Progressive Tumor Formation in Mice with Conditional Deletion of TGF-β Signaling in Head and Neck Epithelia Is Associated with Activation of the PI3K/Akt Pathway

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Abstract
The precise role of transforming growth factor (TGF)-β signaling in head and neck squamous cell carcinoma (SCC) is not yet fully understood. Here, we report generation of an inducible head- and neck-specific knockout mouse model by crossing TGF-β receptor I (Tgfr1) floxed mice with K14-CreER<sup>CreLox</sup> mice. By applying tamoxifen to oral cavity of the mouse to induce Cre expression, we were able to conditionally delete Tgfr1 in the mouse head and neck epithelia. On tumor induction with 7,12-dimethylbenz(a)anthracene (DMBA), 45% of Tgfr1 conditional knockout (cKO) mice (n = 42) developed SCCs in the head and neck area starting from 16 weeks after treatment. However, no tumors were observed in the control littermates. A molecular analysis revealed an enhanced proliferation and loss of apoptosis in the basal layer of the head and neck epithelia of Tgfr1 cKO mice 4 weeks after tamoxifen and DMBA treatment. The most notable finding of our study is that the phosphoinositide 3-kinase (PI3K)/Akt pathway was activated in SCCs that developed in the Tgfr1 cKO mice on inactivation of TGF-β signaling through Smad2/3 and DMBA treatment. These observations suggest that activation of Smad-independent pathways may contribute cooperatively with inactivation of Smad-dependent pathways to promote head and neck carcinogenesis in these mice. Our results revealed the critical role of the TGF-β signaling pathway and its cross-talk with the PI3K/Akt pathway in suppressing head and neck carcinogenesis. [Cancer Res 2009;69(14):5918–26]

Introduction
Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of human cancer (1). Tobacco, alcohol consumption, and viral agents are the major risk factors for development of HNSCC. These risk factors together with genetic susceptibility result in the accumulation of multiple genetic and epigenetic alterations in a multistep process of cancer development (2). However, the underlying cellular and molecular mechanisms that contribute to the initiation and progression from normal epithelia to invasive SCC have not been clearly delineated (3).

There is accumulating evidence that suggests that the transforming growth factor (TGF)-β signal transduction pathway is involved in head and neck carcinogenesis (4, 5). TGF-β is a multifunctional cytokine with diverse biological effects on cellular processes, including cell proliferation, migration, differentiation, and apoptosis. The three mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, exert their functions through a cell surface receptor complex composed of type I (TGFBR1) and type II (TGFBR2) serine/threonine kinase receptors. Intracellular signaling is initiated once TGFBR1 has been phosphorylated by TGFBR2, which in turn phosphorylates Smad2 or Smad3. Phosphorylated Smad2 (p-Smad2) or Smad3 binds to Smad4, and then the complexes translocate from the cytoplasm into the nucleus. This results in the transcriptional activation of TGF-β-responsive genes that mediate the effects of TGF-β at the cellular level. Independent of Smad proteins, receptor activation also induces other downstream targets, including Ras, RhoA, TAK1, MEK1, phosphoinositide 3-kinase (PI3K), and PP2A, to produce the full spectrum of TGF-β responses (6–8).

The effects of TGF-β signaling in carcinogenesis largely depend on the tissue of origin and the tumor type. In most types of human cancer, TGF-β plays a paradoxical role in cancer development by acting as a tumor suppressor in early stages (9) and a tumor promoter in later stages (10, 11). In HNSCC, it is known that TGF-β functions as a potent tumor suppressor (12). However, it is not clear whether TGF-β acts in a pro-oncogenic manner in advanced late-stage HNSCC. A human oral carcinoma cell line, which contained a normal Ras but was growth inhibited by TGF-β1, led to an increase in cell migration and invasion, and metastasis when transfected with dominant-negative TGFBR2 (dn RII) cDNA (13). When TGF-β receptor II (Tgfr2) was conditionally deleted in mouse head and neck epithelia, 35% of the 7,12-dimethylbenz(a)anthracene (DMBA)–initiated Tgfr2<sup>CreLox</sup> mice developed jugular lymph node metastasis, suggesting that TGF-β may actually in fact suppress metastasis rather than promote it (14).

The correlation between TGF-β receptor–mediated signaling and cancer development has been studied extensively. However, much less attention has been paid to the role of TGFBR1 in carcinogenesis when compared with that of TGFBR2. Although several reports have noted that mutations and polymorphisms of TGFBR1 are associated with HNSCC (15–17), the precise molecular nature of TGFBR1-mediated pro-oncogenic effects is still unknown.
In the current study, we conditionally deleted Tgfbr1 in mouse head and neck epithelia using the Cre-LoxP approach to show that deletion of Tgfbr1 alone is not sufficient for spontaneous tumor formation, although it can increase the susceptibility to tumor development initiated by DMBA. The most notable finding of our study is that, in SCCs that developed in the Tgfbr1 conditional knockout (cKO) mice, the PI3K/Akt pathway, one of the most important Smad-independent receptor I signaling pathways, was clearly activated in addition to inactivation of the Smad-dependent TGF-β signaling pathway. Our studies identified the critical role of the TGFBR1-mediated signaling pathway and its cross-talk with the PI3K/Akt pathway in suppressing head and neck carcinogenesis. The Tgfbr1 cKO mouse will be a valuable animal model for studying genetic alterations and signaling pathways that play important roles in HNSCC.

Materials and Methods

**Generation of Tgfbr1 cKO mice.** The Tgfbr1 cKO mice (K14-CreER<sup>tam</sup>;Tgfbr1<sup>f/f</sup>) were generated from crosses between Tgfbr1<sup>f/f</sup> mice (mixed genetic strains of C57BL/6, 129SV/J, and FVB/N; refs. 18, 19) and K14-CreER<sup>tam</sup> mice (genetic strain CD-1; ref. 20). The Tgfbr1 cKO mice and their controls (Tgfbr1<sup>f/f</sup>, Tgfbr1<sup>f/+</sup>, and K14-CreER<sup>tam</sup>;Tgfbr1<sup>f/+</sup> mice) were from the same litter and therefore had exactly the same mixed genetic background. The treatment procedures of tamoxifen and DMBA have been described (14, 20). Additional details are provided in Supplementary Data.

**Histology, immunostaining, and bromodeoxyuridine labeling.** Immunohistochemical staining and quantifications of immunohistochemical slides were performed using a previously published method (21). Intra-tumoral microvessel density (iMVD) was determined as previously described (22). Bromodeoxyuridine (BrdUrd) labeling and primary antibodies are described in Supplementary Data.

**Western blot analysis.** Normal buccal mucosa and tongue from six pairs of Tgfbr1<sup>f/f</sup> and Tgfbr1 cKO mice, together with tumors that developed in DMBA-initiated Tgfbr1 cKO mice, were carefully dissected. A total amount of 40 µg protein from each sample was denatured and then loaded in each lane of NuPAGE 4% to 12% Bis-Tris precast gel. Additional details are provided in Supplementary Data.

**Additional methods.** Information on terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, Cre-mediated recombination assessment, quantitative real-time PCR, and flow cytometry analysis (23) is detailed in Supplementary Data.

**Statistical analysis.** Statistical differences in the levels of mRNA expression between controls and experimental samples were determined using the Student’s t test.

Results

Inducible deletion of Tgfbr1 in head and neck epithelia is not sufficient for SCC formation in mice. We generated an inducible head- and neck-specific knockout mouse model by crossing Tgfbr1 floxed mice with K14-CreER<sup>tam</sup> mice. K14 is expressed in proliferating keratinocytes of the basal layer of the epidermis.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Decreased TGF-β signaling in Tgfbr1 cKO mice. A, Tgfbr1 mRNA significantly reduced in the head and neck epithelia and SCCs of Tgfbr1 cKO mice by qRT-PCR (n = 3). **, P < 0.01; ***, P < 0.001. B, Tgfbr1 and p-Smad2 expression were reduced in the tongue and SCC of Tgfbr1 cKO mice by immunohistochemistry. The dotted lines delineate the adjacent epithelial compartment. The changes in staining patterns are seen in the epithelium (above the dotted line) in which Tgfbr1 was knocked out. Bar, 50 µm. C, Western blot analysis shows that Tgfbr1 and p-Smad2 were reduced in buccal mucosa, tongue, and SCCs of Tgfbr1 cKO mice compared with that of Tgfbr1<sup>f/f</sup> mice.
It is also active in stem cells that regenerate the epidermis, sebaceous glands, hair follicles, and the oral mucosa. Therefore, tamoxifen treatment causes permanent excision of Tgfr1 in both epithelia and epidermis of the head and neck region, including buccal mucosa, tongue, and ears. The Tgfr1 cKO mice and controls (Tgfr1f/f) were dissected 10 days after tamoxifen treatment. Genomic DNA was extracted from all major organs and tissues. Cre-mediated recombination of the Tgfr1f/f allele was assessed using a PCR-based assay. Deletions of Tgfr1 were detected in the buccal mucosa, tongue, and ear but not in the esophagus, forestomach, back skin, or any other nonstratified epithelial organs of Tgfr1 cKO mice (Supplementary Fig. S1). No recombination was detected before tamoxifen administration. Tgfr1 mRNA expression was examined by quantitative reverse transcription-PCR (qRT-PCR). The expression levels of Tgfr1 mRNA in Tgfr1f/f mice were normalized as 1.00 ± 0.23 in the buccal mucosa and 1.00 ± 0.08 in the tongue. The mRNA expression levels were significantly reduced to a mean of 0.65 ± 0.17 in the buccal mucosa (P < 0.01) and 0.07 ± 0.05 in SCC of Tgfr1 cKO mice as well as 0.46 ± 0.05 in the tongue (P < 0.001; Fig. 1A). Using immunostaining, the Tgfr1 protein level was found to be significantly decreased in the tongue of Tgfr1 cKO mice compared with that of Tgfr1f/f mice. A similar decrease was also observed in p-Smad2, an activated mediator of TGF-β signaling (Fig. 1B). However, the expression of both Tgfr1 and p-Smad2 in the back skin of the same mice remained normal (data not shown). This suggests that, on oral administration of tamoxifen, the deletion of Tgfr1 and the inactivation of its downstream signaling were localized only in the head and neck epithelia. These results were further confirmed by Western blot (Fig. 1C).

Of 31 Tgfr1 cKO mice, only 3 (9.7%, 3 of 31) developed spontaneous tumors, including 2 SCCs in the periorbital region and 1 in the upper lateral neck. No significant pathologic changes in the head and neck region were observed in the remaining Tgfr1 cKO mice during 1 year of observation. Thus, our results indicate that inactivation of TGF-β signaling alone is not sufficient to promote tumor formation in head and neck epithelia of these mice.

Deletion of Tgfr1 in the head and neck epithelia together with DMBA initiation-induced SCCs in mice. Because spontaneous tumor formation in Tgfr1 cKO mice was rare, we induced tumors in Tgfr1 cKO mice by applying a single dose (50 μg per mouse) of DMBA to the mouse oral cavity 10 days after the last tamoxifen treatment. DMBA is a commonly used chemical carcinogen, which can induce H-ras mutations in sporadic cells (24). After tumor initiation with DMBA, Tgfr1 cKO mice started to develop SCCs in the head and neck area as early as 16 weeks, and by 1 year after treatment, 19 of 42 (45%) Tgfr1 cKO mice had developed SCCs. The sites of tumors that developed in DMBA-treated Tgfr1 cKO mice included the oral cavity, periorbital region, muzzle area, and skin around the head and neck area (Fig. 2A–E). Sixteen percent (3 of 19) of mice with tumors developed metastases in the jugular lymph nodes and/or lungs by the time the mice were dissected (10–12 months after tamoxifen and DMBA treatment; Fig. 2F and G). No tumors were observed in K14-CreER<sup>tm1 Conditional knockout (cKO) mice (n = 27) or the Tgfr1 floxed homozygous (Tgfr1f/f; n = 34) control littermates (also treated with tamoxifen and DMBA) during the same time period (Fig. 2H). However, only partial excision of Tgfr1 in mouse head and neck epithelia was noted by immunohistochemistry and
Western blot due to relatively low efficiency of the tamoxifen-induced K14-CreER<sup>am</sup> mouse line being used in this study (Fig. 1B and C; ref. 20).

Enhanced cell proliferation, inhibition of apoptosis, and down-regulation of cell cycle inhibitors in the head and neck epithelia of Tgfbr1 cKO mice. TGF-β has effects on both cell growth and apoptosis. Four weeks after DMBA treatment, an increased expression of a proliferative marker, Ki67, was detected in the basal layer of the tongue of Tgfbr1 cKO mice but not in Tgfbr1<sup>f/f</sup> mice. A decreased apoptosis was also observed, indicating that the imbalance between cell proliferation and apoptosis occurs early in the head and neck epithelia of Tgfbr1 cKO mice (Fig. 3A). Using BrdUrd assays, we found a significantly increased number of proliferative cells in Tgfbr1 cKO mouse head and neck epithelia and SCCs when compared with those of Tgfbr1<sup>f/f</sup> mice. However, we did not observe any apoptotic cells in SCCs by TUNEL assays (data not shown). Immunostaining revealed that CDKN1A expression was reduced in tongue and SCCs of Tgfbr1 cKO mice compared with that in Tgfbr1<sup>f/f</sup> mice. In contrast, c-Myc was overexpressed in tongue of Tgfbr1 cKO mice and its expression was even more remarkable in SCCs (Fig. 3B). These results were further confirmed by Western blot analysis (Fig. 3C). Our results indicate the existence of an imbalance between cell proliferation, differentiation, and apoptosis in SCCs that developed in Tgfbr1 cKO mice as well as in normal Tgfbr1 cKO mouse head and neck epithelia.

Enhanced paracrine effect of TGF-β on tumor stroma of Tgfbr1 cKO mice. Increased inflammation and angiogenesis have been found in human HNSCCs (25). Deletion of Tgfbr2 in mouse head and neck epithelia resulted in enhanced paracrine effect of TGF-β on tumor stroma (14). To investigate the paracrine effect of TGF-β in tumor progression in the DMBA-treated Tgfbr1 cKO mice, we analyzed the expression level of cyclooxygenase-2 (Cox-2; ref. 26), endoglin (CD105; ref. 27), and α-smooth muscle actin (SMA) in tumor stroma (28, 29). We found that Cox-2 expression was absent in normal buccal mucosa and tongue of Tgfbr1<sup>f/f</sup> mice, as well as in Tgfbr1 cKO mice, but its expression was significantly increased in SCCs, suggesting increased inflammation in tumors.

**Figure 3.** Enhanced growth promotion and down-regulation of cell cycle inhibitors in Tgfbr1 cKO mice. A, increased expression of Ki67 and loss of apoptosis in the basal layer of tongue of the Tgfbr1 cKO mice 4 wk after tamoxifen and DMBA treatment. B, a significantly increased number of proliferative cells in tongue and SCCs of Tgfbr1 cKO mice by BrdUrd assays. CDKN1A expression was reduced in tongue and SCCs of Tgfbr1 cKO mice compared with that in Tgfbr1<sup>f/f</sup> mice. In contrast, c-Myc was overexpressed in tongue of Tgfbr1 cKO mice and its expression was even more remarkable in SCCs. The dotted lines delineate the adjacent epithelial compartment. Bars, 50 μm. C, the results were further confirmed by Western blot. D, percentage of positive cells in tongue or SCCs of Tgfbr1 cKO mice compared with that of Tgfbr1<sup>f/f</sup> mice. Columns, average of three to five immunostained sections. **, P < 0.01; ***, P < 0.001.
Increased angiogenesis indicated by endoglin (CD105)–stained microvessels in the stroma surrounding SCCs was also observed (Fig. 4A and B). Using immunofluorescent staining, we found that α-SMA, a hallmark of the myofibroblastic phenotype, was strongly expressed in the stroma surrounding SCCs but was not detected in the tongues of Tgfbr1−/− mice (Fig. 4A). To determine whether these enhanced paracrine effects correlate with endogenous TGF-β1 levels in the area surrounding the SCCs, we examined Tgfb1 mRNA expression by qRT-PCR. In comparison with tissues from Tgfbr1−/− mice, the levels of Tgfb1 mRNA expression were 2.42 ± 0.31–fold and 27.08 ± 4.42–fold (P < 0.01) in DMBA-treated Tgfbr1 cKO mice tongues and SCCs, respectively (Fig. 4C). Immunofluorescent staining indicated significantly increased expression of Tgfβ1 located only in the tumor stroma (Fig. 4D).

Evasion of the immune response is one of the most important features of TGF-β–mediated tumor progression (30, 31). We analyzed the immune status of the Tgfbr1 cKO mice using flow cytometry analysis. Compared with their control littermates, Tgfbr1 cKO mice showed significantly reduced numbers of both CD4+ and CD8+ effector T cells in jugular lymph nodes. In contrast, the regulatory T cells (CD4+CD25+Foxp3+) were increased, indicating active immune suppression in Tgfbr1 cKO mice. Gross changes in inflammation within tumors were noted by H&E staining (Supplementary Fig. S2A and B).

**Activation of PI3K/Akt signaling in SCCs of Tgfbr1 cKO mice.** The PI3K/Akt pathway is important in suppressing apoptosis and in promoting cell growth and proliferation. Hyperactivation of PI3K/Akt in HNSCC is induced either by mutations or by enhanced activity of its upstream activators, including the Ras oncogene or inactivation of PTEN (phosphatase and tensin homologue deleted on chromosome 10; ref. 32). PTEN is a potent tumor suppressor gene and a negative regulator of the PI3K/Akt pathway. Mutations of PTEN have been found in a wide range of human cancers (33). In our study, a significantly increased level of unphosphorylated PTEN, an active form of the protein, was detected in all of the tumors that developed in the DMBA-treated Tgfbr1 cKO mice (Fig. 5B). However, despite the elevated PTEN levels, we observed consistently increased levels of the phosphorylated form of Akt (p-Akt) and its downstream target, the mammalian target of rapamycin (mTOR), in all of the tumors analyzed by both immunostaining and Western blot (Fig. 5A and B). These results indicate that in spite of the increased expression of PTEN,
the PI3K/Akt pathway was activated in the SCCs that developed in the DMBA-treated Tgfbr1 cKO mice. Our results suggest that Akt activation in the SCCs is independent of effects on PTEN in this mouse model and that other mechanisms are involved in the activation of this pathway. One of these might be the H-ras mutations caused by DMBA initiation. Indeed, H-ras mutations were detected in 9 of 17 tumors (53%) at codon 61 in exon 2 of the gene. No K-ras mutations were found in any of these tumors (data not shown). However, the mechanisms underlying the activation of the PI3K/Akt pathway on TGFBR1 deletion warrant further investigation. A proposed TGF-β signaling alteration that promotes HNSCC in mice through activation of PI3K/Akt pathway is shown in Fig. 6.

Discussion

TGF-β is a potent growth inhibitor for epithelial cells (34). Inactivating mutations or experimental deletion of components of the TGF-β pathway has been shown to promote tumorigenesis in a variety of organ systems (35, 36). However, the precise role of TGF-β signaling in head and neck carcinogenesis has not been fully understood. As with other organ systems, existing research has been mainly focused on TGFBR2. Inactivation of Tgfbr2 by overexpression of dominant-negative receptor constructs or by targeted deletion promotes tumorigenesis in the mammary gland, prostate, pancreas, anogenital region, as well as in the head and neck area (14, 37–40). With one exception (40), inactivation of Tgfbr2 does not generally result in tumor formation unless cooperating oncogenic lesions are present, suggesting that loss of TGF-β response plays a tumor-promoting rather than initiating role (14, 41). Interestingly, mice that harbored an inactivated Tgfbr2 in stromal cells developed intraepithelial neoplasia of the prostate and invasive SCCs in the forestomach. This suggests that alterations in the TGF-β signaling pathway within cells of the tumor microenvironment can also contribute to cancer development and progression (38). Even in cases where the TGF-β pathway is compromised specifically in the epithelium, the effects of this perturbation seem to extend to the stroma. Thus, mice with inactivated Tgfbr2 in the mammary epithelium show increased recruitment of F4/80+ cells, increased expression of proinflammatory genes, and altered composition of the fibrovascular stroma—all effects that may promote further tumor progression (42). It is clear that perturbations in TGF-β signaling can have far-reaching effects throughout the ecosystem of the tumor.

It is important to note that TGFBR2 not only interacts with TGFBR1 but also forms functional complexes with other type I receptors, such as ActRI/ALK2, ALK3, or ALK1 (43, 44). Signaling through TGFBR2/ALK1 complexes activates Smad1, Smad5, and Smad8, whereas signaling through the TGFBR2/TGFBR1 complex results in phosphorylation of Smad2 and Smad3. In fact, TGF-β signaling through TGFBR1 and ALK1, in a complex with TGFBR2, showed opposing activities in endothelial cell migration and proliferation (45). Importantly, in epithelial cells, TGFBR2 can also directly phosphorylate Par6 without involvement of TGFBR1 and release Par6 from the Par6-TGFBR1 complex. This allows Par6 to trigger the dissolution of tight junctions in the context of epithelial-mesenchymal transitions (46). Therefore, knocking out Tgfbr2 affects not only Smad-mediated TGF-β signaling but also direct receptor II–mediated alternative signaling via Par6. Thus, knocking out TGFBR1 or TGFBR2 individually could affect downstream signaling differently, leading to distinct biological outcomes.

TGFBR1 forms heterotetrameric complexes with TGFBR2 on the cell surface and is critical for the downstream phosphorylation and activation of the Smads. Mutations and polymorphisms of TGFBR1 have been described: TGFBR1(6A), a 9-bp deletion coding for three alanine residues within the nine alanine repeat region of exon 1, has been particularly associated with HNSCC (15–17). In an earlier
study, we showed that 35% of mice with a targeted deletion of Tgfbr1 developed spontaneous SCCs in periorbital and/or perianal regions (19). To specifically study the role of Tgfbr1-mediated signaling in the progression of HNSCCs, we developed a novel inducible knockout mouse model by deleting Tgfbr1 in head and neck epithelium.

Most of our findings on the Tgfbr1 cKO mouse model are consistent with the findings from DMBA-initiated Tgfbr2 cKO mice (14), suggesting that Tgfbr1 functions similarly to Tgfbr2 in the progression of HNSCCs. The lack of spontaneous tumor formation in Tgfbr1 cKO mice, together with the fact that DMBA treatment facilitates tumor development in these mice, suggests that rather than initiation, loss of Tgfbr1 may play a more crucial role in tumor progression in mouse HNSCC. This is also the case for other epithelia, with the sole exception of the anogenital region (40). However, several differences have also been noted in our DMBA-initiated Tgfbr1 cKO mice compared with DMBA-initiated Tgfbr2 cKO mice. For example, none of our DMBA-initiated Tgfbr1 heterozygous mice (K14-CreER<sup>tam</sup>;Tgfbr1<sup+f/-</sup>) developed HNSCCs, whereas ~33% of mice with a heterozygous Tgfbr2 deletion (K5-CrePR1;Tgfbr2<sup)f/-</sup>) in the head and neck epithelia developed HNSCCs after DMBA initiation. Therefore, tumor suppressor activities of TGF-β require a higher threshold level of Tgfbr2 than of Tgfbr1. Furthermore, only 16% of our DMBA-initiated Tgfbr1 cKO mice with tumors developed metastases in jugular lymph nodes and/or lungs by the time the mice were dissected. However, up to 35% of the DMBA-initiated Tgfbr2 cKO mice developed jugular lymph node metastases by 20 to 39 weeks of age. Although this difference between the two mouse models may be attributable to differences in mouse genetic background and/or the Cre mouse line being used in the studies, it may also indicate that Tgfbr1 and Tgfbr2 function differently. For example, Tgfbr2 may have more suppressive effects in later stages of cancer development, possibly due to TGFBR1-independent effects.

It is widely believed that TGF-β can affect cancer progression through both autocrine and paracrine effects. Paracrine effects of TGF-β, which are generally tumor promoting, include stimulation of inflammation and angiogenesis, escape from immunosurveillance, and recruitment of myofibroblasts. Autocrine effects of TGF-β in premalignant epithelial cells are tumor suppressive, whereas more advanced cancer cells with a functional TGF-β receptor complex may exhibit tumor-promoting autocrine effects due to a convergence of TGF-β signaling with other signaling pathways (47). In the current study, we saw evidence for both types of effect. We found that on deletion of Tgfbr1 in mouse head and neck epithelia, there is an enhanced cell proliferation and down-regulation of cell cycle inhibitors due to inactivation of Smad2/3-mediated signaling. An inhibition of apoptosis through activation of the PI3K/Akt pathway in SCCs that developed in Tgfbr1 cKO mice was also observed. These results suggest that in the head and neck epithelia, TGF-β is an early tumor suppressor. In the SCCs that developed in Tgfbr1 cKO mice, we found increased inflammation, angiogenesis, and myofibroblast formation. Similar results have been observed in other mouse models when TGF-β signaling was disrupted (14, 48). Furthermore, elevated levels of endogenous TGF-β1 were detected in tumor stroma of Tgfbr1 cKO mice, as they have been in other studies (14). Therefore, on one hand, the deletion of Tgfbr1 in mouse head and neck epithelia prevents the surrounding increased TGF-β1 from exerting its tumor-suppressive effects. On the other hand, the expression of Tgfbr1 in tumor stroma would certainly enhance its tumor-promoting function through paracrine effects. Consequently, we believe that the elevated level of TGF-β1 in tumor stroma has direct involvement in the creation of microenvironment for tumor progression (4).

Alternative modes of TGF-β signaling have been categorized (8). Recent work showed that TGF-β induces apoptosis through repression of PI3K/Akt signaling, indicating that there may be

![Figure 6](https://cancerres.aacrjournals.org)
negative cross-talk between the TGF-β tumor suppressor and PI3K/Akt pathways (49). The most notable finding of our current study is that in addition to inactivation of the Smad-dependent TGF-β signaling pathway and in spite of increased PTEN levels after deletion of Tgfbr1 in mouse head and neck epithelia and DMBA treatment, the PI3K/Akt pathway is activated in all SCCs that developed in the Tgfbr1 cKO mice. The results from our study indicate that decreased Tgfbr1 expression in Tgfbr1 cKO mice leads to increased cell proliferation and cell survival through PTEN-independent activation of PI3K/Akt pathway. This possibility is due to DMBA-induced H-ras mutation as well as other unknown mechanisms. These changes accompanied by increased TGF-β1 in tumor stroma, which leads to increased invasion, angiogenesis, inflammation, and immune suppression through paracrine effect of TGF-β, switch TGF-β signaling from tumor suppression to normal cells to tumor promotion in head and neck carcinogenesis of Tgfbr1 cKO mice.

In summary, we generated an inducible conditional gene targeting mouse model for head and neck cancer research. We have shown that targeted deletion of Tgfbr1 in the head and neck epithelia is apparently not sufficient for spontaneous tumor formation but could increase susceptibility to tumor development initiated by DMBA. TGF-β is a major tumor suppressor, and inactivation of TGF-β signaling, in the context of ras mutations and aberrant activation of the PI3K/Akt pathway, may contribute cooperatively to the promotion of head and neck carcinogenesis in these mice. Our results underscore a critical role of the TGF-β signaling pathway and its cross-talk with the PI3K/Akt pathway in suppressing head and neck carcinogenesis. These findings have significant implications for the development of effective therapeutic strategies targeting both the TGF-β and the PI3K/Akt pathways for the treatment of HNSCCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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