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Endoglin promotes TGF- β /Smad1 signaling in scleroderma fibroblasts

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Abstract

TGF- β is the primary inducer of extracellular matrix (ECM) proteins in scleroderma (systemic sclerosis, SSc). Previous studies indicate that in a subset of SSc fibroblasts TGF- β signaling is activated via elevated levels of activin receptor-like kinase (ALK) 1 and phosphorylated Smad1 (pSmad1). The goal of this study was to determine the role of endoglin/ALK1 in TGF- β /Smad1 signaling in SSc fibroblasts. In SSc fibroblasts, increased levels of endoglin correlated with high levels of pSmad1, collagen, and connective tissue growth factor (CCN2). Endoglin depletion via siRNA in SSc fibroblasts inhibited pSmad1 but did not affect pSmad2/3. Following endoglin depletion mRNA and protein levels of collagen and CCN2 were significantly decreased in SSc fibroblasts but remained unchanged in normal fibroblasts. ALK1 was expressed at similar levels in SSc and normal fibroblasts. Depletion of ALK1 resulted in inhibition of pSmad1 and a moderate but significant reduction of mRNA and protein levels of collagen and CCN2 in SSc fibroblasts. Furthermore, constitutively high levels of endoglin were found in complexes with ALK1 in SSc fibroblasts. Overexpression of constitutively active ALK1 (caALK1) in normal and SSc fibroblasts led to a moderate increase of collagen and CCN2. However, caALK1 potently induced endothelin 1 (ET-1) mRNA and protein levels in SSc fibroblasts. Additional experiments demonstrated that endoglin and ALK1 mediate TGF- β induction of ET-1 in SSc and normal fibroblasts. In conclusion, this study has revealed an important profibrotic role of endoglin in SSc fibroblasts. The endoglin/ALK1/Smad1 pathway could be a therapeutic target in patients with SSc if appropriately blocked.

INTRODUCTION

Scleroderma, also known as systemic sclerosis (SSc), is a complex autoimmune disease of unknown etiology that affects the connective tissues. SSc is characterized by excessive deposition of extracellular matrix (ECM) proteins in the skin and multiple internal organs, degeneration of the microvasculature, and abnormalities in the cellular and humoral immune system [1]. Fibrosis, the most severe consequence of SSc pathology, culminates in the loss of tissue function and organ failure. TGF- β is a central mediator of SSc fibrosis via activation of downstream Smad and non-Smad signaling pathways. Canonical TGF- β

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signaling occurs through a heteromeric complex of a type I receptor, activin receptor-like kinase (ALK) 5, and a type II receptor, T β RII. Ligand binding to the receptor complex activates ALK5 to phosphorylate Smad2/3 which then complex with Smad4, translocate into the nucleus, and affect target gene expression [2]. It has been demonstrated that in endothelial cells TGF- β signaling can occur through both canonical ALK5 and Smad2/3 as well as through ALK1 type I receptor and Smad1/5/8 [3]. TGF- β phosphorylation of Smad1 in normal epithelial cell lines, epithelium-derived tumor cells, and fibroblasts was recently shown, demonstrating that the TGF- β /Smad1 signaling pathway can occur in various cell types [4]. In endothelial cells, TGF- β signaling through ALK1 and Smad1/5/8 is dependent on the ALK5 kinase and expression of endoglin, a type III TGF- β receptor [5,6].

Endoglin (alternatively named CD105) is an auxiliary transmembrane type III TGF- β receptor that plays an intrinsic role in vessel wall homeostasis [7]. Mutations in endoglin or in the type I receptor ALK1 have been shown to cause the vascular disorder hereditary hemorrhagic telangiectasia (HHT), suggesting that endoglin and ALK1 function in the same pathway. Endoglin is considered a marker for endothelial cells where it is highly expressed but it is also moderately expressed in monocytes, neural crest stem cells, adult bone marrow hematopoietic stem cells, macrophages, stromal cells, and fibroblasts [7,8,9]. Endoglin can bind TGF- β 1 and TGF- β 3 isoforms in the presence of a type II receptor and modulates ligand affinity for the receptor [10,11]. The extracellular and cytoplasmic domains of endoglin interact with T β RII and type I receptors ALK5 and ALK1 in the absence of ligand and alter the phosphorylation state of these TGF- β receptors [12,13].

In addition to the intrinsic role in HHT, endoglin is implicated in several pathological conditions. High circulating levels of soluble endoglin have been detected in the sera of pre-eclamptic women [14]. Endoglin is expressed in tumors associated with the endothelium including breast, prostate, and cervical cancers [7,15,16,17,18]. Interestingly, high levels of endoglin have been demonstrated in liver biopsies and patient serum samples of liver fibrosis and in the interstitium in human renal fibrosis [8,19,20,21]. In SSc, endoglin expression increases with disease progression and is overexpressed in dermal endothelial cells, fibroblasts, and serum strongly suggesting a pathogenic role in SSc [22,23,24,25,26].

In SSc fibroblasts the balance between Smad2/3 and Smad1/5/8 signaling is altered. Activation of Smad1 signaling in SSc is evidenced by increased expression of pSmad1 in SSc skin and cultured fibroblasts [27]. Additionally, siRNA depletion of Smad1 normalized SSc fibroblast production of collagen I and connective tissue growth factor (CCN2) [27]. Thus, high levels of endoglin expression may play an important role in SSc fibrosis by promoting “endothelial like” TGF- β signaling through Smad1 phosphorylation [25]. The goal of this work is to understand the potential contribution of endoglin and ALK1 to TGF- β /Smad1 signaling and the fibrotic phenotype in SSc fibroblasts.

MATERIALS AND METHODS

Fibroblast Culture

Skin biopsy samples were taken from the affected areas of the dorsal forearm of SSc patients who had diffuse cutaneous SSc according to the American College of Rheumatology criteria for SSc [28] and had not undergone any treatment for SSc at the time of biopsy. Normal control biopsies were taken from the dorsal forearm of healthy donors matched with each SSc patient for age, gender, and race. Informed consent was obtained from all study subjects prior to biopsy and studies were performed in compliance with the Institutional Review Board of Human Studies, Medical University of South Carolina. To establish fibroblast cultures, biopsy tissue was rinsed with antibiotic-antimycotic solution (Life Technologies) and then placed in 1 ml of 0.25% collagenase solution (Sigma) for 24-hour incubation at

37°C. The resulting 1 ml was mixed with 5 ml of media (DMEM + 20% FCS) then plated in a 25cm² flask and grown to confluence. The confluent culture was designated passage zero and used for experiments.

Quantitative real time PCR

Total RNA was isolated from confluent fibroblasts using Tri reagent (MRC Inc.) according to the manufacturer's instructions. 1 µg of RNA was used for reverse transcription cDNA synthesis in 20 µl reaction volume using random primers and then diluted to 40 µl. Quantitative real time PCR (qPCR) was carried out using IQ Sybr green mix and an Icyler machine (Biorad) using 1 µl of diluted cDNA in triplicate with β-actin as the internal control. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 1 min. Melt curve analysis of the PCR products confirmed the absence of secondary products.

Western Blotting

Confluent fibroblasts were lysed in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris HCl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P40, 0.5% sodium deoxycholate, 1mM phenylmethylsulfonyl fluoride). Protein concentrations were quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 3% milk in Tris-Buffered Saline-Tween (Collagen I, endoglin, pSmad1, Smad1, pSmad2, pSmad3, ALK1, β-actin) or 2% gelatin (CCN2) for 1 hour at room temperature and probed with primary antibody overnight at 4°C. After TTBS washes, membranes were probed with horseradish peroxidase-conjugated secondary antibody against the appropriate species. Proteins levels were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantified using Image J densitometry software. Antibodies for collagen (Southern Biotechnology), CCN2, collagen I, ALK1 (Santa Cruz Biotechnology), Endoglin (DAKO), pSmad1 (Santa Cruz Biotechnology), Smad1, pSmad2, pSmad3, Smad3 (Cell Signaling) were used at a 1:1000 dilution. β-actin (Sigma) loading control primary antibody was used at a dilution of 1:5000.

Co-Immunoprecipitation (Co-IP)

Cell lysates (100ug) from normal and SSc fibroblasts were prepared in RIPA buffer. Samples were co-immunoprecipitated with α-Eng antibody (Santa Cruz Biotechnology #20632) immobilized to agarose beads according to Pierce Co-Immunoprecipitation Kit (Thermo Scientific #26149) protocol. Resins were washed with IP Lysis/Wash Buffer and the bound protein complexes were eluted with the Elution Buffer. Samples were boiled and separated by the 10% SDS-PAGE followed by western blotting with ALK1 antibody.

Endoglin shRNA adenovirus

The endoglin shRNA adenovirus was made by subcloning GATCCCAGAAAGAGCTTGTTCGCGATTCAAGAGATGCGCAACAAGCTCTTTCTTTGGAAA sequence in the TER vector [29] using the *Bgl*II and *Hind*III restriction sites. H1-RNA promoters and inserts of pTER-shRNA endoglin were ligated into pAdTrap using *Sal*I and *Xba*I restriction sites [30]. After pAd-Track shRNA endoglin recombination with pAdEasy-1, the plasmids obtained were transfected into 293HEK cells for adenovirus amplification. shRNA experiments were performed on confluent fibroblasts. After 24 hours in serum free media, endoglin or scrambled control shRNA virus was added to cells for 72 hours. Serum free media was removed the following day and DMEM+10% serum was added to cells for 48 hours before harvesting and lysing.

Constitutively active ALK1 adenovirus

The constitutively active ALK1 (caALK1) adenovirus was described previously [3]. A glutamine amino acid residue of the ALK1 gene was mutated to aspartic acid to mimic phosphorylation and confer constitutive activation. Experiments were performed on confluent fibroblasts. After 24 hours in serum free media, caALK1 was added to cells for 72 hours before harvesting and lysing.

Transfection with siRNA against human ALK1

The ON-TARGETplus SMARTpool siRNA oligonucleotides specific for human ALK1 were obtained from Dharmacon Inc. (Lafayette, USA). Human skin fibroblasts were transiently transfected with 10nM ALK1 siRNA or Negative siRNA control using DharmaFECT 1 transfection reagent. Briefly, DharmaFECT 1 was added to OptiMEM and incubated for 5 min. siALK1 or non-specific siRNA was added to OptiMEM, and this mix was transferred into DharmaFECT 1 and OptiMEM mix after 5 min. The mix was incubated for another 20 min at room temperature with constant shaking. The siRNA mix was added to the cells for 72 h, followed by cell lysis. Efficiency of gene knockdown was assessed by real-time PCR and Western Blot.

Endothelin (ET-1) bioassay

The ET-1 bioassay was performed according to the protocol supplied with the kit from Assay Designs (#900-020A). Standards and samples were incubated in supplied precoated 96-well plates, washed, incubated with horseradish peroxidase labeled anti-ET1 antibody and washed again before adding the provided tetramethylbenzidine substrate and measuring the absorbance. The limit of detection for ET-1 was 0.41 pg/ml.

RESULTS

Elevated endoglin mRNA and protein levels correlate with profibrotic markers in SSc fibroblasts

Previous studies indicate that endoglin expression is increased in SSc fibroblasts at the mRNA and protein level [25]. To confirm this finding and to determine expression levels of ALK1 in SSc, endoglin and ALK1 mRNA and protein expression was measured in cultured SSc and normal (NS) fibroblasts. Average mRNA expression of endoglin was significantly increased in SSc fibroblasts while mRNA expression of the type I receptor ALK1 was not altered (Figure 1A). TGF- β can moderately activate transcription of endoglin in endothelial cells through a TGF- β response element in the promoter region [31]. To determine if TGF- β signaling can increase endoglin transcription and protein expression, normal fibroblasts were treated with 2.5 ng/ml of TGF- β for 24 hours. Endoglin mRNA and protein expression were measured in untreated and treated fibroblasts using qRT-PCR and western blotting. No significant difference was detected in endoglin mRNA or protein levels following TGF- β treatment indicating that TGF- β signaling does not activate transcription of endoglin in dermal fibroblasts (Figure 1B).

Western blotting was performed on cell lysates to measure protein expression of endoglin and ALK1 in SSc and NS fibroblasts. In the majority of SSc fibroblasts (n=6 out of 9), increased expression of endoglin protein was observed and ALK1 protein levels were not altered (Figure 1C). Extracellular matrix proteins CCN2 and collagen I were elevated in the majority of SSc fibroblasts. The collagen antibody is reactive against two bands for the α 1 (upper) and α 2 (lower) chains of collagen I. Using densitometry software, average expression of endoglin, CCN2, and pSmad1 protein was calculated in normal matched control fibroblasts and in SSc fibroblasts with increased expression of endoglin. In these six

pairs, a significant increase in average endoglin protein expression correlated with a significant increase in average expression of pSmad1, collagen I, and CCN2 (Figure 1D).

Endoglin inhibition in SSc fibroblasts reduces expression of fibrotic genes and proteins

To determine if expression of endoglin is important for TGF- β /Smad1 signaling and ECM production in SSc fibroblasts, endoglin was inhibited using an shRNA adenovirus (siENG). Treatment with a non-silencing adenoviral shRNA was used as a control (siSCR). Increasing doses of siENG in normal dermal fibroblasts produced a dose-response curve of endoglin inhibition at the mRNA and protein level of expression (Figure 2A). Endoglin inhibition in NS fibroblasts resulted in a significant decrease in ENG gene expression while COL1A1, COL1A2, and CCN2 genes were not significantly altered. In SSc fibroblasts endoglin inhibition promoted a significant decrease in ENG, COL1A1, COL1A2, and CCN2 expression (Figure 2B). Protein expression of endoglin, pSmad1, pSmad3, CCN2, and collagen I was measured using western blotting in normal and SSc fibroblast cultures following endoglin inhibition. Collagen I protein was measured in the media in order to detect immediate changes in expression levels and all other proteins were measured in the cell layer (Figure 2C). Following endoglin inhibition a slight decrease in collagen I protein expression was observed in some normal fibroblasts, although overall there were no significant changes in pSmad1, pSmad3, collagen I, and CCN2 protein levels in normal fibroblasts treated with siENG. In the majority of SSc fibroblasts, basal expression of endoglin, pSmad1, pSmad3, CCN2, and collagen was elevated in comparison to NS fibroblasts. In SSc fibroblasts treated with siENG, endoglin, pSmad1, CCN2, and collagen I protein levels were reduced while pSmad3 levels were unchanged. These results suggest that endoglin positively regulates ECM production in SSc fibroblasts.

ALK1 inhibition reduces expression of fibrotic genes and proteins in SSc and normal fibroblasts

To determine the contribution of ALK1 receptor signaling to the expression of profibrotic genes, ALK1 was inhibited using siRNA. Effective inhibition of ALK1 mRNA and protein was observed after 72-hour treatment with 10nM siRNA (Figure 3A). Inhibition of ALK1 resulted in a modest decrease of COL1A1, COL1A2, and CCN2 genes in normal fibroblasts, while a consistently greater decrease of these genes was observed in SSc fibroblasts (Figure 3B). In SSc fibroblasts treated with siALK1, protein levels of ALK1, pSmad1, CCN2, and collagen I were reduced while pSmad2 levels remained unchanged. Inhibition of ALK1 had moderate and variable effects on CCN2 and collagen I protein levels in control fibroblasts. Together, these results indicate that endoglin/ALK1 signaling is responsible for the constitutive activation of Smad1 signaling in SSc fibroblasts. To further corroborate this notion, association of endoglin with ALK1 was examined in unstimulated SSc fibroblasts and in normal fibroblasts stimulated with TGF- β . As shown in Figure 3D, endoglin/ALK1 complexes were present in unstimulated control fibroblasts (lane 1 and 2) and were increased upon TGF- β stimulation (lane 3). Considerably higher levels of ALK1 were associated with endoglin in 3 out of 4 SSc fibroblast strains (lanes 4–7).

Overexpression of constitutively active ALK1 in normal dermal fibroblasts moderately induces a fibrotic response

TGF- β binding to ALK1 has been observed *in vitro* in COS-7 and endothelial cells [32,33,34] and is required for TGF- β /Smad1 signaling in endothelial cells [6]. Additionally, a constitutive association between ALK1 and ALK5 was observed in the adALK5 model of SSc based on overexpression of ALK5 to mimic the SSc phenotype [35]. To determine if activated ALK1 is capable of inducing fibrotic proteins, overexpression of constitutively active ALK1 (caALK1) in normal dermal fibroblasts was performed using adenovirus. Adenovirus expressing GFP alone (AdGo) was used as a control. As expected, caALK1

induced phosphorylation of Smad1 and expression of a Smad1 target gene, Id1 (Figure 4A). In comparison to TGF- β , which robustly stimulated COL1A1, COL1A2, and CCN2 mRNAs, overexpression of caALK1 had only a modest effect on expression of these genes (Figure 4B). SSc fibroblasts were slightly more responsive than control fibroblasts to the effect of caALK1 with respect to induction of ECM genes. Consistent with the mRNA level, a modest increase of CCN2 and collagen proteins was observed (Figure 5C). These results indicate that the presence of constitutively activated ALK1 has only a moderate influence on expression of profibrotic genes in NS and SSc fibroblasts.

caALK1 is a potent inducer of Endothelin-1 (ET-1) in SSc fibroblasts

ET-1 has emerged as important mediator of vascular disease in SSc. In addition, ET-1 is an inducer of collagen in fibroblasts and may work in concert with TGF- β to promote fibrotic changes in SSc fibroblasts [36]. Since expression of caALK1 in endothelial cells results in an increase of ET-1 gene expression [37], ET-1 mRNA and protein were analyzed in control and SSc fibroblasts following overexpression of caALK1. In control fibroblasts caALK1 moderately stimulated ET-1 mRNA and protein levels in a dose-dependent manner (Figure 5A). The stimulatory effects of caALK1 on ET-1 mRNA levels were significantly greater in SSc fibroblasts with the highest doses of caALK1 inducing over 20-fold increase of ET-1 mRNA. In agreement with previous studies [38], we observed elevated expression of ET-1 in SSc fibroblasts in comparison to control fibroblasts. Inhibition of endoglin reduced basal expression of ET-1 mRNA and protein in SSc fibroblasts, but not in normal fibroblasts (Figure 5B). In addition, TGF- β stimulation of ET-1 mRNA and protein was noticeably reduced in normal fibroblasts and reduced to a greater extent in SSc fibroblasts, following endoglin inhibition (Figure 5B). Likewise, inhibition of ALK1 reduced basal and TGF- β induced upregulation of ET-1 in normal and SSc fibroblasts (Figure 5C), suggesting that the endoglin/ALK1 pathway contributes to regulation of the ET-1 gene in dermal fibroblasts. SSc fibroblasts were consistently more responsive to the above treatments, further supporting the profibrotic role of endoglin/ALK1 axis in SSc.

DISCUSSION

This study demonstrates that endoglin is overexpressed in SSc fibroblasts and contributes to activation of the profibrotic gene program in these cells. Elevated expression of endoglin correlated with increased protein levels of pSmad1, collagen I, and CCN2 in SSc fibroblasts indicating that endoglin expression is associated with a fibrotic phenotype. Inhibition of endoglin in SSc fibroblasts led to a reduction in pSmad1 levels while pSmad3 levels were unaltered. Downstream of siENG mediated inhibition of pSmad1, reduction in CCN2 and collagen I protein levels was observed suggesting that endoglin and pSmad1 are required for ECM production in SSc fibroblasts. Consistent with activation of Smad1 signaling, constitutively high levels of Endoglin were found in complexes with ALK1 in SSc fibroblasts. In contrast to SSc fibroblasts, endoglin inhibition in normal dermal fibroblasts had no significant effect on fibrotic protein production.

Published studies suggest that endoglin may be an important modulator of the TGF- β signaling in many different cell types in addition to its well described role as a promoter of ALK1/Smad1 signaling in endothelial cells. Adding further complexity to endoglin signaling is the finding that L- and S-endoglin may play opposing roles in TGF- β -stimulated ECM production. For example, in the L6E9 cell line L-endoglin enhanced the ALK1 pathway and repressed TGF- β -induced collagen I and CCN2 proteins, while S-endoglin enhanced the ALK5 pathway and induced ECM proteins [39]. In contrast to these studies, transient overexpression of endoglin in hepatic stellate cells resulted in a strong increase of pSmad1 and α -smooth muscle cells actin upon TGF- β stimulation [40]. Furthermore, increased endoglin expression was present in transdifferentiating hepatic stellate cells and in

two models of liver fibrosis [40]. In agreement with the latter study, positive induction of Smad1 phosphorylation by endoglin in chondrocytes was also observed [41]. Interestingly, increased levels of endoglin were present in dedifferentiated chondrocytes and in the cartilage of individuals with OA and positively correlated with collagen type I [41]. Another study has shown that radiation-induced fibrosis of the kidneys is reduced in mice heterozygous for endoglin expression, providing further support for a positive role of endoglin in fibrosis [42]. While antifibrotic effects of endoglin were also described in some studies [25,43], the majority of studies support a profibrotic role of endoglin. Specific endoglin-dependent profibrotic mechanisms are still incompletely understood and in addition to activation of the ALK1/Smad1 pathway, may involve modulation of other signaling molecules.

Published studies indicate that endoglin functions as a modulator of the balance between TGF- β /ALK1/Smad1 and TGF- β /ALK5/Smad3 signaling. In this study we have also observed that constitutive phosphorylation of Smad1 in SSc fibroblasts was dependent on the presence of endoglin and ALK1. However, these treatments had no effect on the levels of pSmad2/3. This suggests that the pathways that regulate the balance between ALK1/Smad1 and ALK5/Smad2/3 are disrupted in SSc fibroblasts. Furthermore, depletion of endoglin significantly downregulated CCN2 and collagen in a subset of SSc fibroblasts having elevated levels of pSmad1, suggesting that expression of profibrotic genes in these fibroblasts are less dependent on the TGF- β /Smad3 signaling. This is consistent with a previously published study, which showed that CCN2 expression in SSc fibroblasts is unaffected by the ALK5 kinase inhibitor, SD208 [44]. While inhibition of ALK1 abrogated pSmad1 levels in SSc fibroblasts, the inhibitory effect on ECM genes was less pronounced than that observed by inhibition of endoglin. This suggests that endoglin may have a profibrotic function that is independent of its role in the ALK1 induced Smad1 phosphorylation. SSc fibroblasts are characterized by numerous abnormalities of the TGF- β signaling pathway [45] and it is possible that endoglin also contributes to activation of Smad-independent signaling pathways.

Activation of ALK1/Smad1 signaling in SSc and normal fibroblasts led to a modest increase in production of collagen and CCN2 suggesting that activation of this pathway alone is insufficient to turn on the ECM gene program. This is in agreement with a recent study performed in smooth muscle cells that showed Activin A and TGF- β share many signaling pathways although they diverge with respect to induction of profibrotic genes [46]. Importantly, however, we show that caALK1 potently induced ET-1 in SSc fibroblasts and to a lesser degree in normal fibroblasts. In agreement with previous studies, SSc fibroblasts produced elevated levels of ET-1 [38,47] and were more responsive to TGF- β upregulation of ET-1 gene expression. Inhibition of either endoglin or ALK1 significantly reduced TGF- β stimulation of ET-1 in SSc and normal fibroblasts, suggesting an important contribution of the ALK1/endoglin axis to abnormal ET-1 production in SSc fibroblasts. Previous studies have examined the mechanism responsible for the elevated ET-1 production in lung fibroblasts isolated from SSc patients with pulmonary fibrosis. These studies demonstrated that constitutive ALK5-independent c-Jun N-terminal Kinase (JNK) activation was involved in ET-1 upregulation [47]. Involvement of JNK in the ET-1 upregulation in SSc dermal fibroblasts was not examined in our study, although JNK could be activated by ALK1 [48], it is possible that ALK1/endoglin/JNK pathway regulates ET-1 synthesis in both pulmonary and dermal SSc fibroblasts. Furthermore, activation of the TGF- β /ALK1/endoglin pathway may have an indirect profibrotic effect in SSc via induction of ET-1.

In conclusion, this study has revealed an important profibrotic role of endoglin in SSc fibroblasts. In patients with this signaling phenotype, targeted disruption of endoglin or TGF- β /Smad1 signaling in fibroblasts may inhibit fibrosis without affecting physiological

wound healing. Elucidation of the mechanistic role of endoglin in TGF- β /Smad1 signaling may yield exciting new therapeutic targets to treat SSc fibrosis.

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Figure 1. Endoglin expression is elevated at the mRNA and protein level in SSc fibroblasts

A. Endoglin and ALK1 mRNA expression was measured in cultured normal (NS) and scleroderma (SSc) fibroblasts using qRT-PCR. B. Endoglin mRNA and protein levels in normal dermal fibroblasts treated with 2.5ng/ml of TGF- β for 24 hours after 24 hours of serum starvation. C. Western blotting was performed to measure protein levels of endoglin, ALK1, collagen, CCN2, and pSmad1. D. Quantitative representation of protein expression in all NS and SSc pairs was obtained by densitometric analysis.

Figure 2. Endoglin inhibition in SSc fibroblasts reduces expression of fibrotic genes and proteins
A. NS dermal fibroblasts were grown to confluence and treated with control siRNA (siSCR) or with increasing doses of endoglin siRNA (siENG) for 72 hours in serum. Endoglin mRNA and protein levels were measured using qRT-PCR and western blotting. B. Following siRNA treatment, mRNA expression of endoglin (ENG) and ECM genes were measured in NS and SSc fibroblasts. C. Following siRNA treatment, expression of pSmad1, pSmad3, and ECM proteins was measured in NS and SSc fibroblasts using western blotting. The results are representative of three experiments.

Figure 3. ALK1 inhibition in SSc reduces expression of fibrotic genes and proteins

A. NS dermal fibroblasts were grown to confluence and treated with 10nM of scrambled siRNA (neg siRNA) or with 10nM of ALK1 siRNA (siALK1) for 72 hours. ALK1 mRNA and protein levels were measured using qRT-PCR and western blotting. B. Following siRNA treatment, mRNA expression of ALK1 and ECM genes were measured in NS and SSc fibroblasts. C. Following siRNA treatment, expression of pSmad1, pSmad2, and ECM proteins was measured in NS and SSc fibroblasts using western blotting. The results are representative of three experiments. D. Cell lysates from normal (N) fibroblasts, normal fibroblasts stimulated with 2.5ng/ml of TGF-β for 1 hour, and SSc (S) fibroblasts were immunoprecipitated with endoglin and analyzed for ALK1 by western blotting. β-actin was used as control to verify equal amount of protein in cell lysates before IP.

Figure 4. Overexpression of constitutively active ALK1 in normal and SSc fibroblasts moderately induces ECM genes

A. Normal fibroblasts were grown to confluence and treated with adenovirus expressing caALK1 or GFP (Go) in serum free conditions for 72 hours. Cells were treated with 2.5ng/ml TGF-β for 30 minutes or 24 hours. Expression levels of pSmad1, pSmad2, and Id1 were measured by western blotting. B. qRT-PCR was performed to measure expression levels of fibrotic genes COL1A1, COL1A2, and CCN2 in normal and SSc fibroblasts expressing increasing doses of caALK1. C. Expression levels of fibrotic proteins CCN2, and collagen I were measured by Western blotting in normal and SSc fibroblasts.

Figure 5. Overexpression of constitutively active ALK1 in normal and SSc fibroblasts induces ET-1 expression

A. Normal and SSc fibroblasts were grown to confluence and treated with adenovirus expressing caALK1 or GFP (Go) in serum free conditions for 72 hours. Cells were treated with 2.5ng/ml TGF- β for 24 hours. mRNA levels of ET-1 were measured by qPCR. ET-1 protein was measured by ELISA in the supernatants obtained from the same experimental conditions (right panel). B. Normal and SSc fibroblasts were grown to confluence and treated with control siRNA (siSCR) or with endoglin siRNA (siENG) for 72 hours in serum then treated with TGF- β for 24 hours in serum free medium. ET-1 mRNA and protein levels were measured using qRT-PCR and ELISA. C. Normal and SSc fibroblasts were grown to confluence and treated with control siRNA (neg siRNA) or with ALK1 siRNA (siALK1) for 72 hours in serum, then treated with TGF- β for 24 hours in serum free medium. ET-1 mRNA and protein levels were measured using qRT-PCR and ELISA.