TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers

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Abstract

TGF-β plays a dual role in epithelial malignancies, including head and neck squamous cell carcinoma (HNSCC). Attenuation of canonical TGF-β signaling enhances *de novo* tumor development, while TGF-β overexpression and signaling paradoxically promotes malignant progression. We recently observed that TGF-β-induced growth arrest response is attenuated, in association with aberrant activation of Nuclear Factor-κB (NF-κB), a transcription factor which promotes malignant progression in HNSCC. However, what role cross-talk between components of the TGF-β and NF-κB pathways plays in altered activation of these pathways has not been established. Here, we show TGF-β receptor II and TGF-β-activated kinase 1 (TAK1) are predominantly expressed in a subset of HNSCC tumors with nuclear activation of NF-κB family member RELA (p65). Further, TGF-β1 treatment induced sequential phosphorylation of TAK1, IKK, IκBα, and RELA in human HNSCC lines. TAK1 enhances TGF-β-induced NF-κB activation, as TAK1 siRNA knock-down decreased TGF-β1-induced phosphorylation of IKK, IκB, and RELA, degradation of IκBα, nuclear translocation, and DNA binding of RELA, and NF–κB-induced reporter and target gene transcription. Functionally, TAK1 siRNA inhibited cell proliferation, migration and invasion. Celastrol, a TAK1 inhibitor and anti-inflammatory used in traditional Chinese medicine, also decreased TGF-β1-induced phosphorylation of TAK1 and RELA, suppressed basal, TGF-β1- and TNFα-induced NF-κB reporter gene activity, and cell proliferation, while increasing sub-G0 DNA fragmentation and Annexin V markers of apoptosis. Furthermore, TGF-β and RELA activation promoted SMAD7 expression. In turn, SMAD7 preferentially suppressed TGF-β-induced SMAD and NF-κB reporters when compared with

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constitutive or TNF-α-induced NF-κB reporter gene activation. Thus, cross-talk by TGF-β via TAK1 and NF-κB promotes the malignant phenotype of HNSCC. Moreover, NF-κB may contribute to the downstream attenuation of canonical TGF-β signaling through increased SMAD7 expression. Celastrol highlights the therapeutic potential of agents targeting TAK1 as a key node in this pro-oncogenic TGF-β-NF-κB signal pathway.

Keywords

TAK1; SMAD7; TGF-β; NF-κB; celastrol; head and neck cancer

Introduction

The role of TGF-β signaling in development of epithelial cancer is complex. In normal epithelial cells, TGF-β acts as a potent tumor suppressor through regulation of a variety of physiological processes, including growth inhibition and stimulation of apoptosis.[1] Subsequent inactivation of TGF-β signaling or key target genes can promote the *de novo* development of epithelial cancer. However, overexpression of TGF-β in such established cancer cells or the tumor microenvironment is often observed, and appears to play an important role in progression to a more invasive and metastatic phenotype.[2]

Canonical TGF-β signaling is induced by ligand binding to receptor subunit TβRII, which in turn activates TβRI. TβRI phosphorylates SMAD2 and SMAD3, which associate as a heterodimeric complex with the common signaling mediator SMAD4, and together translocate into the nucleus to regulate gene expression. An inhibitor, SMAD7, may compete for binding with SMAD2 and 3 to the activated TGF-β receptor, thereby suppressing downstream TGF-β signaling.[3] Malignant cells can circumvent the suppressive effects of TGF-β through inactivation of key nodes along this pathway.[4] In human head and neck squamous cell carcinomas (HNSCC) arising from the upper aerodigestive tract, attenuated expression or genetic alteration of TβRII or SMAD4 is common.[5] Further, knockout of the genes encoding canonical pathway components promotes development of HNSCC in mice.[6-8]

The mechanism(s) contributing to pathogenesis of the remaining cancers with intact TGFβRII/I-SMAD activation remains less clear. We recently observed that the TGF-β-induced growth arrest response is attenuated in the subset of HNSCC cell lines retaining TGF-β receptor II expression.[9] These HNSCC exhibited aberrant activation of signal-activated transcription factor Nuclear Factor-κB (NF-κB) target genes. Similar to the pro-oncogenic role of TGF-β, activation of NF-κB/RELA (p65) is implicated in cell proliferation, survival, malignant progression and poor prognosis of HNSCC, as well as other cancers.[10] We and others previously showed that NF-κB/RELA may be partially inhibited by blockade of autocrine IL-1α and TNF-α,[11,12] suggesting that other factor(s), may also contribute to NF-κB activation.

Interestingly, a TGF-β activated kinase, TAK1, was previously shown to mediate responses to cytokines TNFα or IL-1, and directly phosphorylate the Inhibitor-KappaB Kinase (IKK) complex that promotes activation of NF-κB.[12-14] TAK1 activation by TGF-β and these

cytokines involves complex formation with associated linkers.[14-16] In turn, the IKK complex, formed by IKKα and IKKβ catalytic subunits and a scaffold subunit, IKKγ/NEMO, phosphorylates Inhibitor-κBα (IκB), which is ubiquitinylated and degraded by the proteasome.[17] IKK-mediated degradation of IκB promotes nuclear translocation and DNA binding of NF-κB, while IKK phosphorylation of the RELA(p65) subunit is required for transactivation of target genes.[18,19] Of further potential interest, NF-κB as well as SMADs 2/3 can upregulate SMAD7, a negative feedback inhibitor of TGF-β mediated activation of both canonical TGF-β-SMAD and TGF-β-TAK1 activation.[20-22] However, what role cross-talk and negative feedback between these components of the TGF-β and NF-κB pathways play in altered activation of these pathways in HNSCC and other cancers is not well established.

Here, we examined the hypothesis that TGF-β activation via TAK1 contributes to aberrant NF-κB activation in HNSCC. We further explored the effects of TAK1 siRNA and a known TAK1 inhibitor, Celastrol,[23] to inhibit TAK1 mediated NF-κB signaling and the malignant phenotype in HNSCC. As NF-κB can induce SMAD7, we examined the potential role of NF-κB and SMAD7 in the cross-talk between NF-κB and TGF-β pathway, and suppression of TGF-β induced signaling and gene expression. Our findings support a model whereby TGF-β-induced TAK1 enhances NF-κB activation, while SMAD7 can attenuate canonical and non-canonical TGF-β signaling, thereby promoting the malignant phenotype of a subset of HNSCC.

Results

Differential Expression of TGF-β Receptor II and correlation with phosphorylated canonical TGF-β signaling SMAD components in HNSCC tumor tissue and cell lines

To examine the prevalence and relationship of TβRII expression to canonical TGF-β-SMAD signaling in HNSCC *in situ*, we performed immunostaining for TβRII, and phosphorylated (activated) SMAD2 and SMAD3 using a tissue array containing 20 human HNSCC specimens in triplicate, and six normal oral mucosa specimens in duplicate, as summarized in Fig. 1A. All normal oral mucosal samples exhibited strong (++) TβRII and phosphorylated SMAD2 staining. Strong (++) staining for TβRII was observed in 47% (28/60) of HNSCC tumor specimens (Fig. 1A, higher magnification, Suppl Fig. 1), and was significantly correlated with staining for activated p-SMAD2 and p-SMAD3 (p=0.0001). Conversely, 53% (32/60) of the tumor specimens showed decreased or absent (+/-) expression of TβRII protein, associated with decreased levels of phosphorylated SMAD2 and SMAD3. Together, these observations indicate that expression of TβRII and phosphorylation of immediate downstream canonical signaling substrates are related, in subsets of HNSCC tumors.

To identify human HNSCC lines that exhibit similar patterns of expression and phosphorylation of TGF-β signaling components, we compared expression and phosphorylation of TβRII and SMADs in primary human oral keratinocytes (HOK) and a panel of nine UM-SCC cell lines (Supplementary Table S1)[24] by Western blot (Fig. 1B). When compared with HOK, expression of TβRII was similar or greater in 5/9 (56%; UMSCC1-11B) and attenuated in 4/9 (44%; UMSCC22A-46) lines, as reported

previously. [9] Expression of TβRII, SMAD2, p-SMAD2, SMAD3, p-SMAD3 and SMAD7 was detectable in HOK and 8/9 of the UM-SCC lines, providing evidence for functional activation of TGF-β receptor and canonical SMAD signal components. An exception was UM-SCC 46, in which decreased expression of the TGFβR2, p-SMAD3 and SMAD7 protein was found to be due to mutation in TβRII by sequence analysis (J. Burnett, unpublished data, not shown).

Canonical TGF-β signaling in HNSCC cell lines is intact but attenuated

As previously shown, HNSCC cell lines appear to be refractory to TGF-β-mediated growth arrest, which can be attributed in part to defects or attenuated expression of signaling components.[9,25] To determine the functional responsiveness of canonical TGF-β-SMAD signaling in HNSCC lines, we compared the effect of TGF-β1 on SMAD phosphorylation in HOK and three UMSCC lines. In HOK cells, treatment with TGF-β1 for a short interval of 1h strongly induced phosphorylation of SMAD2 and SMAD3 signal pathway component activation, without significantly modulating total SMAD 2, 3 or 7 proteins (Fig 1B). For UM-SCC 6 and UM-SCC 22B, treatment with TGF-β1 induced detectable but reduced phosphorylation of SMAD2 (Fig. 1C), and weak induction of TGF-β-inducible PAI1 reporter gene activity (Fig. 1D, left and middle panels). By contrast, UM-SCC 46, which shows reduced expression of a mutant TβRII, treatment with TGF-β1 neither induced phosphorylation of SMAD2, nor increased *PAI1* mRNA, as revealed by Western Blotting and RT-PCR, respectively (Fig 1C and D, right panel, empty vector control). However, if wild-type TβRII was reintroduced into UM-SCC 46 cells by transient transfection, *PAI1* mRNA expression was clearly inducible by TGF-β1 treatment (Fig. 1D, right panel). The differences in TGF-β1-induced SMAD phosphorylation and *PAI1* modulation are consistent with the weak inhibitory effect of TGF-β on cell proliferation of UMSCC 6 and 22B relative to UMSCC 46, reported previously.[9]

Relationship between TβRII, TAK1 and RELA/p65 in HNSCC tumors and TGF-β1-induced TAK1-NF-κB signaling in HNSCC lines

NF-κB subunit RELA (p65) exhibits aberrant nuclear activation in a major subset of oral premalignant lesions and HNSCC in association with poor prognosis,[26] but the signal pathway(s) responsible for NF-κB activation in HNSCC remain to be fully defined.[10] TGF-β and TβRs can also potentially activate and stabilize expression of TAK1 protein and activate NF-κB.[16, 27-30] Thus, we examined the possible relationship between TβRII, TAK1 expression, and phosphorylated RELA (p65) using the HNSCC tissue array. As shown in representative and overall analysis of immunostained tumors (Fig. 2A; higher magnification, Suppl. Fig. 2A), a highly significant correlation between TβRII, TAK1 (p=0.0011), and phospho-p65 (p=0.0006) immunostaining intensity was revealed in a subset of HNSCC. Furthermore, the highly significant correlation between staining for TAK1 and phospho-p65 (p=0.0001), indicate that TAK1 might be an important link between TGF-β and NF-κB signaling.

Western blot analyses in Fig. 2B revealed that, compared with Heka, most of the 9 HNSCC cell lines expressing TβRII in Fig. 1B also show increased basal phosphorylation of TAK1 and NF-κB subunit RELA (p65) serine-536 protein when cultured in serum. Conversely,

UMSCC46, with the defect in TGFβRII, showed relatively weaker p-TAK1 and p-p65, suggesting the possible role of TGF-β-mediated activation of TAK1 in IKK-dependent NF-κB signal phosphorylation. Further supporting this possibility, UM-SCC 6 cells exhibit basal p-TAK1, p-IKK, and p-p65 (Fig. 2B, C), and addition of recombinant TGF-β1 for various time intervals sequentially induced further phosphorylation of TAK1, IKKα/β, and IKK-dependent p-IκBα and p-p65 at serine-536 over 2 hours (Fig. 2C, independent experiment, Suppl. Fig. 2B). Functionally, TGF-β1 also induced a significant increase in NF-κB luciferase reporter gene activity in two independent UM-SCC lines by 24 h (Fig. 2D). Taken together, these findings support the hypothesis that TGF-β is able to activate canonical IKK-NF-κB signaling through activation of TAK1.

Knockdown of TAK1 by siRNA suppresses NF-κB signal activation, cell proliferation, migration and invasion

To further establish if TAK1 mediates constitutive, TNFα- and TGF-β-induced TAK1-IKK-NF-κB activation, UM-SCC 6 cells were treated with TAK1 siRNA, and cultured for 48h in serum containing medium alone, with added TNF-α for final 8h, or with TGF-β for final 24h (Fig. 3A), based upon optimal effects of TGF-β upon NF-κB-luciferase reporter activity (Fig. 2D). TAK1 siRNA similarly depleted TAK1 expression in UM-SCC 6 cells cultured without or with added TNF-α, and less completely with the longer exposure to TGF-β (Fig. 3A), consistent with TGF-β-induced stabilization of TAK1 protein.[30] In untreated, TNFα- and TGF-β-treated cells, TAK1 depletion inhibited phosphorylation of IKKα/β, IκBα and p65-ser-536 compared to control siRNA. TNFα and TGF-β markedly induced degradation of total IκB, and increased cytoplasmic to nuclear translocation of NF-κB subunit p65 (Fig. 3A; densitometry, Suppl Fig 3A), even though the TGF-β-induced increase in p—p65 observed at earlier timepoints (i.e, Fig. 2C; Suppl Fig. 2B), was attenuated by 24h exposure. Conversely, TAK1 siRNA partially inhibited IκB degradation and cytoplasmic-nuclear p65 translocation. As only a small increase in resynthesis of IκB was detectable with TAK1 depletion in cells continuously exposed to both factors, we transfected a plasmid expressing an IκB-luciferase fusion protein which can serve as a quantitative reporter of IKK kinase-induced degradation of IκBα protein.[31] Relative to IκB-luc signal in cells with no treatment, TGF-β and TNF-α treatment induced IκB-luc protein degradation (Fig. 3B, left panel). Conversely, knockdown of TAK1 significantly attenuated constitutive, TGF-β-and TNFα-induced degradation of the IκBα-luc fusion protein (Fig. 3B, left panel). TAK1 siRNA also suppressed constitutive, TNFα- and TGF-β-induced activation of an NF-κB specific reporter gene (Fig. 3B, right panel), consistent with inhibitory effects of TAK1 siRNA on p65-ser-536 phosphorylation (Fig. 3A), which is required for NF-κB gene transactivation.[18,19] Conversely, transient transfection of exogenous TAK1 protein, further enhanced constitutive, TGF-β1- and TNFα-induced NF-κB reporter gene transactivation (Suppl Fig 3B). Knockdown of TAK1 also lowered constitutive, TNFα- and TGF-β-induced nuclear NF-κB p65 binding activity (Fig. 3C, left panel), and NF-κB inducible target gene IL-8, as revealed by RT-PCR (Fig. 3C, right panel). Together, the effects of TAK1 depletion on total IκB, nuclear and DNA bound p65 observed were relatively smaller than effects on IKK-dependent phosphorylation of p65 and NF-κB reporter gene transactivation, consistent with previous findings that modification of nuclear p65 is most critical for its functional activity.[19]

Examination of the effects of TAK1 siRNA knockdown on the malignant phenotype of cells demonstrating TGF-β and TAK1 signaling in serum-containing medium (Fig, 1B, 2B), revealed that TAK1 promotes cell proliferation (Fig. 3D, left panel). As further supplementation of TGF-β present in serum did not further increase, but slightly inhibited proliferation (Suppl Fig. 3C, upper and lower panels), we examined if the canonical SMAD and TAK1 pathways mediate opposing effects on proliferation in 10% serum -/+ supplemental TGF-β, by knockdown with TAK1, SMAD2, or both siRNAs. TAK1 siRNA alone inhibited proliferation, while SMAD2 siRNA enhanced proliferation, and combination with TAK1 siRNA inhibited this SMAD2 siRNA-related increase in proliferation in serum alone, or with additional TGF-β, consistent with residual canonical SMAD inhibitory signaling observed in UMSCC6 cells. We confirmed the efficiency of TAK1 and SMAD2 knockdown by qRT-PCR Suppl. Figure 3D. Similar partial inhibitory effects were observed with anti-TGF-β antibody attributable to TGF-β in 10%FBS, and addition of TGF-β partially overcame the inhibitory effect of anti-TGF-β antibody and increased proliferation, without further increasing proliferation above that attributable to TGF-β in 10%FBS. (Suppl. Fig. 3E). Together, these observations support a role for TGF-β and TAK1 in promoting proliferation and opposing the inhibitory effects of SMAD mediated-canonical signaling. TAK1 depletion also partially inhibited matrigel invasion and migration in wound assay (Fig. 3D, right panel; Suppl Fig. 3F). Thus, these effects of TAK1 depletion are similar to those observed previously with inhibition of NF-κB p65 in HNSCC.[10]

Celastrol, a TAK1 inhibitor, inhibits NF-κB signaling and induces apoptosis in HNSCC

Celastrol, used as an anti-inflammatory drug in traditional Chinese medicine,[32] has been shown to inhibit TAK1.[23] We examined effects of Celastrol treatment on TGF-β-induced phosphorylation of TAK1 and p65 by Western blot analyses in UM-SCC 6 deficient for wtTP53, and UMSCC 22B with mutant TP53 (Fig. 4A, densitometry Suppl. Fig. 4A). Treatment with 1.0 or 2.5 μM Celastrol for 1 h clearly reduced levels of phosphorylated TAK1 and p65 in both cell lines. Celastrol 2.5μM could also reduce p-TAK1 and p-p65 over 1-2 hours, without reducing total TAK1 (Suppl. Fig. 4B). Furthermore, Celastrol treatment reduced constitutive, TGF-β1- and TNFα-induced NF-κB reporter gene activity (Fig. 4B). Further, celastrol significantly inhibited proliferation of both cell lines in a concentration-dependent manner with EC_{50} values ranging from 1.1 to 1.3 μM after 72h incubation (Fig. 4C). We next analyzed whether Celastrol treatment would affect cell cycle distribution or fragmentation of DNA and Annexin V, which are markers of cell death, by flow cytometry. Celastrol at an inhibitory concentration of 2.5 μM induced accumulation in G2/M, sub-G0 DNA fragmentation and Annexin V over 12 to 24 hours in UM-SCC 22B, indicative of growth arrest and apoptotic cell death, respectively (Fig. 4D, Suppl. Fig. 4C). Similar effects were observed for UM-SCC 6 (data not shown).

NF-κB subunit p65 induces SMAD7 expression and represses TGF-β-SMAD regulated gene PAI1 in HNSCC

SMAD7 is a downstream target of TGF-β signaling, that associates with TGF-βRI and competes with receptor activated SMADS to inhibit their activation, providing a negative feedback mechanism.[3,33] Several past studies have reported that SMAD7 may also be induced by or modulate other pathways, including TAK1 and NF-κB.[15,22,34-36] Therefore

we further hypothesized that SMAD7 might be involved in the cross-talk between TGF-β and NF-κB signaling in HNSCC. To examine the potential relation between SMAD7 and NF-κB signaling in HNSCC *in situ*, we performed immunostaining for SMAD7 and the phosphorylated/activated NF-κB subunit p65 using a tissue array (Fig. 5A, higher magnification, Suppl. Fig 5A). Seventy-three percent of the tumor specimens with strong (++) p-p65 staining (22/30) also showed strong (++) expression of SMAD7 protein. Conversely, only 8% of the tumors with weak (+/-) p-p65 expression had strong (++) staining of SMAD7. This correlation between expression of p-p65 and SMAD7 was highly significant (p=0.0007), suggesting that NF-κB signaling might contribute to SMAD7 expression.

To examine this hypothesis, we first used TAK1 inhibitor Celastrol, which could potentially modulate TAK1-p65-NF-κB dependent SMAD7 expression. Treatment of UM-SCC 6 cells with 2.5 μM of Celastrol for 6h abolished phosphorylation/activation of p-65, and inhibited SMAD7 protein levels (Fig. 5B). As Celastrol also inhibited p-SMAD2 (Fig. 2B), and TAK1 has also been implicated in modulating signal SMADs,[37] these findings suggest that phospho-p65 and/or signal SMADs could modulate SMAD7 expression. To further examine the specific role of p65 in SMAD7 expression, UM-SCC 6 cells were transiently transfected with control or p65 siRNA, which reduced SMAD7 mRNA levels (Fig. 5C, left panel; p<0.05), implicating p65 in SMAD7 expression. Depletion of p65 and decreased SMAD7 was also associated with increased expression of canonical TGF-β-SMAD regulated gene PAI1 (Fig. 5C, right panel). Taken together, these results indicate that p65-NF-κB activation may contribute to SMAD7 expression and reciprocal repression of canonical signal SMAD regulated gene PAI1 in HNSCC.

SMAD7 preferentially suppresses TGF-β-induced SMAD and NF-κB activation over constitutive and TNF-α-induced NF-κB activation

SMAD7 has been implicated in inhibition of TAK1 activation and canonical TGFβ signaling.[15,22] As we have shown that TGF-β and NF-κB signaling induce SMAD7 expression (Fig. 5B, C; Supplemental Fig. 5B), we explored the effects of SMAD7 modulation on both pathways. UM-SCC 6 cells were transiently transfected with control or SMAD7 vector and treated with 10 ng/ml TGF-β1 for 24h. Cytoplasmic and nuclear extracts were immunoblotted for TGF-β signaling components (Fig. 5D; separation controls, Suppl. Fig. 5C). Treatment with TGF-β1 induced phosphorylation of SMAD2, especially in the nuclear fraction. Furthermore, overexpression of SMAD7 clearly reduced both cytoplasmic and nuclear phosphorylation of SMAD2 in untreated and TGF-β1 treated cells, indicating an inhibitory effect of SMAD7 on TGF-β signaling.

In addition, SMAD7 overexpression markedly diminished TGF-β reporter gene activity by 60% in untreated cells and by 75% in TGF-β1 treated cells, respectively (Fig. 5E, upper panel). While overexpression of SMAD7 also reduced TGF-β1 induced NF-κB reporter gene activity by 64%, it reduced NF-κB reporter gene activity by only 20% in untreated cells and 24% in TNFα treated cells (Fig. 5E, lower panel). Together, these results indicate that SMAD7 has an inhibitory effect on both TGF-β-induced SMAD and NF-κB signaling, providing a negative feedback mechanism as both pathways induce its expression. However,

the inhibitory effect of SMAD7 on TGF-β-induced SMAD or NF-κB signaling is greater than that observed for constitutive or TNFα-induced NF-κB signaling, providing a basis for preferential activation of NF-κB and inhibition of the downstream canonical SMAD pathways.

Discussion

In the present study, we provide evidence for a novel crosstalk between TGF-β signaling and the NF-κB pathway involving TAK1 and SMAD7 in HNSCC (Figure 6). Tissue microarray studies provide evidence linking residual upstream TβRII-SMAD signaling with increased TAK1 expression, and NF-κB activation, in the same subset of HNSCC tumors. We demonstrate that TGF-β1 treatment results in sequential phosphorylation of TAK1 and the canonical NF-κB pathway comprised of IKKα/β, IκB-α and p65 in HNSCC lines. TAK1 depletion blocked activation of NF-κB, cell proliferation, migration and invasion, implicating TAK1 as a key node in aberrant activation of NF-κB and the malignant phenotype of HNSCC. Further studies supported a role for TGFβ and TAK1 in countering the inhibitory effects of SMAD2 mediated-canonical signaling upon proliferation. Furthermore, we show that NF-κB is linked to SMAD7 expression in the same HNSCC subset, and that both TGF-β and NF-κB induce SMAD7 expression, and that SMAD7 preferentially suppresses constitutive and TGF-β-induced canonical p-SMAD2 signaling and reporter gene activation, relative to effects upon constitutive and TNFα-inducible NF-κB reporter gene activation.

It is well accepted that loss of growth-inhibitory responses to TGF-β is an important event in early malignant transformation of epithelial cells,[38] including most HNSCC.[25] Subsets of epithelial tumor cells escape from TGF-β-SMAD-dependent effects through defects at different levels of the canonical signal transduction pathway, such as decreased expression of TβRII or SMAD4 in HNSCC.[5,7,9,39] However, only ~50% of HNSCC tumors (Fig. 1A) and cell lines (UM-SCC 22A, 22B, 38 and 46, Fig. 1B) investigated in this study demonstrated relative reduction in TβRII, which may result from repression by mutant TP53 [9] or TβRII mutation (J. Burnett, unpublished data). TGF-β1 treatment induced detectable phosphorylation of SMAD2, but attenuated TGF-β reporter gene activity (Fig. 1C, D) and growth arrest in most of the HNSCC lines examined.[9] Previously, p-SMAD2 was also detected in a majority of HNSCC lines and tumors.[39] Hence, mechanisms other than defects in expression and phosphorylation of upstream canonical TβR-SMAD components must account for the loss of growth-inhibitory response, and augmentation of the malignant phenotype by TGF-β in most HNSCC.

Along with attenuation of TGF-β tumor suppressive effects, aberrant nuclear activation of NF-κB subunit RELA (p65) is observed in a major subset of early premalignant lesions and associated with risk of malignant progression and decreased prognosis. [10,26] Our results provide evidence that endogenous TβRII and TAK1 expression is associated with nuclear NF-κB activation in a subset of HNSCC tumors (Fig. 2A). Further, we provide direct evidence that TGF-β induces NF-κB activation through phosphorylation and activation of TAK1 in HNSCC lines. In addition, we have identified TGF-β-induced TAK1 as an upstream mediator of IKKα/β phosphorylation and activation, leading to phosphorylation

and degradation of the NF-κB inhibitor IκBα, and nuclear translocation and transactivation of NF-κB. TAK1 was detected in nucleus as well as cytoplasm (Fig. 2A, Suppl Fig 2A), consistent with similar distribution in embryonic epithelia[40] and with substrate IKKα/β in HNSCC. [41] Previously, TAK1 has been implicated in NF-κB activation in response to bacterial lipopolysaccharide, TNFα, or IL-1.[13,14,42] Here we show that TAK1 is essential for TGF-β1- as well as TNFα-induced NF-κB activation, where ectopic expression of TAK1 kinase inactive mutant attenuated TNFα- and IL-1-induced NF-κB activation.[43] Conversely, we also demonstrate that overexpression of TAK1 augments both TGF-β1- and TNFα-induced NF-κB activation, highlighting its importance as a key node in regulation of NF-κB in HNSCC.

We further investigated the effect of celastrol, known for its ability to inhibit inflammation and TAK1 activation.[23] Celastrol is a quinone methide triterpene extracted from the root bark of *Tripterygium wilfordii* Hook F, also known as "Thunder of God Vine" and has been used in traditional Chinese medicine for decades.[32] Celastrol is reportedly used for its anti-inflammatory activity for the treatment of asthma, rheumatoid arthritis and neurodegenerative disease.[44-46] Our results indicate that celastrol significantly reduced not only constitutive and TNFα-induced but also TGF-β1-induced NF-κB activation. This inhibition was mediated through reduced phosphorylation and activation of TAK1 and phosphorylation of NF-κB transactivating subunit p65 (Fig 4). Celastrol has been shown to suppress TNFα-induced NF-κB activation by inhibition of TAK1 and IKK activation in human myeloid KBM-5 cells.[23] Furthermore, we showed that celastrol reduced cell density in a dose dependent manner, which was likely due to growth arrest and induction of cell death, as indicated by G2/M accumulation, sub-GO DNA fragmentation, and increase in Annexin V by fluorescence flow cytometric analysis. Recently, it was shown that celastrol treatment could significantly inhibit tumor growth in a human prostate tumor xenograft model.[47] Although the target effects on NF-κB were not defined as in our study, these results indicate that celastrol may hold potential as a therapeutic agent targeting the pro-oncogenic TGF-β-TAK1-NF-κB pathway.

Functional crosstalk between TGF-β and NF-κB signaling in tumor cells has been reported in previous studies. On the one hand, TGF-β was reported to suppress NF-κB signaling, through increased activation and expression of IκBα and retention of NF-κB in the cytoplasm in B cells, hepatocytes, and certain breast cancer cell lines.[48-51] Conversely, we recently showed that attenuated canonical TGF-β signaling was nonetheless linked with NF-κB activation in another subset of HNSCC.[9] Consistent with our current findings, previous reports have implicated a tumor-promoting effect of TGF-β in the context of prolonged exposure of cells to high levels of TGF-β.[52] In addition, TGF-β2 functions as a potent activator of NF-κB signaling in prostate cancer PC3 cells.[53] TGF-β1 also induced NF-κB, however levels of TGF-β2 secreted by the prostate cancer PC3 cells were much higher than of TGF-β1. These findings together with our results showing that TAK1 depletion modulates NF-κB and related proliferative, prosurvival, and invasive phenotypes support the hypothesis that TGF-βs may directly contribute to NF-κB activation and related processes implicated in tumorigenesis.

TGF-β signaling may act through the conventional SMAD-dependent pathway or via alternative pathways independent of SMADs. Generally, SMAD-dependent TGF-β signaling has been implicated in tumor suppressing effects (Hahn et al 1996), while SMAD-independent pathways have been correlated with tumor promoting events.[54] Previous studies have revealed that TGF-β can also activate JNK, Erk and p38 MAPK kinase pathways independent from SMADs.[21,34-36] TGF-β1 may activate these pathways by signaling via TAK1, through its function as a mitogen-activated protein kinase kinase kinase (MAPKKK).[16] Thus, several alternative SMAD-independent TGF-β pathways besides NF-κB demonstrated herein could also contribute to a switch in signal transduction towards the pro-oncogenic activities of TGF-β signaling observed with cancer progression.

Previous studies have suggested an important role of SMAD7 in the interplay between TGF-β and NF-κB signaling. The NF-κB subunit p65 may suppress TGF-β-SMAD signaling through upregulation of SMAD7.[21] Conversely, SMAD7 can induce IκBα expression, thereby inhibiting NF-κB activation.[55] Our results suggest a more complex role of SMAD7. We found evidence that the inhibitory effect of SMAD7 on canonical TGF-β-SMAD signaling is greater than that upon constitutive and TNFα-induced TAK1-NF-κB signaling previously defined in HNSCC.[12] This provides a means for preferential inhibition of canonical tumor TGF-β-SMAD signal mediated tumor suppression, relative to effects on constitutive and TNFα-inducible TAK1-IKK-NF-κB activation, favoring the malignant phenotype. As celastrol inhibits TAK1-mediated NF-κB activation, and promotes apoptosis in HNSCC cells, the alternative TAK1-NF-κB pathway could serve as a potential drug target for developing effective therapies to treat HNSCC.

MATERIALS AND METHODS

Cell lines

The patient characteristics (Supplemental Methods, Table S1), molecular and phenotypic characteristics typical of HNSCC tumors and culture conditions have been described for the panel of genotyped University of Michigan squamous cell carcinoma (UM-SCC) lines kindly provided by Dr. T. E. Carey (University of Michigan, Ann Arbor, MI).[24,56,57] Primary human oral keratinocytes (HOK; ScienCell Research Laboratories, Carlsbad, CA) or human epidermal keratinocytes (HEKA) used as controls were cultured following the manufacturer's protocol and used within 5 passages.

Reagents

Recombinant Factors and antibodies are described in Supplemental methods.

Immunohistochemistry

Formalin-fixed and paraffin-embedded human HNSCC tissue arrays obtained from Cybrdi were previously described.[58] Detailed immunohistochemistry methods are described in Supplemental Methods.

Western Blot analysis

Proteins were separated and immunoblotting were performed using standard methods described in supplemental methods

Transfection

Vectors, siRNAs and Methods used are described in Supplemental Methods.

Luciferase Reporter Assays

Transfection and reporter assays were done as described previously, [38] and in Supplemental Methods. The TGF-β-inducible reporter p3TP-Luc plasmid used [60] which provides specific readout for TGF-β-mediated signaling [61] was kindly provided by Dr. Stuart Yuspa (National Institutes of Health, Bethesda, MD). The IκBα-luciferase fusion protein serves as an indicator of IKK mediated IκBα degradation and was kindly provided by Dr. Louis M. Staudt. [31]

NF-κB DNA binding assay

NF-κB DNA binding activity was assessed using TransAM NF-κB Family Transcription Factor Assay Kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol as described in Supplemental Methods.

Real-time QRT-PCR

RNA isolation and cDNA synthesis and QRT-PCR were performed as previously described [57] and in Supplemental Methods.

Cell Proliferation Assay

Cell proliferation assay with control or TAK1 siRNA, and without and with Celastrol was performed as detailed in Supplemental methods.

Migration and Invasion assays

Scratch migration and matrigel invasion assay with control or TAK1 siRNA were performed as described in Supplemental methods.

Flow cytometry analysis

UM-SCC 6 and 22B cells were plated in 6-well plates and cultured for 24 hours. Media was replaced with celastrol at 2.5 μM concentration. At 12 and 24 hours, cells were harvested, counted, and labeled using the Cycletest Plus DNA Reagent Kit (BD Biosciences, San Jose, CA). Samples were run on a FACS Canto machine within 1 hour after labeling, and the data were analyzed using Flow-Jo analysis software (Tree Star, Inc, Ashland, OR).

Statistical Analysis

Statistical differences between two groups of data were analyzed using the Student's t-test. The data are presented as mean ± standard deviation (SD). Immunhistochemistry results were analyzed using Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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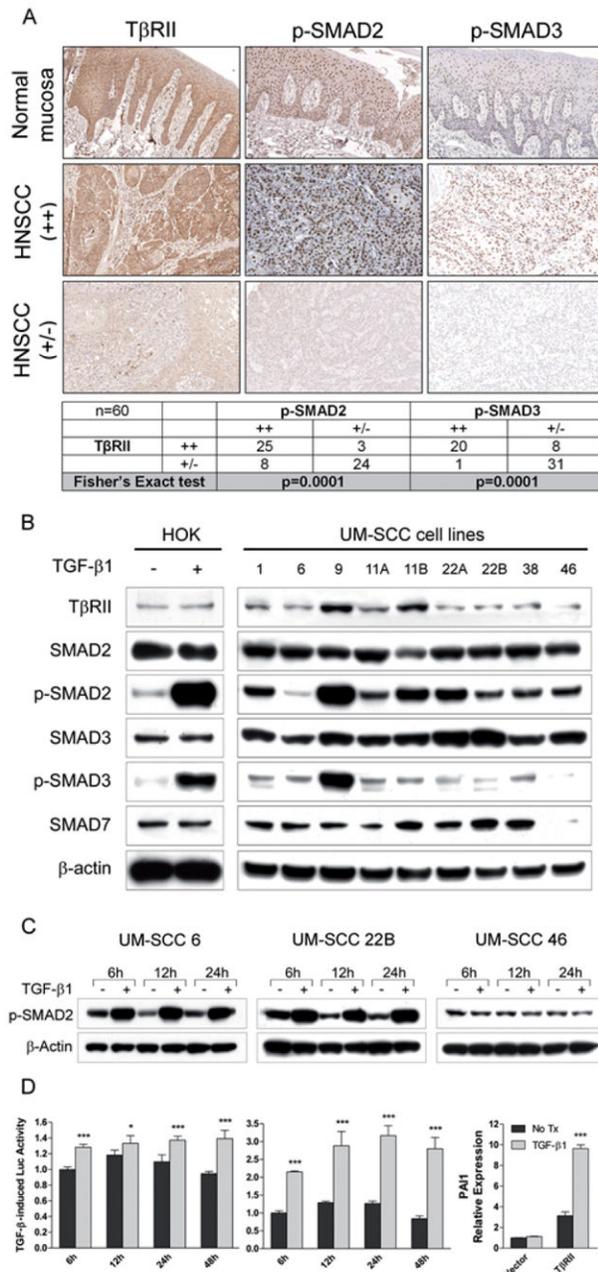
Figure 1.
Expression and correlation between TGF-β signaling components in HNSCC tumors and cell lines. (A) Representative immunostaining for TβRII, p-SMAD2 and p-SMAD3, showing decreased TβRII expression in approximately 50% HNSCC tumor specimens (lower panels). Expression of TβRII is statistically correlated with expression of p-SMAD2 and p-SMAD3 by Fisher Exact test (p<0.0001). Original magnification 200X. (B) Immunoblot of TGF-β signaling components protein expression in HOK and UM-SCC cell lines. HOK cells treated with 10 ng/ml TGF-β1 for short interval of 1 hour demonstrate increased phosphorylation of SMAD2 and 3 without affecting total SMAD 2, 3 or 7.

HNSCC cell line UM-SCC 46 shows diminished TβRII, p-SMAD3 and SMAD7 protein levels, indicating a defective TGF-β signaling pathway. (C) UM-SCC 6, 22B and 46 cells were treated ± 10 ng/ml TGF-β1 for different time intervals, and p-SMAD2 was examined in whole cell protein extracts by Western blot. Detectable TGF-β signal phosphorylation of SMAD2 was induced in UM-SCC 6 and 22B, but not in 46 cells. (D) TGF-β reporter gene activity was examined in UM-SCC 6 and 22B through cotransfection of TGF-β-inducible and β-galactosidase reporter plasmids ±10ng/ml TGF-β-1. Luciferase values are normalized to β-galactosidase activity. TGF-β1 treatment did not induce mRNA expression of TGF-β target PAI1 in UM-SCC 46 due to the TGF-βRII defect. However, UM-SCC 46 cell transfection with a TβRII expression vector restored TGF-β signaling. Columns, mean of triplicate or quadruplicate samples; bars, SD. *, p<0.05, ***, p<0.001.
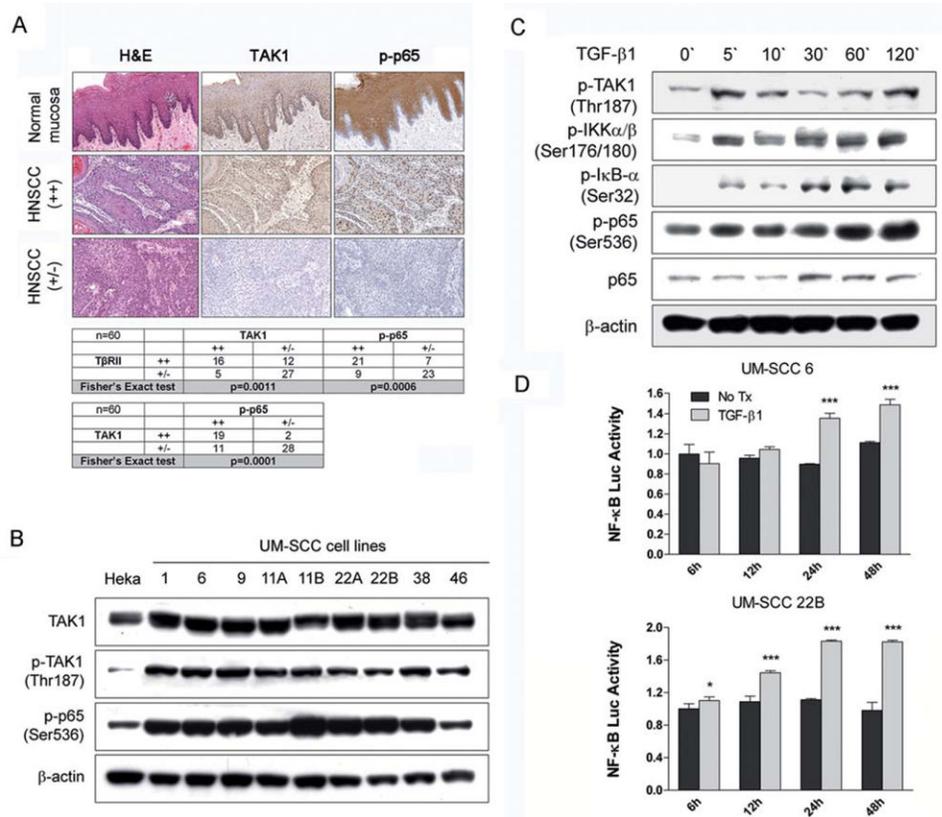
Figure 2.
Relationship between TAK1 and phospho-RELA/p65 in HNSCC tumors and lines, and TGF-β1 induced phosphorylation of TAK1, IKK, IκBα, RELA/p65 and NF-κB reporter activation (A) Immunostaining of TβRII is significantly correlated with expression of TAK1 and p-p65 (p<0.001 and p=0.0006, respectively) in HNSCC tumor specimens. In addition, correlation between TAK1 and NF-κB p-p65 was highly significant (p=0.0001). Original magnification 200X. (B) Protein Expression of TAK-1, p-TAK1 and p65 exhibited increased phospho-activation in UM-SCC cells when compared to Heka, except UM-SCC 46. (C) UM-SCC 6 cells were treated with 10 ng/ml TGF-β1 for different time intervals, and increased p-TAK1, p-IKKα/β, p-IκB-α and p-p65 were observed in whole cell protein extracts by Western blot. (D) NF-κB reporter gene activity was examined in UM-SCC 6 and 22B cell lines through co-transfection of NF-κB and β-galactosidase reporter plasmids +/-10 ng/ml TGF-β1 for different time intervals. Luciferase values are normalized to β-galactosidase activity. The 6h timepoint without TGF-β1 treatment was taken as 1.0 representing basal NF-κB activation and all other values were normalized to that. Columns, mean of triplicate or quadruplicate samples; bars, SD. *, p<0.05, **, p<0.01, ***, p<0.001.
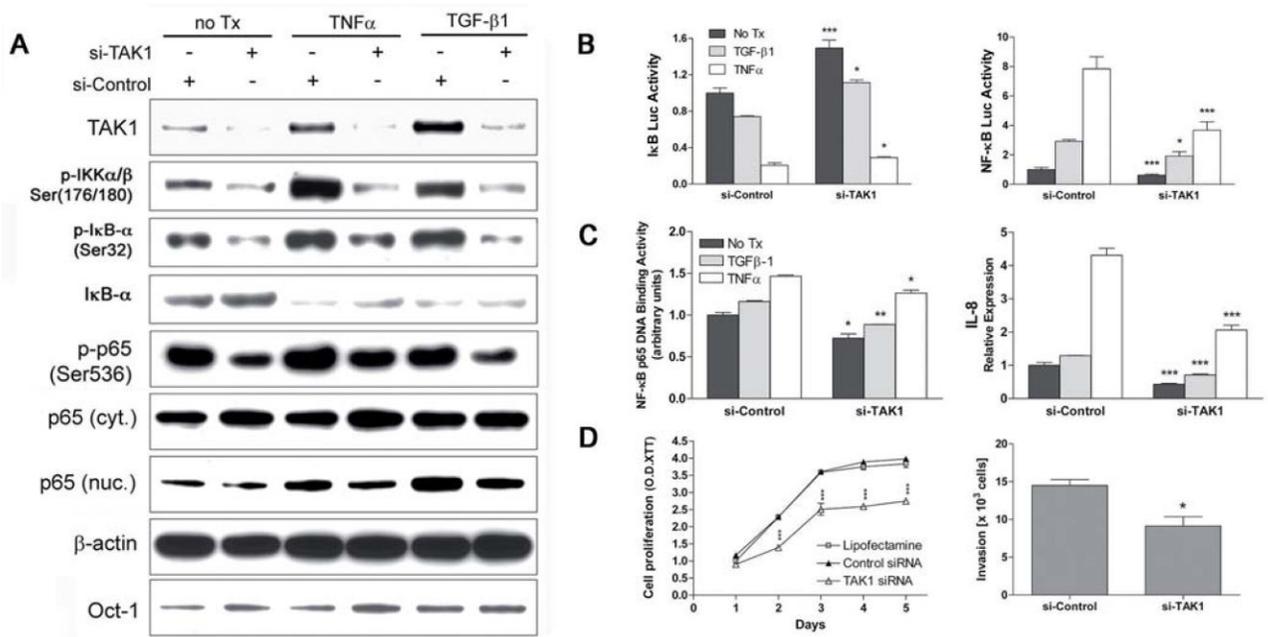
Figure 3.
Knockdown of TAK1 by siRNA suppresses NF-κB signaling (A) UM-SCC 6 cells were
transfected with TAK1 or control siRNA and treated with 10 ng/ml TNFα for 8h or with 10
ng/ml TGF-β1 for 24h, respectively, that induce comparable levels of TAK1 activation, and
NF-κB reporter activation by 24h. Cytoplasmic and nuclear extracts were immunoblotted
with indicated antibodies. Knockdown of TAK1 decreased p-IKK, p-IκBα, and p-p65,
Baseline, TNFα and TGF-β1 induced degradation of IκBα and nuclear translocation of NF-
κB p65 is inhibited by TAK1 depletion. (B) TAK1 knockdown in UM-SCC 6 cells
attenuated basal, TGF-β1 and TNFα- induced degradation of IκBα-luciferase fusion protein
and NF-κB reporter gene activity. (C) TAK1 knockdown in UM-SCC 6 cells decreased
TNFα- and TGF-β-induced NF-κB p65 DNA binding activity and mRNA expression of NF-
κB downstream target IL-8. Columns, mean of triplicate or quadruplicate samples; bars, SD.
*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. (D) TAK1 siRNA inhibits UM-SCC 6 proliferation in
5d XTT assay (left panel) and matrigel invasion (right panel). Student t test, ***, $p<0.001$; *
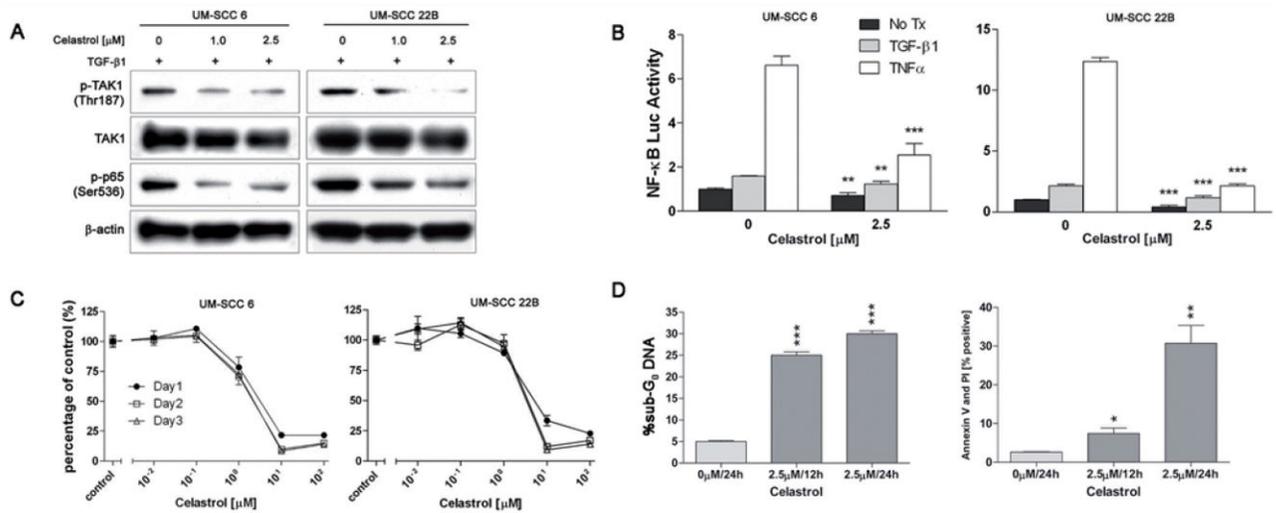$p<0.05$.

Figure 4.
Celastrol, a TAK1 inhibitor, inhibits NF-κB signaling and induces apoptosis in HNSCC cells. (A) UMSCC-6 and 22B cells were pre-treated with different concentrations of Celastrol for 1h and then treated with 10 ng/ml TGF-β1 for 1h. p-TAK1, TAK1 and p-p65 were examined in whole cell extract by Western blot. Celastrol suppressed TGF-β1 induced phosphorylation of TAK1 and p65 in a dose dependent manner. (B) NF-κB reporter gene activity was examined in UM-SCC 6 and 22B cell lines through cotransfection of NF-κB and β-galactosidase reporter plasmids +10 ng/ml TGF-β1 or TNFα. Luciferase values are normalized to β-galactosidase activity. Celastrol decreased basal, TGF-β1 and TNFα induced NF-κB reporter gene activity. (C) Celastrol reduced cell growth as measured by XTT assay, IC50=1.2μM. (D) Celastrol increased sub-G0 DNA fragmentation and Annexin V in UM-SCC 22B cells, markers of apoptosis. Columns, mean of triplicate or quadruplicate samples; bars, SD. *, $p<0.05$, **, p0.01, ***, $p<0.001$.
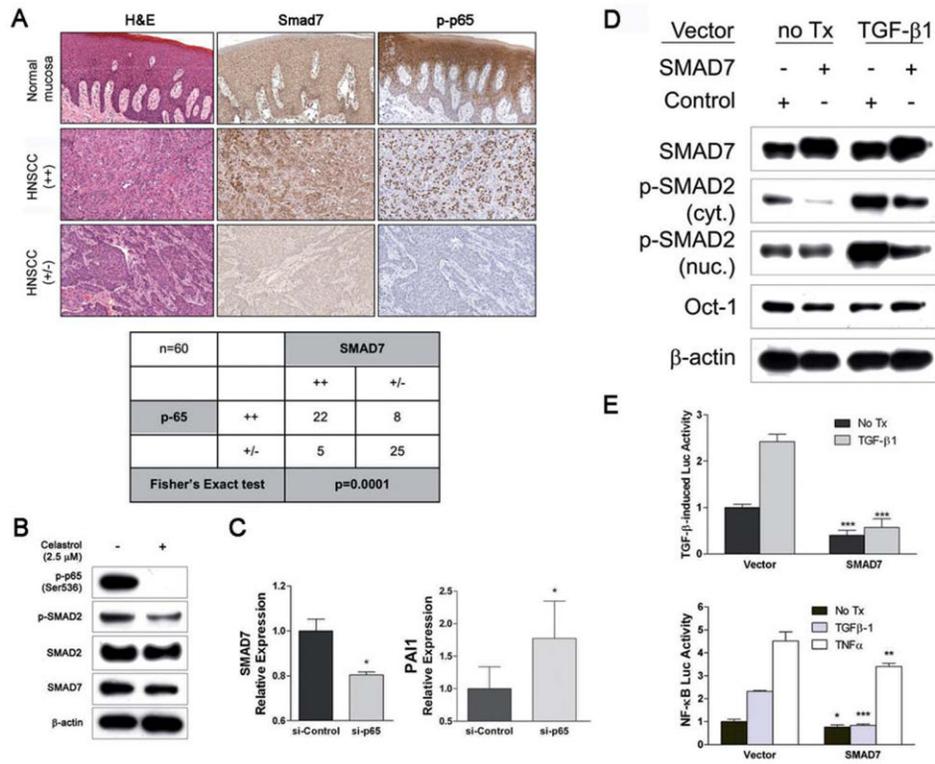
Figure 5.
NF-κB subunit p65 is correlated with and induces SMAD7 expression in HNSCC. (A) Immunostaining for p-p65 and SMAD7 in 20 HNSCC tumor specimens in triplicate showing a statistical correlation between p-p65 expression and SMAD7 (p=0.0007). (B) UMSCC-6 cells were treated with 2.5 μM of Celastrol for 6h and p-65, p-SMAD2 and SMAD7 were examined in whole cell extract by Western blot. Celastrol diminished phosphorylation of p65, SMAD2 and decreased SMAD7 expression. (C) UM-SCC 6 cells were transfected with p65 or control siRNA and mRNA was extracted after 48h for qRT-PCR. Knockdown of p65 decreased SMAD7, while increasing PAI1 mRNA. Columns, mean of triplicate samples; bars, SD. *, p<0.05. (D) UM-SCC 6 cells were transfected with SMAD7 or control Vector and treated with 10 ng/ml TGF-β1 for 24h, respectively. Cytoplasmic and nuclear extracts were immunoblotted with indicated antibodies. Overexpression of SMAD7 reduced both cytoplasmic and nuclear phosphorylation of SMAD2 in untreated and TGF-β1 treated cells, indicating attenuated TGF-β signaling. (E) SMAD7 overexpression significantly decreased TGF-β reporter gene activity by 60% in untreated cells and by 75% in TGF-β1 treated cells, respectively. Overexpression of SMAD7 reduced NF-κB reporter gene activity by only 20% in untreated cells and 24% in TNFα treated cells. However, SMAD7 overexpression reduced TGF-β1 induced NF-κB reporter gene activity by 64%. Columns, mean of quadruplicate samples; bars, SD. *, p<0.05, ***, p<0.01, ***, p<0.001.
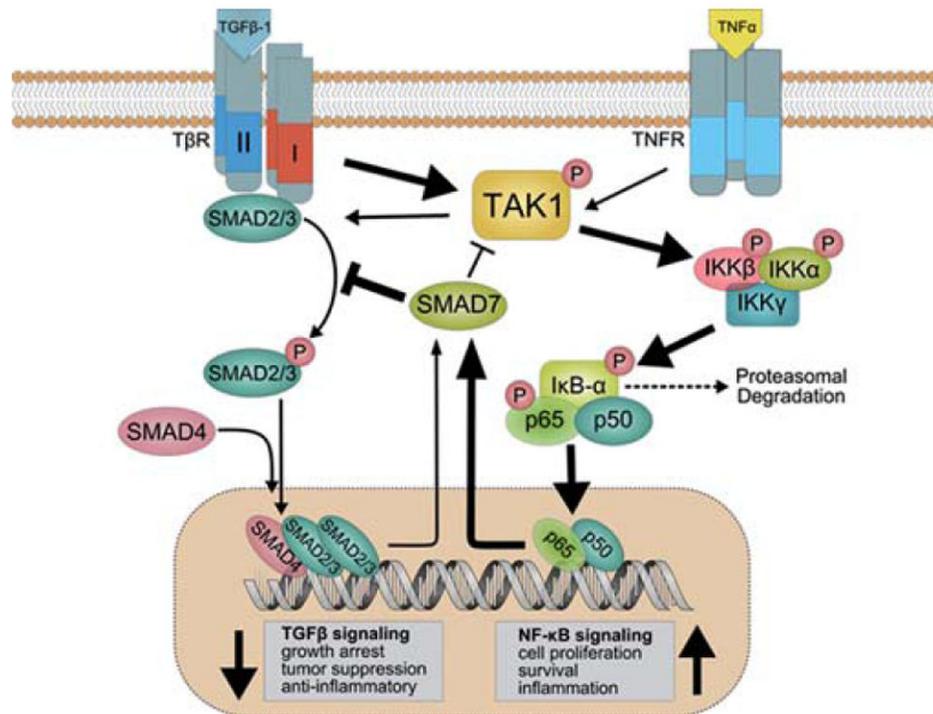
Figure 6.
Proposed model for attenuation of canonical TGF-β SMAD and activation of non-canonical TGF-β-NF-κB signaling pathways in HNSCC. TGF-β activates NF-κB signaling through a sequential regulation of TAK1 and IKK kinases leading to phosphorylation of IκBα, nuclear translocation and phosphorylation of NF-κB subunit p65 and activation of NF-κB downstream targets. Both TGF-β and NF-κB signaling induce expression of SMAD7, which in turn preferentially suppresses TGF-β induced canonical and NF-κB signaling, relative to TNF-α-induced NF-κB signaling.