of TTF-1 and E-cadherin. We
have further shown that exogenous expression of
TTF-1 in A549 cells inhibits TGF-
induced EMT, decreases MMP-2 activity, cell migration,
and cellular invasive capacity, and restores the ep-
ithelial phenotype through high E-cadherin expres-
sion. Conversely, TGF-
induces the expression of
Snail and Slug in A549 cells, and silencing of
TTF-1 in H441 cells enhances TGF-
mediated EMT. TTF-1 has been reported to interact physically
with Smad3 (28) and may inhibit Smad3 function.
We have also shown that TGF-
down-regulates
TTF-1 expression in A549 cells and that TGF-1
inhibits the expression of TGF-
3, which is expressed in epithelial cells at the tip of the distal airway
during lung morphogenesis. Thus, TTF-1 may exert a
tumor-suppressive effect through antagonizing the
effect of TGF-
. These findings indicate a function-
ally inverse relationship between TTF-1 and TGF-
signaling in the progression of lung adenocarcinoma
through regulation of EMT.

**TGF-β signaling in vascular tissues and angiogenesis**

New blood vessel formation in tumor tissues (tumor angiogenesis) is essential for the growth and metas-
tasis of tumor cells. Although TGF-β potently inhibits
the growth of endothelial cells in *vitro*, it functions
as a pro-angiogenic factor and stimulates angiogenesis in *vivo*. Increased expression of TGF-β is
correlated to increased vascular density in some types
of tumors.

For induction of tumor angiogenesis, TGF-β
induces the expression of angiogenic factors, includ-
ing connective tissue growth factor (CTGF) and
vascular endothelial growth factor (VEGF) (29).
Additionally, TGF-β stimulates the synthesis of
MMP-2 and MMP-9 and down-regulates the expres-
sion of tissue inhibitors of metalloproteinase (TIMPs)
in tumor tissues. Increased MMP activity leads to
stimulation of migration and invasion of vascular
endothelial cells, resulting in accelerated tumor
angiogenesis.

However, TGF-β suppresses angiogenesis in cer-
tain types of tumors through reduced expression of
some angiogenic factors or increased expression of
angiogenic inhibitors. In diffuse-type gastric carci-
noma, TGF-β induces the production of some angi-
ogenic inhibitors, including thrombospondin-1 and
TIMP-2, and perturbations of TGF-β signaling
may thus lead to induction of angiogenesis and tumor
growth in *vivo* (30,31).

In addition to induction of tumor angiogenesis,
TGF-β acts on vascular endothelial cells and may
disrupt cell–cell junctions and support the coloniza-
tion of tumor cells to establish metastasis. Using
endothelial cells derived from mouse embryonic stem (ES) cells, we showed that TGF-β suppresses
the expression of claudin-5 and disrupts sheet
formation in *vitro* (32). We also showed that
TGF-β induces differentiation of certain endothelial
cells into mesenchymal cells, resulting in the loss of
tight cell–cell contacts in *vitro* (33). Moreover,
through disruption of endothelial cell–cell junctions
by inducing angiopoietin-like 4 (Angptl4) expression,
TGF-β has been shown to increase the permeability
of blood vessels and stimulate the trans-endothelial
movement of cancer cells (34).

**Acceleration of cancer metastasis by TGF-β signaling**

TGF-β facilitates metastasis of certain types of cancer
in advanced stages, including breast cancer (35).
Inhibition of TGF-β signaling may thus be a potential
strategy for preventing metastasis of advanced can-
cers. Though not discussed in detail in this review,
TGF-β regulates tumor development by regulating
immune functions (36,37). Wakefield and colleagues
reported that inhibition of TGF-β function prevents
the progression of breast cancer by enhancing various
immune functions (38).
We have shown that Smad7, an I-Smad that inhibits TGF-β and BMP signaling, efficiently inhibits lung and liver metastasis of mouse breast cancer JygMC(A) cells (39). We subcutaneously inoculated JygMC(A) cells, which spontaneously metastasize to the lung, liver, and other organs in 3 to 4 weeks, in nude mice. Ten days after subcutaneous inoculation, adenoviruses containing Smad7 or LacZ were intravenously administered to the mice once weekly. Mice bearing JygMC(A) tumors and treated with LacZ adenovirus developed numerous metastases to the lung and liver, and all mice died by 50 days (median survival time, 41 days) after inoculation of JygMC(A) cells. In contrast, mice treated with Smad7 adenovirus showed a significant decrease in metastases of tumors in both the lung and liver, and the median survival time of Smad7-treated mice was 55 days. JygMC(A) cells treated with Smad7 showed increased expression of components involved in adherence and tight junctions, including E-cadherin, and decreased expression of mesenchymal markers, including N-cadherin. Smad7 also inhibited the migration and invasion of cells, indicating that Smad7 leads to prevention of the EMT process. Interestingly, Smad6, which preferentially inhibits BMP signaling, failed to show significant effects on the metastasis of JygMC(A) cells in nude mice, whereas c-Ski adenovirus showed effects similar to Smad7. Thus, inhibiting TGF-β signaling using Smad7 or c-Ski prevents the EMT process and eventually inhibits lung and liver metastasis of JygMC(A) cells (Figure 3).

In addition to preventing EMT, TGF-β appears to inhibit metastasis of JygMC(A) cells by some other mechanisms. Although TGF-β induces apoptosis of many different types of cells by inducing specific genes, it stimulates survival of certain types of cells in a context-dependent manner through activation of the PI3K-Akt signaling pathway. We have identified Dec1 (differentially expressed in chondrocytes 1, also known as SHARP2 and Stra13) as a downstream target of TGF-β–Smad signaling by DNA microarray analysis (40). Dec1 is a bHLH transcription factor, which is widely expressed in many tissues and overexpressed in certain types of cancer cells. Dec1 prevented the apoptosis of JygMC(A) as well as 4T1 cells, and a dominant-negative mutant of Dec1 suppressed lung and liver metastases of JygMC(A) cells in nude mice (Figure 3). Dec1 has been reported to induce the expression of an anti-apoptotic protein, survivin, in certain types of cells (41); however, we failed to show induction of survivin by TGF-β in JygMC(A) cells. Mechanisms of Dec1 induction of cell survival in JygMC(A) cells should be examined in the future.

We also found that inhibiting endogenous TGF-β signaling by a TβRI inhibitor, SB431542, induces the expression of the BH3-only protein, Bim (also known as Bcl2-like 11), in JygMC(A) and stimulates apoptosis in these cells (42). We showed that suppression of
Bim expression by TGF-β is mediated by repression of a FOX family transcription factor, Foxc1, in JygMC(A) cells, thus suggesting an important role of the TGF-β–Foxc1–Bim axis in the survival of certain types of cells. Further studies are needed to determine whether the TGF-β–Foxc1–Bim axis is involved in lung and liver metastases of this type of cancer (Figure 3).

TGF-β also plays critical roles in bone metastasis, during which functional interaction between cancer cells and the bone microenvironment is important. In-vivo experimental models using intracardiac injection of cancer cells have been widely used to study the mechanisms of bone metastasis. Several studies revealed that TGF-β and its target molecules, such as parathyroid hormone-related protein (PTHrP) and interleukin-11 (IL-11), play critical roles in the development of bone metastasis of breast cancers (29,43), which occurs in a Smad-dependent fashion (44). PTHrP stimulates the expression of the RANK ligand (RANKL) in osteoblasts and induces differentiation of osteoclast precursors and resorption of bone. We studied the effects of a TβRI inhibitor, Ki26894, on bone metastasis in the human breast cancer cell line MDA-MB-231–5a-D (MDA-231-D), which is a highly metastatic variant of MDA-MB-231 cells. Ki26894 suppressed induction of PTHrP and IL-11 mRNA in MDA-231-D cells stimulated by TGF-β (45). When MDA-231-D cells were injected into the left ventricle of nude mice and treated with systemic administration of Ki26894 (treatment with Ki26894 was started 1 day before tumor cell inoculation), X-ray radiography showed that treatment with Ki26894 decreased bone metastasis of breast cancer cells and prolonged the survival of MDA-231-D-bearing mice compared to vehicle treatment. These findings suggest that inhibition of TGF-β signaling may be useful for preventing bone metastasis of advanced breast cancers.

TGF-β maintains stemness of certain cancer-initiating cells

Cancer-initiating cells show increased tumor-initiating ability and often exhibit stem cell-like properties such as self-renewal, multipotency, and expression of specific stem cell markers. The concept of cancer-initiating cells reveals a new strategy of therapy against intractable cancers, though it remains unclear how cancer-initiating cells can be specifically eradicated. It is important to investigate the differences between cancer-initiating cells and normal stem cells and to identify specific molecules to target cancer-initiating cells without affecting the function of normal stem cells. Recent studies have also revealed critical roles of TGF-β signaling in the maintenance of stem cell-like properties of certain cancer-initiating cells, including glioma-initiating cells (GICs) (46,47), breast cancer-initiating cells (48), and leukemia-initiating cells in chronic myeloid leukemia (CML) (49).

Glioma cells produce TGF-β1 and TGF-β2, and autocrine TGF-β signaling plays a pivotal role in maintaining the stem cell-like properties and tumorigenic activity of GICs (46,47). GICs obtained from patients with glioblastoma multiforme exhibit sphere-forming ability in a self-renewal medium containing EGF and FGF-2. Although TGF-β did not significantly affect the sphere-forming ability of GICs, a TβRI inhibitor, SB431542, efficiently reduced this ability in GICs. Moreover, SB431542 dramatically reduced the number of CD133-expressing cells and induced differentiation of GICs, leading to the appearance of cells expressing neural or glial cell markers. Analyses of TGF-β target genes using quantitative RT-PCR and by searching public datasets showed that TGF-β induces expression of the Sry-related HMG box (Sox) transcription factors Sox2 and Sox4. We showed that Sox4 is a direct target of Smad proteins activated by TGF-β and that it induces the expression of Sox2, which plays a critical role in the maintenance of GIC stemness. We also confirmed that in intracranial transplantation assays using immunocompromised mice, GICs pre-treated with SB431542 showed decreased lethal potency. These results indicate that the TGF-β–Sox4–Sox2 pathway is essential for retaining the stemness of GICs, and inhibition of TGF-β signaling may be a potential method for treating glioma through targeting GICs (Figure 4).

Westerman and his colleagues (50) reported that another Sox family protein, Sox21, is expressed in glioma cells. Sox21 is an antagonizing partner of Sox2 and negatively regulates the expression of Sox2 in glioma cells. They showed that reduction in Sox2 expression using Sox2 siRNA or Sox21 over-expression reduced the cell number by inducing apoptosis.

In addition to the Sox4–Sox2 pathway, TGF-β also induces the expression of leukemia inhibitory factor (LIF) in a Smad-dependent fashion. LIF activates the downstream JAK-STAT pathway, leading to increased tumorigenesis of GICs (47). Anido et al. have shown that TGF-β inhibitors target GICs with high levels of CD44 and Id1 and that CD44^hi/Id1^hi GICs are generally localized in the perivascular niche (51).

Conclusion and perspectives

As proposed by Roberts and Wakefield (2), it is now well known that TGF-β exhibits both positive and
negative effects on cancer progression. The bidirectional roles of TGF-β can be observed at the molecular, cellular, and tissue levels. Although we described the positive effects of TGF-β on maintaining the stemness of cancer-initiating cells, TGF-β has also been shown to decrease the number of specific types of cancer-initiating cells, including diffuse-type gastric carcinoma cells (52). Moreover, TGF-β induces maintenance of stem cell-like properties of certain breast cancer-initiating cells (48), while suppression of the TGF-β pathway leads to an increase in breast cancer-initiating cells in other types of breast cancer cells (53), thereby suggesting that the response to TGF-β varies depending on the type of cancer-initiating cells.

Mani et al. (48) reported that TGF-β maintains stem cell-like properties of certain cancer-initiating cells through induction of EMT. They showed that normal and transformed mammary epithelial cells acquired stem cell-like properties with high tumorigenic activity when EMT was induced in these cells by TGF-β. Although we have not determined whether the sizes of cancer-initiating cell compartments are affected by EMT in the pancreatic carcinoma Panc1 cells and lung adenocarcinoma A549 cells described above, these findings suggest a functional connection between EMT and cancer-initiating properties of certain epithelial cells.

Recent findings based on genome-wide analyses of Smad-binding sites in some types of cells, which were performed using ChIP-sequencing analyses, revealed that the binding profiles of Smads differ remarkably depending on the cell types and are affected by interaction with transcription factors expressed in each cell type and by cell-specific differences in baseline chromatin accessibility patterns (7,9,54). It is thus possible that the response of cells to TGF-β may be differentially affected by coexisting transcription factors and chromatin assembly patterns. Further studies examining global gene expression profiles and genome-wide maps of protein binding sites or epigenetic marks using high-throughput sequencing may be valuable for elucidating the mechanisms of differential cellular responsiveness to TGF-β.

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