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Review

Review

Paradoxical roles of TGF-β signaling in suppressing and promoting squamous cell carcinoma

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Abstract

Transforming growth factor β (TGF-β) signaling either promotes or inhibits tumor formation and/or progression of many cancer types including squamous cell carcinoma (SCC). Canonical TGF-β signaling is mediated by a number of downstream proteins including Smad family proteins. Alterations in either TGF-β or Smad signaling can impact cancer. For instance, defects in TGF-β type I and type II receptors (TGF-βRI and TGF-βRII) and in Smad2/3/4 could promote tumor development. Conversely, increased TGF-β1 and activated TGF-βRI and Smad3 have all been shown to have tumor-promoting effects in experimental systems of human and mouse SCCs. Among TGF-β/Smad signaling, only TGF-βRII or Smad4 deletion in mouse epithelium causes spontaneous SCC in the mouse model, highlighting the critical roles of TGF-βRII and Smad4 in tumor suppression. Herein, we review the dual roles of the TGF-β/Smad signaling pathway and related mechanisms in SCC, highlighting the potential benefits and challenges of TGF-β/Smad-targeted therapies.

Key words: TGF-β signaling, Smad proteins, SCC, tumor suppression, tumor promotion, therapeutic targets

Introduction

In mammals, transforming growth factor β (TGF-β) signaling has been extensively studied and is known to impact diverse cellular processes including differentiation, proliferation, migration, extracellular matrix remodeling, and apoptosis, all of which could be involved in various biological events including embryogenesis, immunity regulation, fibrosis, wound healing and tumor progression [1]. Typically, TGF-β ligands bind to TGF-β type II receptor (TGF-βRII), which can phosphorylate TGF-β type I receptor (TGF-βRI). The binding of TGF-βRII and TGF-βRI propagates signaling by phosphorylating cytoplasm mediators, Smad2 and Smad3, which then complex with Smad4 and translocate into the nucleus. In the nucleus, the phosphorylated Smad2/3–Smad4 complex binds to specific DNA sequence known as Smad binding elements (SBE), subsequently regulating transcription of TGF-β target genes [2] (**Fig. 1A**).

The functions of the TGF-β signaling pathway in cancer suppression and progression have also been extensively studied. Studies regarding the suppressive role of TGF-β signaling in cancers suggest that it could inhibit tumor formation mainly through inhibition of proliferation and by inducing growth arrest and apoptosis [3,4]. However, TGF-β also acts as a potent inducer of angiogenesis, inflammation, epithelial–mesenchymal transition (EMT) and immune suppression thereby promoting tumor progression and metastasis. Furthermore, depletions or mutations in genes encoding TGF-β receptors and Smads can cause spontaneous tumor development in mouse models and correlate to poor survival in human cancer [5,6]. However, novel molecular targets of TGF-β signaling that mediate tumor suppression and promotion effects, especially the ones that may serve as druggable targets, still remain to be identified.

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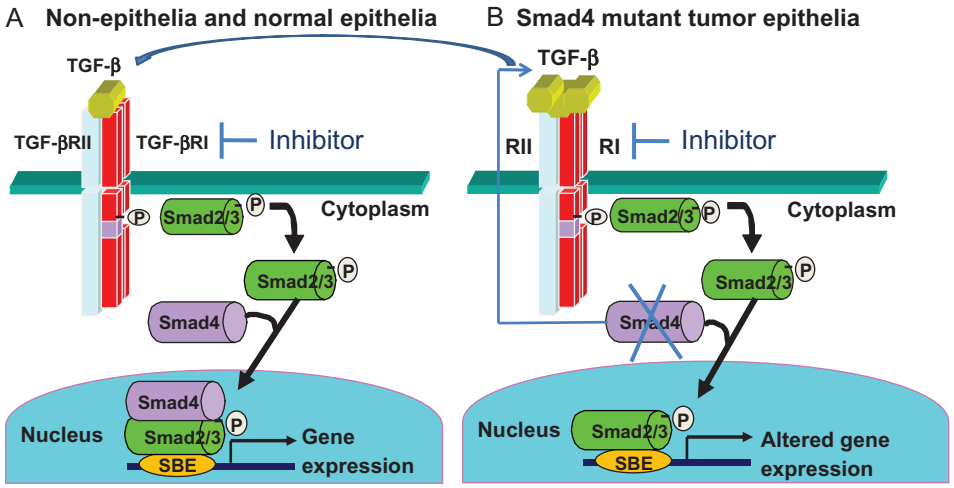

Figure 1. Normal and altered Smad4 signaling (A) Canonical TGF-β signaling in normal cells or stromal cells. (B) Smad4 loss in tumor epithelia causes compensatory TGF-β overproduction that signals through Smad2/3 in tumor cells and paracrine TGF-β signaling in stromal cells through Smad2/3/4. TGF-β signaling can be blocked by a TGF-βRI inhibitor.

Squamous cell carcinomas (SCC), as one of the most common cancers, mainly derives from stratified squamous epithelial cells in the upper digestive track and skin, causing more than 1 million deaths each year worldwide [7]. This review will focus on the paradoxical roles of TGF-β/Smad signaling in SCC in an effort to highlight the consequences of the signaling and how a better understanding of these outcomes may be utilized to design more targeted therapeutic approaches for patients with SCC.

TGF-β1 and Its Roles in SCC

TGF-β1 expression in SCC

TGF-β1 is a potent inhibitor of epithelial proliferation. Hence, it was unexpected when Akhurst *et al.* identified that TGF-β1 was overexpressed in chemical carcinogen-induced mouse skin SCCs [8]. Further studies from her laboratory have shown that TGF-β1 exhibits biphasic actions in murine skin SCC: suppressing the benign tumor growth but enhancing malignant conversion [9]. By creating a transgenic mouse model in which TGF-β1 can be inducibly expressed at discrete stages of skin carcinogenesis, we have further defined that the tumor suppression and promotion effects are stage-specific: inducing TGF-β1 overexpression prior to tumor formation suppresses benign papilloma formation [10], whereas inducing TGF-β1 overexpression after benign tumor formation promotes malignant transformation and metastasis [11]. We have also found that in human head and neck SCCs (HNSCCs) and skin SCCs, TGF-β1 is also overexpressed in ~78% and 52.9% of specimens, respectively [12,13]. Similarly, other laboratories have also identified elevated expression of TGF-β1 in esophageal SCC (ESCC) (**Table 1**). However, Logullo *et al.* argued that increased TGF-β1 expression exhibited no significant correlation with clinicopathological parameters in HNSCC [14]. Given the stage-specific effects of TGF-β1 found in experimental models described above, attempts of using TGF-β1 as a prognostic marker would need careful considerations for the stages of cancer samples being examined.

Autocrine and paracrine effects of TGF-β1 and the tumor suppressive roles/mechanisms in SCC

Studies described above have revealed that the functions of TGF-β1 largely depend on tumor stage; it predominantly acts as a tumor suppressor during the early stage of tumorigenesis, while exerts a promotive role at the late stages of tumor development. During early tumorigenesis, components of TGF-β signaling pathway such as TGF-βRII, Smad2 and Smad4 have not yet become depleted or mutated; thus, endogenous TGF-β1 overexpression exerts a growth inhibitory effect. In epithelial cells, after the secretion and activation of endogenous TGF-β1 ligands, the ligands suppressed tumor development by inducing cell cycle inhibitory genes including $p15^{InK4b}$ and $p21^{Waf1/Cip1}$. Furthermore, the TGF-β1 ligands downregulate c-Myc expression, and this downregulation is involved in proliferation inhibition [15,16]. Similarly, the increased TGF-β1 induced by inhibition of ANRIL (CDKN2B-AS1), a 3.8-kb long noncoding RNA, augments $p15^{InK4b}$ expression, thus inhibiting cellular proliferation in ESCC cell lines [17]. Finally, in a tongue SCC study, endogenous TGF-β1 slightly upregulated Smad4-induced p21 expression and delayed matrix metalloproteinase-2 (MMP-2) expression to promote apoptosis and inhibit proliferation of tumor cells [18]. However, we have shown that TGF-β1-induced inflammation in mouse oral mucosa overrides TGF-β1-mediated growth arrest [12]. Therefore, growth inhibition could not solely explain tumor suppressive effects of TGF-β1 in SCCs. Glick *et al.* have identified a critical role for TGF-β1 in DNA damage repair [19], which could be critical to prevent cancer formation at early stages.

Autocrine and paracrine effects of TGF-β1 and the tumor-promoting roles/mechanisms in SCC

We have shown that the non-malignant tissue adjacent to human HNSCC or skin SCC exhibits TGF-β1 overexpression [12,13]. To understand the role of TGF-β1 in this early stage of SCC carcinogenesis, we have generated transgenic mice in which TGF-β1 is overexpressed in keratinocytes of the skin or oral cavity [12,20]. TGF-β1 overexpression elicited profound inflammation in the skin and oral cavity, including the increased secretions of inflammatory cytokines including IL-1, IL-6, and IL-8 [20]. We have found that dramatic epithelial hyperplasia and increased expression of IL-1β, tumor necrosis factor α, and NF-κB are all present in TGF-β1-transgenic stroma and epithelium [12]. Our data also suggest that through paracrine signaling to endothelial cells, TGF-β1 transgene induction resulted in angiogenesis through upregulated expression of ALK1/pSmad1/5/8 [12]. Increased inflammation and angiogenesis in turn

Table 1. Expression of TGF-β/Smad signaling components in human SCC

		Skin SCC		Oral SCC		Esophageal SCC		References
		N/T	%	N/T	%	N/T	%	
TGF-β1	Up	18/34	52.9%	29/79	36.7%	110/258	42.6%	[13,14,25,55]
						29/80	36.3%	
TGF-βRI	Up	49/61	80.3%	0/68	0%			[29,30]
	Down					43/80	53.8%	[25]
TGF-βRII	Down	19/34	55.9%	36/68	52.9%	23/80	28.8%	[13,25,30,53]
				71/108	65.7%			
Smad2	Up			19/48	39.6%			[48]
	Down	58/83	69.9%			7/80	8.8%	[40,47]
Smad3	Up			19/48	39.6%			[48]
	Down	4/83	4.8%			2/80	2.5%	[40,47]
Smad4	Down	58/83	69.9%	66/108	61.1%	175/258	67.8%	[40,53,55]

N/T: number of positive cases in total cases; Up: overexpression in mRNA or protein level; Down: decreased or loss in mRNA, protein or genetic level.

increase keratinocyte proliferation [20]. These mice never develop spontaneous SCC, suggesting that angiogenesis, profound inflammation and associated epithelial proliferation are insufficient for SCC initiation. However, these TGF-β1 effects play an important role in SCC progression [11]. We have also found that TGF-β1-induced EMT plays a critical role in early onset SCC metastasis [11]. Additionally, endogenous TGF-β1 secreted by stromal cells can also facilitate tumor progression in SCC. For instance, TGF-β1 secreted by cancer-associated fibroblasts (CAFs) increased matrix stiffness through activation of Yap1 and MMPs, subsequently facilitating invasion in OSCC [21,22].

In sum, the studies of molecular mechanisms described above illustrate that TGF-β1 plays dual roles in facilitating tumor inhibition and promoting tumor progression.

TGF-βRI and Its Roles in SCC

Although TGF-βRI mutation has been detected in ~19% of HNSCC patients with metastasis [23], its mutation or loss is quite rare in all cases of human HNSCC overall [24] (**Table 1**). However, in a study of human ESCC, ~53.8% of patients exhibited reduced expression of TGF-βRI, and this reduced expression correlated to depth of invasion, metastasis, and pathological stage [25] (**Table 1**), illustrating that TGF-βRI could play a suppressive role in SCC.

To understand the suppressive function of TGF-βRI in tumors, Yasuyuki *et al.* have generated a mouse model with conditional knockout of TGF-βRI in neurons by neurofilament (NF-H) Cre and found that ~35% TGF-βRI-knockout mice developed SCC in the periorbital or perianal regions 6 months after birth [26]. However, these spontaneous SCCs were negative for the neuronal marker (neuron specific enolase) and did not harbor TGF-βRI deletion, suggesting the SCCs were not derived from TGF-βRI null neural cells [26]. Instead, it is likely the SCCs were derived from TGF-βRI wild type skin epithelial cells as the result of crosstalk between TGF-βRI knockout neurons and epithelial stem cells [26]. In their study, 33% of SCCs from TGF-βRI-knockout mice exhibited IL-13Rα2 and its expression might be involved in the tumorigenesis of SCC, probably enhancing paracrine effects of TGF-β in escaping from immunosurveillance [26]. Another study also showed that TGF-βRI depletion in head and neck epithelia alone is insufficient to initiate spontaneous HNSCC development but accelerates carcinogen 7,12-dimethybenz (a)anthracene (DMBA) initiated SCC in mice [27]. Interestingly, Goudie *et al.* have found that TGF-βRI mutation causes multiple self-healing squamous epithelioma, an autosomal dominant skin cancer characterized by spontaneous regression [28]. Together, these data indicate that abrogating tumor suppressive effects of TGF-βRI can contribute to the development of both malignant SCC as well as benign squamous tumors.

On the other hand, TGF-βRI can also be overexpressed beyond a physiological level. For example, 80.3% of patients with skin SCC exhibited overexpressed TGF-βRI [29] (**Table 1**), and continuous expression of TGF-βRI correlated with high pSmad2/3 in skin SCC compared to the surrounding epidermis with the strongest TGF-βRI expression in tumors on sun-exposed skin [29]. However, to date, there is not an experimental model to assess the role of TGF-βRI overexpression in SCC.

TGF-βRII and Its Roles in SCC

The decreased expression and inhibitory roles of TGF-βRII in human SCC have been identified (**Table 1**). We have found that decreased or lost expression of TGF-βRII occurs in 35.3% of human OSCC on the protein level and in more than 70% of human HNSCC by mRNA levels [30,31]. The fact that TGF-βRII reduction occurs only in HNSCCs but not in adjacent mucosa suggests that loss of TGF-βRII is a relatively late-stage event of SCC carcinogenesis. In late-stage OSCC, the E221V/N238I mutation of TGF-βRII enhanced TGF-β signaling and delayed the internalization of TGF-βRII, subsequently leading to more invasive phenotypic changes [32]. To define the role of TGF-βRII in SCC, several mouse models with keratinocyte-specific TGF-βRII deletion have been established. In oral keratinocytes with TGF-βRII deletion, we have found no spontaneous tumor formation in mice, which is consistent with TGF-βRII loss in human HNSCCs but not in early lesions [31]. These data suggest that TGF-βRII loss in oral keratinocyte is not an initiation event. Indeed, when we introduced a Kras or Hras mutation in this model as an SCC initiation event, these mice developed HNSCC [31]. This model represents the first genetically engineered mouse model with full penetrance of HNSCC. Of note, when TGF-βRII is deleted in mouse airway epithelial cells, it causes increased size and number of Kras-initiated lung SCC [33]. Contrary to our findings, Guasch *et al.* have shown that K14-Cre/TGF-βRII$^{-/-}$ mice could develop spontaneous anal and genital SCC derived from the transition zone between mucosal epithelium of large intestine and stratified squamous epithelium of anal skin [34,35], suggesting that such transition zones are uniquely susceptible to tumorigenesis in

contrast to the more refractory oral and skin epithelium. These data illustrate that TGF-βRII loss can be a tumor-initiating event but exhibits tissue specificity. All these studies support the notion that TGF-βRII loss promotes SCC progression *in vivo*, and can also act as tumor initiator with tissue and temporal specificity.

Mechanistically, we showed that TGF-βRII depletion could increase the expression of endogenous TGF-β1 secreted by both the epithelia and stroma, though the increase was more significant in the stroma [31]. Consequently, the resultant overexpression of TGF-β1 may increase angiogenesis and inflammation, subsequently promoting tumor progression in HNSCC [31]. Similarly, a mouse model with TGF-βRII knockdown in airway epithelia also exhibited increased TGF-β1 ligand expression, enhancing lung tumor development through increased proliferation and local inflammation but without increasing angiogenesis [33]. From these studies, it is clear that TGF-βRII deletion in keratinocytes causes inflammation during tumor progression. To further examine its role in inflammation, Cohen *et al.* have developed an oral-specific TGF-βRII-mutant model, and have shown that mutant TP53 might serve as an upstream repressor of TGF-βRII expression and TGF-βRII depletion in tumor epithelial cells results in activated NF-κB1/RelA (p50/p65)[36]. Conversely, we have shown that TGF-β1 overexpression activates NF-κB [20], and increased NF-κB activation in TGF-βRII depletion tumor cells could be via a mechanism independent of TGF-β signaling. Additionally, TGF-βRII deletion increases tumor cell migration and invasion in human bronchial epithelial cell line [33]. Further supporting the suppressive role of TGF-βRII in SCC, and confirmed by both *in vitro* and *in vivo* studies, TGF-βRII ablation in epidermal keratinocytes, in coordination with oncogenic mutations in Hras, promotes hyperproliferation and maintains low apoptosis, thereby leading to destabilized homeostasis and tumorigenesis in stratified epithelium [35]. In addition, TGF-βRII deficiency also enhances cell migration and invasion, mainly through integrin-FAK-Src signaling [35]. Furthermore, tumor-initiating stem cells or cancer stem cells (CSCs) from the anal canal and rectum transition zone with TGF-βRII loss enhance tumor cell invasion and metastasis in SCC through de-repression of ELMO1, a RAC-activating guanine exchange factor specifically located in CSCs of anorectal SCC [34]. Taken together, these data demonstrate that angiogenesis, inflammation, proliferation, apoptosis and tumor cell migration and/or invasion can be involved in TGF-βRII-deficient SCC initiation or progression.

Smad2 and Its Roles in SCC

Smad2 is located on chromosome 18q21, near the Smad4 site in the human genome [37]. Smad2 point mutations are infrequent in human primary HNSCC and HNSCC cell lines; only one study reported a Smad2 mutant HNSCC cell line [38,39]. However, we have shown that ~67% of poorly differentiated skin SCCs exhibit loss of heterozygosity (LOH) at the Smad2 locus [40]. Similarly, in another study, Smad2 LOH was detected in 63% of HNSCC cell lines [41]. Further, 94% and 70% of poorly differentiated human skin SCCs had a Smad2 reduction in mRNA and protein levels, respectively [40] (**Table 1**). These studies suggest that Smad2 LOH is a common event in pre-transcriptional, transcriptional, and post-transcriptional levels during the SCC progression. With regard to the correlation between decreased or loss of Smad2 and clinical tumor behavior, Smad2 protein loss was most common in poorly differentiated human HNSCC [39]. Intriguingly, in a study related to posttranscriptional regulation of Smad2, epigenetically decreased disabled homolog 2 (DAB2) in SCC cell lines inhibits Smad2 phosphorylation and its activation, thereby promoting tumor progression [42]. Conversely, re-expression of DAB2 in SCC cell lines with DAB2-downregulation results in renewed growth prohibitive responses to TGF-β [42]. Taken together, these two studies suggest that DAB2 loss could act as a switch to transition TGF-β pathway signaling from tumor suppressive to promoting, and the critical protein for this transition may be Smad2.

To better understand the role of Smad2 loss in stratified epithelia *in vivo*, we have created a model with inducible and keratinocyte-specific Smad2-knockout mice driven by a keratin-5 promoter (K5.Smad2$^{-/-}$) [40,43]. Neither the homozygous (K5.Smad2$^{-/-}$) nor heterozygous (K5.Smad2$^{+/-}$) loss mice developed spontaneous skin tumors [40], thus Smad2 loss alone is not sufficient for tumor initiation. However, both K5.Smad2$^{-/-}$ and K5.Smad2$^{+/-}$ mice exhibited accelerated tumor formation and malignant conversion when subject to a two-stage chemical skin carcinogen exposure compared to wild type mice [40,44], indicating that Smad2 loss promotes susceptibility to skin tumorigenesis and promotes malignant progression.

Smad2 loss-associated EMT and angiogenesis are the two main processes contributing to tumor progression in SCC. We have found that Smad2 loss recruits Smad4 binding to the SBE of Snail, subsequently leading to Snail expression and contributing to the loss of E-cadherin [40]. Additionally, skin SCC with Smad2 ablation increased the expression of hepatocyte growth factor (HGF), a potent angiogenic factor and a promoter for tumor epithelial cell migration, resulting in activation of the HGF receptor, c-Met, on the endothelial cells [40,44]. These data suggest that EMT and angiogenesis induced by Smad2 loss contribute to SCC susceptibility and progression.

Smad3 and Its Roles in SCC

Smad3 expression in SCC

In human SCCs, Smad3 missense mutations are at a low frequency in HNSCC [13] and Smad3 loss or reduction in protein level is also uncommon (0%–4.8%) in HNSCC, skin SCC or ESCC [40,45,46,47] (**Table 1**). Increased Smad3 expression at the mRNA level, however, has been reported in 39.6% of OSCCs [48].

Tumor suppressive roles/mechanisms of Smad3 in SCC

In a mouse model with conditional Smad3 knockdown, Bae *et al.* found that v-Ras^{Ha}-transduced Smad3$^{-/-}$ keratinocytes developed SCC, while v-Ras^{Ha}-transduced Smad3$^{+/+}$ keratinocytes only exhibited papillomas [49]. Similarly, in another study, Vijgayachandra *et al.* grafted the primary keratinocytes with v-Ras^{Ha}-transduced Smad3 loss onto nude mice and found that 50% of the Smad3$^{-/-}$ grafts underwent malignant conversion, while 85.7% of the Smad3$^{+/+}$ ones exhibited benign papillomas [50]. These data indicate that expression of Smad3 can suppress SCC carcinogenesis. Indeed, Smad3 expression can abrogate tumor progression through inducing senescence and regulating inflammation. For instance, overexpression of Smad3 in v-Ras^{Ha}-transduced keratinocytes increased senescent cells and S phase cells [50].

Tumor promotion roles/mechanisms of Smad3 in SCC

In contradiction to the above v-Ras^{Ha}-transduced Smad3$^{-/-}$ spontaneous tumor data [49,50], in our study, neither Smad3$^{+/-}$ nor Smad3$^{-/-}$

mice developed spontaneous skin tumors [46]. Surprisingly, Smad3$^{-/-}$ mice have attenuated inflammation and fewer tumor associated macrophages but increased apoptosis [46]. Furthermore, we have found that more than 90% of Smad3$^{+/+}$ papilloma cells were positive for NF-κB, while only ~50% or less were identified in Smad3$^{+/-}$ and Smad3$^{-/-}$ mice respectively [46], suggesting that inducing a pro-inflammatory response is one of the potential mechanisms of Smad3 acting as a tumor promotor in SCC. In addition to alterations in NF-κB, Smad3 can function in other capacities to promote tumor progression by antagonizing the tumor suppressive roles of TGF-β1. For instance, Smad3 knockdown blocks the ability of TGF-β1 to induce either MMP-9 or *uPA* gene expression, consequently inhibiting tumor invasion and metastasis [49]. Additionally, Park *et al.* have found that death-associated protein kinase-related apoptosis-inducing kinase 1 (DRAK1) inhibits TGF-β1 tumor suppressor activity by binding Smad3 in HNSCC, consequently blocking Smad3–Smad4 complex formation [16]. These studies demonstrate that Smad3 might mediate tumor promotion in SCC.

Smad4 and Its Roles in SCC

Smad4 is localized to 18q21–22 chromosome, near the Smad2 locus. Notably, chromosome 18q LOH is a common event in HNSCC and it occurs in 56% of the primary and secondary HNSCC cell lines [5,51,52]. Furthermore, we have also found that 86% of tumors and 67% of adjacent non-malignant mucosa show more than 50% reduction of Smad4 mRNA expression in HNSCC [5], suggesting that Smad4 reduction is an early event during the HNSCC progression. However, by immunostaining, studies of Smad4 loss at a protein level vary significantly from as low as 12% to as high as 61.12% in HNSCC [53,54] (**Table 1**). These differences might have been caused by different tumor locations, different methods in measurement or even different races. In accordance, by immunostaining, 51.2% of the patients with ESCC exhibited Smad4 loss and 67.8% had a Smad4 reduction at protein level [55,56]. In both human HNSCC and ESCC, attenuated Smad4 is associated with more advanced tumor characteristics including invasion and poor prognosis [53,55–57]. Taken together, these observations reveal that Smad4 loss or reduction is a common event even in the early stage of SCC and Smad4 mainly plays a suppressive role in SCC progression. In fact, Smad4 ablation alone causes spontaneous SCC in the skin, oral cavity and stomach of mice [5,58–60]. Intriguingly, Smad4/Dpc4 conditional knockout in mouse mammary glands causes SCC development, representing a trans-differentiation of tumor type [61].

There are a variety of potential mechanisms related to carcinogenesis induced by Smad4 loss in SCC (**Fig. 1B**). For example, we have shown that Smad4 loss increases cell proliferation and reduces apoptosis in Smad4$^{-/-}$ mucosa and Smad4$^{-/-}$ SCC when compared to Smad$^{+/+}$ mucosa [11]. These alterations effectively abrogate the early tumor suppression induced by TGF-β via relieving the TGF-β-mediated growth arrest. However, these changes do not explain why Smad4 depletion alone is an initiation event for SCC, as similar changes were found in our study on TGF-βRII ablation which required combination with Kras or Hras mutation for HNSCC tumorigenesis [31]. Intriguingly, we have identified that Smad4 loss downregulates the expression of Brca/Fanc (Breast cancer susceptibility/Fanconi anemia complementation) genes, which are critical for double-stranded DNA repair [5]. Furthermore, decreased expression of Brca/Fanc induced by Smad4 loss is essential for accumulation of DNA damage to initiate SCC formation [5]. In support of this, clinical data indicate that Fanconi anemia patients with Brca/Fanc mutations have markedly increased susceptibility to HNSCC compared to general population [62]. Interestingly, Brca1 depletion leads to development of SCC in the skin, the inner ear canal and the oral epithelium [63,64]. In addition, Smad4 loss could increase the overexpression of TGF-β1 and activate Smad3, subsequently leading to inflammation [5]. Intriguingly, leukocyte infiltration in Smad4$^{-/-}$ tissue was decreased significantly when these mice were bred into the Smad3$^{+/-}$ background [5]. These results suggest that Smad3 contributes to TGF-β1-associated inflammation during abrogation of Smad4 in SCC. A study using human HNSCC cells showed that Smad4 downregulation induces EMT while enhancing cetuximab resistance in HNSCC [65]. Similarly, we have shown that when Smad4 deletion is targeted to K15^{+} stem cells, SCCs have a high incidence of EMT [66]. Conversely, studies have also shown that Smad4 is required for TGF-β-mediated EMT [40,67]. Therefore, EMT in HNSCCs with low Smad4 may be an indirect effect of Smad4 loss or independent of TGF-β signaling. To this end, Ozawa *et al.* have found that activated JNK and MAPK pathways contribute to cetuximab resistance in human HNSCC cell lines with Smad4 loss [68]. Importantly, the use of JNK and MAPK inhibitors sensitized the Smad4-loss HNSCC cell lines to cetuximab [68]. Lastly, changes in Smad4 can impact the surrounding stromal microenvironment. For example, Smad4 abrogation in the oral mucosa increases infiltration of macrophages, granulocytes and T lymphocytes in the stroma adjacent to Smad4$^{-/-}$ mucosa and the tumor stroma of Smad4$^{-/-}$ SCC, consequently increasing inflammation [5]. Furthermore, SCCs with Smad4 loss escape CD8^{+} T cell-mediated immune surveillance by activation and exhaustion of CD8^{+} T cells with co-expression of programmed cell death-1 (PD-1) and lymphocyte activation gene-3 (LAG-3), and dual inhibition of PD-1 and LAG-3 on CD8^{+} T cells suppresses tumor growth in SCC with Smad4 loss [69]. In sum, these data demonstrate that Smad4 loss in the epithelium promotes tumor formation through its direct effects in the epithelium and indirect effects in the stroma.

Potential Strategies for Targeting TGF-β/Smad Signaling in Cancer Therapy

Considering the paradoxical roles of TGF-β/Smad signaling pathway in tumor suppression and promotion of SCC, careful considerations are warranted in the development of cancer therapies targeting TGF-β signaling. Many therapeutic agents including neutralizing antibodies, antisense oligonucleotides (ASOs), and receptor kinase inhibitors that block TGF-β/Smad signaling have already been developed for suppressing tumor progression after tumors have lost early TGF-β-mediated tumor suppression. For example, in mouse models, two neutralizing antibodies, 2G7 and 1D11, can bind all three TGF-β isoforms and reduce their biological activity in tumors [70,71]. Another strategy to reduce TGF-β ligand synthesis is achieved by ASOs. ASOs are designed to hybridize to TGF-β isoforms' complementary sequence in RNA and increase the mRNA degradation [72]. ASOs to TGF-β1 and TGF-β2 have already been investigated as an approach for cancer therapy. Clinically, the suppression of TGF-β2 production by ASOs (AP12009) has been employed in clinical trials for glioblastoma and astrocytoma [73]. Although ASOs inhibit the activities of TGF-β ligands, they are unable to block receptor signaling directly, suggesting that inhibiting receptors may be more effective at tumor suppression. For instance, unlike ASOs, miR-211 and miR-17/20a can bind to TGF-βRII directly, thereby attenuating the phosphorylation

of Smad2 or Smad3 and promoting SCC progression [74,75]. In addition, TGF-βRI/ALK5 inhibitors such as Ki26894 and LY364937 block TGF-β signaling in pancreatic, hepatocellular cancers and glioblastoma [72,76]. Finally, in skin SCC, LY2109761, another ALK5 inhibitor, exhibited tumor suppression through reducing carcinoma myofibroblasts and disrupting vascular integrity [77]. Due to the early tumor suppressive actions of TGF-β signaling, TGF-β inhibitor clinical trials are all at the late-stage/metastasis setting. To date, it is unknown if treatment regimens of TGF-β inhibitor are effective in SCC, as no such a clinical trial exist to date in SCCs. For clinical trial designs, any therapeutic strategies developed to exploit the dual roles of TGF-β1, TGF-βRI and Smads should be aware of the temporal transition from tumor suppressor to promotor to optimize treatment efficacy.

Conclusion

The TGF-β/Smad signaling pathway exhibits paradoxical roles by exhibiting both tumor-suppressing and tumor-promoting functions. TGF-β1 and TGF-βRI are identified as tumor suppressors during the early stage of tumorigenesis, while they exert promotive roles in later stages. Smad2, TGF-βRII, and Smad4 mainly act as tumor suppressors in SCC. However, these TGF-β signaling components are also required for tumor-promoting effects of TGF-β signaling. Only TGF-βRII or Smad4 deletion in the epithelium could develop spontaneous SCC in mouse models, indicating that TGF-βRII and Smad4 play a key role in the suppression of SCC. Any therapeutic strategies designed to inhibit the tumor-promoting role of TGF-β, TGF-βRI, TGF-βRII, and Smads should focus on, or be aware of, the mechanism and timing of the switch from tumor suppressor to promoter. Notably, efforts in drug development of TGF-β inhibitors are now gearing towards selectively blocking the tumor-promoting effects of TGF-β/Smad signaling, while avoiding toxicity.

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