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SMAD3 expression and regulation of fibroplasia in vocal fold injury

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Abstract

Objectives/Hypothesis—Recent reports highlight the efficacy of small interfering RNA (siRNA) targeting *SMAD3* to regulate transforming growth factor β (TGF- β)-mediated fibroplasia in vocal fold fibroblasts. The current study sought to investigate *SMAD3* expression during wound healing *in vivo* and quantify the downstream transcriptional events associated with *SMAD3* knockdown *in vitro*.

Study Design—*In vivo* and *in vitro*

Methods—Unilateral vocal fold injury was created in a rabbit model. *SMAD3* and *SMAD7* mRNA expression was quantified at 1 hour and 1, 3, 7, 14, 30, 60, and 90 days following injury. *In vitro*, multi-gene analysis technology was employed in our immortalized human vocal fold fibroblast cell line following TGF- β 1 stimulation +/- *SMAD3* knockdown across time points.

Results—*SMAD3* mRNA expression increased following injury; upregulation was significant at 3 and 7 days compared to control (both $p < 0.001$). *SMAD7* mRNA was also upregulated at 3, 7, and 14 days ($p = 0.02$, $p < 0.001$, and $p < 0.001$, respectively). *In vitro*, *SMAD3* knockdown reduced the expression of multiple pro-fibrotic, TGF- β signaling, and extracellular matrix metabolism genes at 6 and 24 hours following TGF- β 1 stimulation.

Conclusions—Cumulatively, these data support *SMAD3* as a potential master regulator of TGF- β -mediated fibrosis. *SMAD3* transcription peaked 7 days following injury. Multi-gene analysis indicated that the therapeutic effectiveness of *SMAD3* knockdown may be related to regulation of downstream mediators of fibroplasia and altered TGF- β signaling.

Keywords

Vocal fold; fibrosis; siRNA; SMAD3; transforming growth factor- β ; PCR array

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INTRODUCTION

Vocal fold (VF) fibrosis associated with mucosal injury is characterized by altered quantity and organization of extracellular matrix (ECM) components within the superficial lamina propria. This disrupted architecture frequently results in recalcitrant dysphonia due to the loss of vocal fold viscoelasticity and vibratory function.^{1,2} The tissue response following injury is complex and involves various cellular and acellular components, such as cytokines and chemokines. Transforming growth factor (TGF)- β is a pro-fibrotic mediator and TGF- β signaling underlies numerous fibroproliferative diseases across organs.^{3–6} Given that TGF- β is ubiquitous in pathological fibrosis, many have posited that therapeutics targeting TGF- β signaling represent an ideal strategy for the prevention and/or treatment of fibrosis.

Receptor-activated SMAD2 and SMAD3 are intimately related to TGF- β signaling. SMAD2 and SMAD3 are phosphorylated via TGF- β 1 binding to its receptor and heterodimerize with SMAD4. This complex then translocates to the nucleus and is a potent regulator of transcription. Conversely, SMAD7 is a competitive inhibitor of SMAD activation, and therefore, is thought to be anti-fibrotic. SMAD3 has been hypothesized by our group and others to be the master-regulator of TGF- β mediated fibrosis. As such, RNA interference-based methods to alter *SMAD3* transcription have emerged as a potentially powerful gene therapy for fibrotic diseases. Most research in this field has focused on the use of small interfering RNA (siRNA), which are short sequences of double-stranded messenger RNA (mRNA) that cause sequence-specific mRNA degradation, briefly decreasing expression of targeted genes.^{7,8} For example, a topical siRNA gel effectively attenuated *Smad3* expression in a mouse model, leading to resistance to radiation-induced cutaneous fibrosis.⁹ Regarding the vocal fold, our laboratory first described increased *SMAD3* mRNA expression following vocal fold injury, and recently achieved local VF knockdown of *SMAD3* expression *in vivo*.^{10,11} *In vitro*, our laboratory also confirmed that siRNA targeting *SMAD3* suppressed collagen gene expression in human vocal fold fibroblasts under stimulation with exogenous TGF- β 1,¹⁰ and knockdown of *SMAD3* limited TGF- β -induced contraction.¹² Cumulatively, these data suggest that gene therapy-mediated knockdown of *SMAD3* may have the potential to be an efficacious therapeutic to alter wound healing following vocal fold injury.

The vocal folds are an ideal tissue system for localized gene therapy, given their accessible anatomic position and improved methods for injection to the vocal fold mucosa. Current treatment approaches for VF injury include collagen or hyaluronic acid injection^{13,14} and/or fat or fascia implantation lateral to the vibratory margin to improve glottic valving.^{15,16} These treatments largely ignore the cellular and molecular mechanisms underlying the fibrotic tissue response. Biologically-based therapeutic strategies are likely to yield more favorable outcomes. Globally, we hypothesize that therapeutic manipulation of *SMAD3* signaling following vocal fold injury is likely to enhance patient outcomes, and specifically, localized gene therapy to the vocal fold mucosa represents a significant shift in clinical care paradigms.

In the current study, we sought to expand upon our previous, preliminary investigation¹⁰ and more comprehensively describe the temporal dynamics of *SMAD3* and *SMAD7* mRNA expression following vocal fold injury. Additionally, we sought to characterize the

downstream effects of *SMAD3* knockdown in human vocal fold fibroblasts as a means to elucidate both the role of *SMAD3* in the development of fibrosis and to enhance our understanding of the mechanisms underlying the potential therapeutic effects of *SMAD3* manipulation in the context of vocal fold wound healing.

MATERIALS AND METHODS

In Vivo

Vocal fold injury—The current protocol was approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. Unilateral vocal fold injury was induced in 32 adult New Zealand rabbits (2–3kg). Four uninjured animals served as a control cohort. Following paralytic anesthesia, the larynx was visualized by inserting a Karl Storz Benjamin slotted laryngoscope (Size 1; Karl Storz, Flanders, NJ). Under the guidance of 2.7mm, 0° or 30° telescope (Karl Storz) coupled to a camera and video monitor, the right vocal fold was injured by separating the lamina propria from the thyroarytenoid muscle via insertion of a 22-gauge needle at the lateral portion of right vocal fold. The lamina propria of the right vocal fold was removed with microforceps. Four animals were sacrificed at 1 hour and then 1, 3, 7, 14, 30, 60, and 90 days following injury. The larynges were harvested and the bilateral vocal folds were dissected under magnification.

Real-Time Polymerase Chain Reaction (qRT-PCR)—Total RNA was extracted from the tissue via the RNeasy Mini Kit (Qiagen, Valencia, CA). The quantity and quality of mRNA were evaluated via 260/280 ratio. RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction (PCR) employed the TaqMan Gene Expression kit (Life Technologies, Waltham, MA) and StepOne Plus (Applied Biosystems). Taqman probes for *SMAD3* and *SMAD7* employed as well as *GAPDH* as an endogenous control. mRNA expression levels were calculated compared to control using $\Delta\Delta Ct$ method.

Quantitative Statistical Analyses—For *in vivo* experimentation, a two-way factorial analysis of variance (ANOVA) was employed to determine a significant main effect. Bonferroni/Dunn post-hoc comparisons were then employed to investigate differences in gene expression across time points. If an interaction was observed between the treatment side and experimental time point, a one-way factorial ANOVA followed by a Bonferroni/Dunn post hoc test was performed. All data are expressed as means $\pm$ standard error.

In Vitro

Cell culture and transfection—An immortalized human vocal fold fibroblast cell line created in our laboratory was employed for all *in vitro* experimentation. This cell line, referred to as HVOX, has been shown to be stable through multiple population doublings;¹⁷ cells at passages 20–30 were employed in the current study. As described previously by our laboratory, siRNA was used to knockdown the expression of *SMAD3*.¹⁸ HVOX were transfected with 100pmol of *SMAD3* siRNA (Ambion, Grand Island, NY) in 5 μ l of Lipofectamine 2000 (Invitrogen, Grand Island, NY) for 6 hours. Transfection with nonsense siRNA (Ambion) served as a negative control. Transfection media was replaced with fresh

Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) $\pm$ TGF- β 1 (Life Technologies, Carlsbad, CA) at 10ng/mL for 6 hours and 24 hours. Cells cultured without TGF- β 1 stimulation served as the control at each time point. Recombinant human TGF- β 1 was employed for cell stimulation.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)—Total RNA was extracted at the appropriate study endpoint via the RNeasy Mini Kit (Qiagen, Valencia, CA). The quantity and quality of mRNA were evaluated via 260/280 ratio. RNA (5 μ g) was then reverse transcribed with the RT² first-strand kit (Qiagen). The Human Fibrosis RT² Profiler PCR Array (Qiagen) was employed, according to the recommended manufacturer's protocol. The array profiled 84 genes involved in the reparative response to tissue injury including genes encoding extracellular matrix remodeling enzymes, TGF- β signaling molecules and inflammatory cytokines, as well as additional genes involved in the development of fibrosis. Data output from this array were analyzed with the RT² Profiler PCR Array Data Analysis Software (v3.5). The genes included in this array is categorized by role in the fibrotic process and shown in Table 1. To examine condition and time effects, the gene profiling assay employed the generalized estimating equation (GEE) method to take into account differences at each time point. When reporting statistically significant findings, the Bonferroni correction was used to adjust for multiple comparisons.

RESULTS

***SMAD3* and *SMAD7* gene expression peaked 7 days following injury**

To investigate changes in gene expression during both the acute and more chronic phases of vocal fold injury and repair, qRT-PCR was performed for *SMAD3* and *SMAD7*. *SMAD3* gene expression in injured VF's increased continuously from 1 hour following injury to its peak at day 7. *SMAD3* mRNA was significantly increased on days 3 and 7 compared to control (~7 times baseline, $p < 0.0001$; ~17 times baseline, $p < 0.0001$, respectively). Following this peak, *SMAD3* remained upregulated on post-injury day 60; this increase, however, did not achieve statistical significance. Regarding *SMAD3* gene expression of the uninjured contralateral VF, a similar qualitative temporal distribution was observed. Interestingly, significantly increased *SMAD3* mRNA was observed on day 3 ($p = 0.02$; Figure 1A). *SMAD7* expression following injury was upregulated to a greater extent than *SMAD3*. Similarly to *SMAD3*, *SMAD7* expression peaked on day 7; significant differences were observed on days 3, 7, and 14 after injury ($p = 0.02$, $p < 0.0001$, and $p = 0.0002$, respectively). *SMAD7* expression in the contralateral vocal fold varied slightly, but did not achieve significant differences at any time point (Figure 1B).

SMAD3* knockdown was associated transcriptional changes *in vitro

Human vocal fold fibroblasts were stimulated with exogenous recombinant TGF- β 1 with or without *SMAD3* siRNA (*si-SMAD3*) and gene profiling was performed using a commercial assay. Significant shifts in transcription were noted (Table 2). Knockdown efficacy of *si-SMAD3* treatment was verified by suppression of *SMAD3* expression at both time points in this study. The most prominent differences between TGF- β 1 stimulation and treatment with *si-SMAD3* + TGF- β 1 were seen in genes related to fibrosis and TGF- β signaling (Figure 2).

Specifically, upregulation of *CTGF* and *SNAIL* induced by TGF- β 1 were attenuated by *si-SMAD3*. In addition, *ACTA2* was significantly downregulated at less than 0.5-fold at 6 hours and returned to baseline at 24 hours. Among the TGF- β superfamily genes, upregulation of *LTBP1*, *SMAD7*, *TGFB2*, *TGFB3*, *TGFBR1*, *THBS1*, and *THBS2* under TGF- β 1 stimulation decreased or returned to baseline levels with *si-SMAD3* at 6 hours or 24 hours. In addition, *CAV1*, *DCN* and *GREM1* were significantly downregulated. With regard to extracellular matrix genes, *COL3A1*, *LOX*, *MMP2*, *SERPINE1*, *SERPINH1*, *ITGA1*, *ITGB3*, *ITGB5*, and *ITGB6* were upregulated with 24-hour TGF- β 1 stimulation. This response was reduced with *si-SMAD3*; *SERPINE1* mRNA decreased from 22 times baseline to 4 times baseline. *COL1A1* expression also decreased with *si-SMAD3* treatment, but this change did not achieve significance (data not shown). Altered transcriptional patterns were also observed for growth factors related to wound healing; *EGF* and *PDGFA* decreased from 4 times baseline to normal baseline at 24 hours and from 3 times baseline to normal baseline at 6 hours, respectively. *HGF* expression was upregulated with *si-SMAD3* without TGF- β 1 stimulation, but downregulated under TGF- β 1 stimulation regardless of *si-SMAD3*.

DISCUSSION

Vocal fold fibrosis and the resulting vocal impairment are challenging clinical issues further complicated by the lack of effective treatment options. VF fibrosis affects both glottic closure and pliability of the vocal fold mucosa through a network of signaling cascades that culminate in the deposition of stiff, disorganized collagen. This collagen disrupts the native VF stroma throughout the layers of the lamina propria.¹⁹ Current treatment modalities primarily attempt to improve the mechanics of glottic closure via tissue bulking agents.

Our laboratory and others have identified SMAD3 as a principle mediator of fibrotic VF wound healing due to its role in TGF- β 1 signaling, which in turn, regulates numerous fibroblast activities including migration, proliferation, and production of extracellular matrix (ECM) proteins.^{12,17,20–23} SMAD7 is a homolog of SMAD3 and serves as an anti-fibrotic regulator opposing the actions of SMAD3 through competitive inhibition.^{24,25} Preliminary investigation conducted by our group demonstrated that *SMAD3* expression remained elevated 7 days after VF injury in a leporine model,¹⁰ but the full course of *SMAD3* and *SMAD7* expression through VF wound healing has not yet been reported. Our current data demonstrated a qualitative, but statistically insignificant increase in *SMAD3* mRNA expression 1 hour following injury and continuing *SMAD3* upregulation out to 60 days post-injury; however, this response did not achieve statistical significance. Peak *SMAD3* expression was observed on day 7 following injury. These findings have meaningful implications for the continued development of locally delivered therapeutics targeting *SMAD3*, and the translation of this technology to the clinical milieu. Specifically, therapeutics administered within one week of injury are likely to yield the greatest physiologic effects.

Upregulation of *SMAD7* follows a similar course as *SMAD3*, peaking at post-injury day 7. Of note, *SMAD7* expression remained elevated longer than *SMAD3*. Cumulatively, these findings support the concept that SMAD7 is a competitive inhibitor of SMAD3. Given the central role of SMAD3 in fibrosis, several laboratories have attempted to knockout or

knockdown its expression. Mice deficient in *Smad3* have been found to be resistant to multiple fibrotic processes, including carbon tetrachloride-induced hepatic fibrosis, glomerular fibrosis, bleomycin-induced pulmonary fibrosis, and others.^{3,26–29} In this regard, we hypothesize that manipulation of *SMAD3* via siRNA represents a promising potential therapeutic option for the treatment of vocal fold fibrosis. Lee et al recently demonstrated that suppression of *Smad3* using a topical siRNA gel conferred resistance to radiation-induced cutaneous fibrosis in a murine model.⁹ Our laboratory has also recently demonstrated that *SMAD3* siRNA reduced the fibrotic phenotype in vocal fold fibroblasts *in vitro*.¹² Most recently, our laboratory described successful knockdown of *SMAD3* via local siRNA injection in a leporine model of vocal fold injury.¹¹ Despite these encouraging findings, little is known regarding the effects of siRNA-mediated *SMAD3* knockdown on the complex signaling associated with the fibrotic response. To explore these issues with the ultimate goal of increasing insight into the mechanisms underlying the efficacy of *SMAD3* knockdown, expression of 84 genes involved in fibrosis was assayed in our human vocal fold fibroblast cell line after incubation with *SMAD3* siRNA and/or TGF- β 1. Two incubation time points (6 hours and 24 hours) were selected to examine the near-immediate and more delayed transcriptional responses without compromised cell health. Many significant changes in gene expression were noted; comprehensive review of these findings likely exceeds the scope of this manuscript. However, discussion of the most relevant transcriptional events is provided.

Consistent with previous reports, *SMAD3* was downregulated in response to TGF- β 1.^{30,31} Constitutive or over-expression of *SMAD3* during TGF- β 1 stimulation has been reported to induce apoptosis;^{31,32} this cell death can be avoided by downregulation of *SMAD3*.³³ This observation likely represents a feedback mechanism intended to limit excessive TGF- β 1 signaling. Similarly, TGF- β 1 curtails induction of its target genes via *SMAD7* upregulation.³⁴ In the current study, upregulation of *SMAD7* was observed in response to TGF- β 1. As expected, *SMAD3* expression decreased concurrently with *SMAD7* upregulation in HVOX treated with *SMAD3* siRNA and TGF- β 1. This finding is significant in that one potential morbidity of *SMAD3* knockdown could be a reduction in the inherent anti-fibrotic feedback system following injury; in HVOX, *SMAD3* knockdown did not alter *SMAD7* signaling.

Differentiation of fibroblasts into myofibroblasts is characterized by upregulation of alpha smooth muscle acting (α -SMA, encoded by ACTA2) in response to TGF- β 1 and other pro-fibrotic growth factors.³⁵ Myofibroblasts have increased contractile properties compared to fibroblasts, and prolonged myofibroblast presence and activity in the wound bed is a hallmark of fibrosis. Previous work from our laboratory demonstrated that *SMAD3* knockdown reduced the contractile properties of human vocal fold fibroblasts stimulated with TGF- β 1.¹² In the current study, α -SMA mRNA expression increased in response to TGF- β 1 stimulation. This response was wholly blunted in response to *SMAD3* knockdown. This result can likely be viewed as favorable and has the potential to be exploited clinically. Similarly, connective tissue growth factor (CTGF) is a pro-fibrotic connective tissue mitogen secreted by fibroblasts in response to TGF- β . CTGF stimulates proliferation of connective tissue cells and the production of extracellular matrix proteins, and has been found to be expressed in concurrently with TGF- β in a wide range of fibrotic disorders.³⁶ In vocal fold

fibrosis, CTGF expression has been shown to be *SMAD3*-dependant, raising the possibility that *SMAD3* regulates TGF- β 1-driven fibrosis both directly through its own actions, and indirectly through other factors.¹² CTGF expression decreased significantly in response to *SMAD3* knockdown.

Apoptosis of fibroblasts and myofibroblasts is an indicator of the resolution of the wound healing response. TGF- β 1 protects fibroblasts from plasmin-induced apoptosis via upregulation of *SERPINE1* (also known as plasminogen activator inhibitor-1) which reduces plasminogen activation.³⁷ This mechanism is thought to promote prolonged survival of fibroblasts and myofibroblasts in numerous chronic fibrotic disorders, but has never been examined in the vocal folds. In HVOX, *SERPINE1* was upregulated in response to 24 hours of TGF- β 1 stimulation. This increased expression was suppressed by *SMAD3* knockdown. These data support the possibility that vocal fold fibroblast apoptosis following injury is *SMAD3*-dependent.

Extracellular matrix (ECM) is dynamic and an imbalance between proliferation and degradation underlies fibrotic disorders. Lysyl oxidase (*LOX*) and collagen type III (*COL3A1*) are key ECM components, both of which were upregulated in TGF- β 1 stimulated HVOX. This response was reduced with *SMAD3* knockdown. LOX is an amine oxidase necessary for collagen cross-linking that contributes to fibrosis by increasing the resistance of collagen and extracellular matrix components to proteolytic degradation.³⁸ LOX has been found to be upregulated in animal models of pulmonary fibrosis, although its precise role in vocal fold fibrosis has not yet been described.^{38–40} Collagen type III has been found to be increased after vocal fold injury *in vivo*.⁴¹ TGF- β 1 exerts an extensive influence on ECM dynamics, aspects of which appear *SMAD3* dependent.

Integrins are transmembrane receptors responsible for cell adhesion to the extracellular matrix that influences the wound healing response. One such molecule is α V β 6 integrin (integrin β 6, encoded by *ITGB6*), which activates latent TGF- β by releasing it from its propeptide complex.⁴² Mice homo- and heterozygous for deletions of this gene have demonstrated robust resistance to pulmonary fibrosis, as have animals receiving anti-Itgb6 therapy.^{42,43} In the current investigation, integrin β 6 expression increased in response to TGF- β 1. Knockdown of *SMAD3* reduced this response. The precise role of this integrin in the vocal folds is not clear and likely warrants further investigation.

The current study described the temporal dynamics of both *SMAD3* and *SMAD7* expression following iatrogenic vocal fold injury. In addition, many previously unknown regulatory effects of *SMAD3* knockdown were elucidated *in vitro* via qRT-PCR array technology. This work is, however, not without limitations. For instance, no functional data were obtained *in vivo*. Furthermore, the extent of fibrosis following injury was not quantified. In addition, *in vitro* array technology, although interesting, is inherently exploratory rather than hypothesis-driven and the data yield can be overwhelming. As such, we attempted to highlight only those findings most clinically applicable and mechanistically feasible, but future studies are required to verify the impact of these transcriptional events.

CONCLUSION

The current study presents a detailed examination of the temporal dynamics of *SMAD3* and *SMAD7* expression from 1 hour through 90 days following vocal fold injury. Based on these data, *SMAD3* modulating therapeutics are likely to have the greatest impact if administered within the first week after injury and local administration of therapy to the injured vocal fold may be sufficient to reduce *SMAD3* upregulation during wound healing. Additionally, modulation of *SMAD3* expression had marked effects on transcriptional events related to fibroplasia. This exploratory work not only provides fundamental, mechanistic insight into the regulatory role of SMAD3 in vocal fold injury and repair, but also provides data regarding other potential targets for physiologically-based therapeutics.

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Figure 1.

SMAD3 and *SMAD7* mRNA expression changes following rabbit vocal fold injury.

SMAD3 gene expression of injured right vocal fold increased at 1 hour post-injury and peaked at day 7; significant differences were observed at days 3 and 7 compared to control (A) (both $p < 0.001$). *SMAD7* expression peaked on day 7; significant differences were observed at days 3, 7, and 14 after injury (B) ($p = 0.02$, $p < 0.001$, and $p < 0.001$, respectively). Data presented as mean $\pm$ SEM ($n = 4$ per group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Figure 2.

Clustergram of eight representative genes. mRNA expression was examined 6 and 24 hours following TGF-β1 stimulation +/- siRNA for *SMAD3*; the average of each condition is displayed. TGF-β1 = transforming growth factor-β1.

Table 1Functional groups of genes related to TGF- β 1 stimulation +/- Smad3 siRNA

<u>Pro-Fibrotic</u>	
<i>ACTA2</i>	Actin, alpha 2, smooth muscle, aorta
<i>CTGF</i>	Connective tissue growth factor
<u>TGF-β Superfamily Members</u>	
<i>BMP7</i>	Bone morphogenetic protein 7
<i>CAV1</i>	Caveolin 1, caveolae protein, 22kDa
<i>DCN</i>	Decorin
<i>ENG</i>	Endoglin
<i>GREM1</i>	Gremlin 1
<i>INHBE</i>	inhibin, beta E
<i>LTBP1</i>	Latent transforming growth factor beta binding protein 1
<i>SMAD2</i>	SMAD family member 2
<i>SMAD3</i>	SMAD family member 3
<i>SMAD4</i>	SMAD family member 4
<i>SMAD6</i>	SMAD family member 6
<i>SMAD7</i>	SMAD family member 7
<i>TGFB1</i>	Transforming growth factor, beta 1
<i>TGFB2</i>	Transforming growth factor, beta 2
<i>TGFB3</i>	Transforming growth factor, beta 3
<i>TGFBRI</i>	Transforming growth factor, beta receptor 1
<i>TGFBRII</i>	Transforming growth factor, beta receptor II (70/80kDa)
<i>TGFI1</i>	TGFB-induced factor homeobox 1
<i>THBS1</i>	Thrombospondin 1
<i>THBS2</i>	Thrombospondin 2
<u>ECM Structural Constituents</u>	
<i>COL1A2</i>	Collagen, type III, alpha 1
<i>COL3A1</i>	Collagen, type I, alpha 2
<u>ECM Remodeling Enzymes</u>	
<i>LOX</i>	Lysyl oxidase
<i>MMP1</i>	Matrix metalloproteinase 1 (interstitial collagenase)
<i>MMP13</i>	Matrix metalloproteinase 13 (collagenase 3)
<i>MMP14</i>	Matrix metalloproteinase 14 (membrane-inserted)
<i>MMP2</i>	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
<i>MMP3</i>	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)
<i>MMP8</i>	Matrix metalloproteinase 8 (neutrophil collagenase)
<i>MMP9</i>	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
<i>PLAT</i>	Plasminogen activator, tissue
<i>PLAU</i>	Plasminogen activator, urokinase
<i>PLG</i>	Plasminogen
<i>SERPINA1</i>	Serpin peptidase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 1

<i>SERPINE1</i>	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
<i>SERPINH1</i>	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
<i>TIMP1</i>	TIMP metalloproteinase inhibitor 1
<i>TIMP2</i>	TIMP metalloproteinase inhibitor 2
<i>TIMP3</i>	TIMP metalloproteinase inhibitor 3
<i>TIMP4</i>	TIMP metalloproteinase inhibitor 4

Cell Adhesion Molecules

<i>ITGA1</i>	Integrin, alpha 1
<i>ITGA2</i>	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
<i>ITGA3</i>	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
<i>ITGAV</i>	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
<i>ITGB1</i>	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
<i>ITGB3</i>	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
<i>ITGB5</i>	Integrin, beta 5
<i>ITGB6</i>	Integrin, beta 6
<i>ITGB8</i>	Integrin, beta 8

Inflammatory Cytokines and Chemokines

<i>CCL11</i>	Chemokine (C-C motif) ligand 11
<i>CCL2</i>	Chemokine (C-C motif) ligand 2
<i>CCL3</i>	Chemokine (C-C motif) ligand 3
<i>CCR2</i>	Chemokine (C-C motif) receptor 2
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4
<i>IFNG</i>	Interferon, gamma
<i>IL10</i>	Interleukin 10
<i>IL13</i>	Interleukin 13
<i>IL13RA2</i>	Interleukin 13 receptor, alpha 2
<i>IL1A</i>	Interleukin 1, alpha
<i>IL1B</i>	Interleukin 1, beta
<i>IL4</i>	Interleukin 4
<i>IL5</i>	Interleukin 5 (colony-stimulating factor, eosinophil)
<i>ILK</i>	Integrin-linked kinase
<i>TNF</i>	Tumor necrosis factor

Growth Factors

<i>AGT</i>	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
<i>EDN1</i>	Endothelin 1
<i>EGF</i>	Epidermal growth factor
<i>HGF</i>	Hepatocyte growth factor (hepapoietin A; scatter factor)
<i>PDGFA</i>	Platelet-derived growth factor alpha polypeptide
<i>PDGFB</i>	Platelet-derived growth factor beta polypeptide
<i>VEGFA</i>	Vascular endothelial growth factor A

Transcription Factors

<i>CEBPB</i>	CCAAT/enhancer binding protein (C/EBP), beta
<i>JUN</i>	Jun proto-oncogene

<i>MYC</i>	V-myc myelocytomatosis viral oncogene homolog (avian)
<i>NFKB1</i>	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
<i>SP1</i>	Sp1 transcription factor
<i>STAT1</i>	Signal transducer and activator of transcription 1, 91kDa
<i>STAT6</i>	Signal transducer and activator of transcription 6, interleukin-4 induced

EMT

<i>AKT1</i>	V-akt murine thymoma viral oncogene homolog 1
<i>SNAIL</i>	Snail homolog 1 (Drosophila)

Other Fibrosis Genes

<i>BCL2</i>	B-cell CLL/lymphoma 2
<i>FASLG</i>	Fas ligand (TNF superfamily, member 6)

ECM = extra cellular matrix; TGF- β = transforming growth factor- β ; EMT = epithelial-to-mesenchymal transition.

Table 2

Transcription was examined 6 and 24 hours following culture under TGF- β 1 stimulation +/- *SMAD3* siRNA in HVOX. Genes that increased over 2 times or decreased to less than 0.5 fold compared to control are listed (all assays were performed in triplicate; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; downregulation: $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, and $\dagger\dagger\dagger p < 0.001$). RE=relative mRNA expression to control; TGF- β 1 = transforming growth factor- β 1.

Gene	si-SMAD3			TGF- β 1			si-SMAD3 + TGF- β 1		
	6 hr	RE	P	24 hr	RE	P	6 hr	RE	P
Pro-Fibrotic									
<i>ACTA2</i>	0.3745	$\dagger$			7.9269	*	0.4988	$\dagger$	
<i>CTGF</i>	0.2929	$\dagger$			4.9401	**			2.2444 *
Signal Transduction									
TGFβ Superfamily Members									
<i>CAV1</i>	0.3853	$\dagger$					0.2368	$\dagger$	
<i>DCN</i>		0.4761	$\dagger$					0.4637	$\dagger$
<i>GREM1</i>		0.3324	$\dagger$				0.496	$\dagger\dagger$	0.3399 $\dagger$
<i>LTBP1</i>						2.4092 *			
<i>SMAD3</i>	0.0299	$\dagger\dagger$		0.0255 $\dagger\dagger\dagger$		0.3051 $\dagger\dagger$	0.0277 $\dagger\dagger$	0.0179 $\dagger\dagger\dagger$	
<i>SMAD6</i>					0.4199 $\dagger$	0.2333 $\dagger$	0.4522 $\dagger$	0.4247 $\dagger$	
<i>SMAD7</i>					4.7848 **	8.4034 *		3.1747 *	
<i>TGFB2</i>						9.6053 *			
<i>TGFB3</i>						3.0571 **			
<i>TGFBR1</i>						3.0099 *			
<i>THBS1</i>	0.2261	$\dagger$		0.3032 $\dagger$		11.4125 **			
<i>THBS2</i>						3.5577 **			
Transcription Factors									
<i>JUN</i>						3.1276 **			
<i>MYC</i>						4.4466 **			
<i>NFKB1</i>	0.4614	$\dagger\dagger$							
<i>SPI</i>							0.4768	$\dagger$	
Extracellular Matrix(ECM) & Cell Adhesion Molecules									

Gene	si-SMAD3			TGF- β 1			si-SMAD3 + TGF- β 1		
	6 hr	P	RE	24 hr	P	RE	6 hr	P	RE
ECM Structural Constituents									
<i>COL3A1</i>						2.0156		**	
ECM Remodeling Enzymes									
<i>LOX</i>						3.1992		**	
<i>MMP13</i>	4.5242	*							
<i>MMP2</i>						3.0494		*	
<i>MMP3</i>	2.3036	*	3.9438	*			2.5803	*	
<i>PLAT</i>							0.4191	†	
<i>PLAU</i>			2.4158	*		0.1722	†††		
<i>SERPINE1</i>						22.5233		*	4.0333
<i>SERPINH1</i>						3.5018		**	
Cell Adhesion Molecules									
<i>ITGA1</i>						2.9919		**	
<i>ITGB3</i>						2.8005		*	
<i>ITGB5</i>						3.0017		**	
<i>ITGB6</i>	0.1671	†				11.4155	*		
<i>ITGB8</i>						0.3758	†	0.1086	†
							0.2591	†	0.2435
Inflammatory Cytokines & Chemokines									
<i>CCL2</i>						0.4671		†	
<i>IL1B</i>	0.2927	††					0.2905		†
Growth Factors									
<i>EDN1</i>						5.8535		**	3.1676
<i>EGF</i>						3.9084		**	0.3494
<i>HGF</i>	2.5281	*	3.8214	**		0.1349		†	0.426
<i>PDGFA</i>						3.6352	*		
Epithelial-to-Mesenchymal Transition(EMT)									
<i>SNAIL</i>						3.9446	*	**	3.1134