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Role of Smad2/3 and p38 MAP kinase in TGF- β 1-induced epithelial-mesenchymal transition of pulmonary epithelial cells

Irina Kolosova¹, David Nethery², and Jeffrey A. Kern^{3,*}

¹Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

²Case Western Reserve University Department of Medicine, Cleveland, OH

³National Jewish Health, Denver, CO

Abstract

Idiopathic pulmonary fibrosis is characterized by myofibroblast accumulation, extracellular matrix (ECM) remodeling and excessive collagen deposition. ECM-producing myofibroblasts may originate from epithelial cells through epithelial to mesenchymal transition (EMT). TGF- β 1 is an inducer of EMT in pulmonary epithelial cells *in vitro* and *in vivo*, though the mechanisms are unclear. We hypothesized that TGF- β 1 induced EMT through Smad dependent and independent processes. To test this hypothesis, we studied the roles and mechanisms of TGF- β 1 induced Smad and p38 Mitogen Activated Protein Kinase (MAPK) signaling in EMT-related changes in pulmonary epithelial cells. Exposure of pulmonary epithelial 1HAEO⁻ cells to TGF- β 1 resulted in morphological and molecular changes of EMT over a 96-hour period; loss of cell-cell contact, cell elongation, down-regulation of E-cadherin, up-regulation of fibronectin, and up-regulation of collagen I. Both Smad2/3 and p38 MAPK signaling pathways were activated by TGF- β 1. However, neither Smad2/3 nor p38 MAPK were required for the down-regulation of E-cadherin, yet p38 MAPK was associated with fibronectin up-regulation. Both Smad2/3 and p38 MAPK had a role in regulation of TGF- β 1 induced collagen expression. Furthermore, these data demonstrate that Smads and p38 MAPK differentially regulate EMT-related changes in pulmonary epithelial cells.

Keywords

Smad2/3; p38 MAPK; EMT; collagen; fibrosis

INTRODUCTION

Pulmonary fibrosis is characterized by mesenchymal cell proliferation and transdifferentiation to myofibroblasts, leading to excessive production and accumulation of collagen in the alveolar and interstitial compartments of the lung. One form of pulmonary fibrosis, Idiopathic pulmonary fibrosis (IPF) is a rapidly progressive fibrotic illness of unknown cause that leads to death from respiratory failure in 40% of patients and occurs with a prevalence ranging from 5 to 15 per 100,000 persons, and above 175 per 100,000 in the older population. Treatment at present remains largely supportive, with no drug therapy clearly shown to benefit patients with IPF. The median survival after the diagnosis of biopsy-confirmed IPF is less than three years (Bjoraker et al. 1998). Therefore a better

* Address correspondence to: Jeffrey A. Kern, MD National Jewish Health 1400 Jackson St Denver, CO 80206 KernJ@NJHealth.org.

understanding of the pathogenesis of fibrosis, especially in IPF, that will ultimately lead to novel therapeutics is needed.

The cytokine and chemokine milieu in the lung tissue is essential for the development of pulmonary fibrosis. Numerous cytokines and chemokines have been identified in bronchoalveolar lavage fluid or lung tissue of patients with fibrosis that possibly contribute to the initiation and maintenance of fibrosis (Agostini et al., 2006). Transforming Growth Factor- β (TGF- β) appears to be a key factor in the development of pulmonary fibrosis. In silica and bleomycin induced animal models of pulmonary fibrosis, strategies directed against TGF- β 1, such as peroxisome proliferator receptor gamma ligands, and soluble TGF- β receptors, have decreased fibrosis (Yamada et al., 2007; Wang et al., 1999; Burgess et al., 2005). The pulmonary epithelial cell response to TGF- β is the conversion to a fibroblast-like phenotype, or epithelial-to-mesenchymal transition (EMT).

Recently much attention has been given to the process of EMT and its role in the pathogenesis of fibrotic diseases. During EMT, epithelial cells undergo cytoskeletal remodeling with loss of cell-cell contacts, acquire a mesenchymal phenotype with loss of epithelial-specific markers and excessive extracellular matrix deposition. During epithelial injury and subsequent repair, EMT may be important in the formation of scar tissue and fibrosis. Recently EMT has been observed in pulmonary epithelial cells (Kasai et al., 2005; Willis et al., 2006) as well as in the lung *in vivo* (Kim et al., 2006). Alveolar epithelial cells *in vitro* undergo EMT in response to TGF- β 1 (Yao et al., 2004; Kasai et al., 2005; Willis et al., 2006). EMT of alveolar cells *in vivo* has also been shown in transgenic mice that express β -galactosidase exclusively in alveolar type II cells under the direction of the surfactant protein C promoter (Kim et al., 2006). In these transgenic animals, TGF- β 1 delivered to the lung through TGF- β 1 gene containing adenovirus, β -galactosidase positive cells expressing mesenchymal markers were identified 3 weeks after TGF- β 1 expression identifying epithelial cells as the main source of mesenchymal expansion. In humans, lung tissue from patients with idiopathic pulmonary fibrosis co-localize myofibroblast and epithelial cell markers in alveolar epithelial cells overlying fibroblastic foci, a key feature of the disease, further supporting an active EMT process *in vivo* (Willis et al., 2006).

These studies suggest that the majority of myofibroblast-like cells after experimental lung injury are the result of alveolar epithelial cells undergoing EMT (Kim et al., 2006). TGF- β is a major inducer of EMT during development, carcinogenesis, and fibrosis (Nawshad et al., 2005). Furthermore, TGF- β 1 has been implicated as an important mediator of fibrosis in a number of disease states. Therefore, TGF- β 1-dependent EMT might be central to the process of collagen production and ultimately fibrosis.

Regulation of TGF- β 1-induced EMT may prove complex, since TGF- β 1 signals through different pathways including Smads, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K). Binding of TGF- β 1 to heterotetrameric complexes of type I and type II TGF- β receptors leads to the phosphorylation/activation of the type I receptor by the constitutively active type II receptor. In turn, the activated type I receptor kinase phosphorylates Smad2 and Smad3 (Shi et al., 2003). Smads 2 and 3 then form complexes with Smad4 allowing the complex to translocate to the nucleus, where the receptor components regulate gene expression. While both Smads 2 and 3 mediate signals from TGF- β 1, they have different mechanisms of action and different functions (Moustakas et al., 2001). The mechanisms which determine whether the TGF- β receptor will activate signaling through Smad2 or Smad3 in a particular cell type are not known. Recent studies suggest that the majority of TGF- β target genes are controlled through Smad3-dependent transcriptional regulation (Yang et al., 2003). Experimental *in vivo* models of EMT and fibrosis, using Smad3 knockout mice, demonstrated that Smad3 signaling was important for

fibrosis (Sato et al., 2003; Saika et al., 2004) whereas the functions of Smad2 in fibrosis in response to TGF- β 1 are not clear.

TGF- β 1 can also signal independently of Smads. The mitogen-activated protein kinase (MAPK) signaling pathway is induced by TGF- β 1, and can modulate the outcome of TGF- β 1-induced responses (Javelaud and Mauviel, 2005). In particular, p38 MAPK has been shown to mediate Smad-independent TGF- β responses (Yu et al., 2002).

Although there are data confirming the importance of both Smads (Kasai et al., 2005; Yu et al., 2002; Valcourt et al., 2005; Phanish et al., 2006; Zavadil et al., 2004) and p38 MAPK (Bhowmick et al., 2001) in EMT, the contribution of each pathway has not been elucidated. In the present study, we aimed to investigate signaling mechanisms governing EMT-related changes in pulmonary epithelial cells. Our studies show that Smad2/3 and p38 MAPK differentially regulate EMT-related changes, while collagen I expression is dependent on both signaling pathways.

METHODS

Reagents and Antibodies

Recombinant human TGF- β 1 was obtained from R&D Systems (Minneapolis, MN), SB203580 was obtained from EMD Chemicals (Gibbstown, NJ). The following antibodies were used for the study: E-cadherin mouse mAb, fibronectin mouse mAb (BD Biosciences, San Jose, CA), phospho-p38 MAPK (Thr180/Tyr182) mouse mAb, Smad2 rabbit mAb, Smad3 rabbit mAb, phospho-Smad2 (Ser465/467) rabbit mAb, phospho-Smad3 (Ser423/425) rabbit mAb, Snail rabbit mAb (Cell Signaling Technology, Danvers, MA), β -Actin mouse mAb clone AC-15 (Sigma-Aldrich, St. Louis, MO).

Cell culture and treatment

1HAEO⁻ cells, SV40 immortalized human airway epithelial cells whose properties have been well characterized (Gruenert et al., 1995; Dorscheid et al., 1999) were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Inhibition of p38 MAPK was accomplished by preincubating cells for 1 h with the p38 MAPK inhibitor SB203580 (5 μ M) before treatment with TGF- β 1. The cells were stimulated with 10 ng/mL of TGF- β 1 in the growth medium for 48–96 hours and then harvested for future analysis.

F-actin staining

1HAEO⁻ cells grown on poly-lysine coated coverslips were exposed to experimental conditions, fixed with 3.7% formaldehyde, and permeabilized with 0.25% Triton X-100. After blocking with 2% BSA, cells were incubated with Texas Red-conjugated phalloidin (Molecular Probes, Carlsbad, CA) for 1 h. The coverslips were washed and mounted using ProLong Gold antifade reagent (Molecular Probes). Images were acquired by microscopy (Nikon UK Ltd, Surrey), using a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI), and Image-Pro Plus software (Media Cybernetics UK, Berkshire).

RNA Extraction, Purification, and Real-Time PCR Analyses

Total RNA extraction was performed using an RNA Easy kit (Qiagen, Valencia, CA). RNA samples were eluted with sterile DEPC-treated water, and the final concentration was spectrophotometrically determined. Real-time PCR was carried out by the Gene Expression and Genotyping Facility at Case Western Reserve University. RNA was quantified using a nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Archive cDNA was made for all samples by means of a reverse transcription reaction using an Applied

Biosystems High-Capacity cDNA archive kit (ABI, Carlsbad, CA), starting with 3 µg total RNA in a 100µL reverse transcription reaction in an ABI 9700 PCR unit (ABI). 384-well plates were set up to accommodate triplicate reactions for all assays. An endogenous control assay was always used to control for RNA loading. Gene expression assays for E-Cadherin, Collagen type 1 alpha, and Fibronectin were purchased from ABI (Taqman assays Hs00170423_m1; Hs00164004_m1 and Hs01549940) and were set up in accordance with the manufacturer's instructions for a 40 cycle run. GAPDH was used as a RNA loading control. Spectral data gathered during the PCR run were converted into numerical data using ABI SDS 2.2 software. Results are presented as relative fold changes versus a designated calibrator sample. The software algorithms used the delta-delta Ct method to calculate relative quantification (RQ) values. Results are within 95% confidence limits. Experiments were performed in triplicate.

RNA Interference and Transfection

Silencer pre-designed SMAD2 siRNA (Ambion, Austin, TX) and SMARTpool SMAD3 siRNA (Dharmacon, Chicago, IL) were transfected using siPORT Amine Transfection Reagent (Ambion). Cells were grown in 12-well plates in normal medium to reach 50% confluence. siRNA-transfection reagent complexes were prepared as described by the manufacturer. Briefly, 5 µL of siPORT was mixed with either siRNA or negative control siRNA in 100 µL Opti-MEM medium (Invitrogen, Carlsbad, CA) to bring the final concentration of RNA in a well to 20 nM. The cells were then transfected with siRNA/siPORT complexes in 1 mL normal medium and incubated for 48 h at 37°C in a CO₂ incubator. Following incubation, the cells were stimulated with 10 ng/mL of TGF-β1 for 96 hours, then harvested for analysis. Depletion efficiency of Smad2 or Smad3 was evaluated by immunoblotting.

RESULTS

TGF-β1 induces EMT-related changes in pulmonary airway epithelial cells

To define TGF-β1 EMT in pulmonary epithelial cells *in vitro*, 1HAEo⁻ cells were exposed to TGF-β1 and biochemical and morphological changes indicative of EMT were examined. Early biochemical signs of EMT are the loss of the adherence junction protein E-cadherin, as well as synthesis of extracellular matrix proteins fibronectin and collagen I. As shown in Figure 1A, TGF-β1 decreased E-cadherin mRNA expression from baseline values by over 30% at 48H (p<0.05) and increased fibronectin and collagen gene expression in a time-dependent manner. Fibronectin transcript levels increased 4-fold at 24H and almost 7-fold by 48H (p<0.005), while collagen transcript levels increase over 50-fold at 24H and over 150-fold at 48H (p<0.005). Consistent with the gene expression data, upon TGF-β1 stimulation fibronectin protein expression was induced from no detectable expression at baseline, and E-cadherin protein expression decreased (Fig. 1B). Thus TGF-β1 has the ability to induce biochemical changes consistent with EMT including collagen production. In addition, TGF-β1 stimulation induced changes in cellular morphology. Confluent 1HAEo⁻ cells in culture have a cobble-stone morphology. However, after 72–96 hours of TGF-β1 exposure the cells expressed a morphological phenotype characteristic of EMT, with loss of cell-cell contact, an elongated shape (Fig. 1C), and cytoskeletal alterations with development of thick F-actin stress fibers (Fig. 1D).

TGF-β1-induced production of collagen I is Smad-dependent

Smad transcription factors are well-known mediators of TGF-β1 signaling. Therefore, we hypothesized that TGF-β1 induced EMT through Smad activation. TGF-β1 exposure activated Smad2 and Smad3 as evidenced by their phosphorylation within minutes of TGF-β1 exposure (Fig. 2A). In order to investigate whether Smad2 and Smad3 were involved in

TGF- β 1-mediated EMT, siRNAs were used to deplete Smad2 and Smad3. Western blot analysis showed that Smad2 and Smad3 protein expression was significantly and specifically reduced by siRNA gene silencing after 48 hours of incubation with the respective siRNAs, while a non-specific siRNA serving as a negative control had no effect on Smad2/3 protein expression (Fig. 2B). After 48 h of Smad depletion cells were stimulated with TGF- β 1. Fig. 3B shows that depletion of Smads persisted over the entire time period of TGF- β 1 stimulation (96 h). Interestingly, independently from Smad siRNA, TGF- β 1 stimulation for 4 days caused reduction of Smad2 and Smad3 compared to unstimulated cells (Fig 3B), possibly due to proteasome-dependent degradation in the nucleus, as it has been established earlier (Lo et al., 1999).

TGF- β 1-induced fibronectin gene expressions was unaffected by Smad depletion, while collagen gene expression was suppressed by both Smad2 (32% decrease) or Smad3 silencing (37% decrease) (Fig. 3A). Fig. 3B shows that depletion of either Smad2 or Smad3 had no effect on TGF- β 1 mediated changes in in fibronectin and E-cadherin protein expression or TGF- β 1 stimulated morphological changes in airway epithelial cells (Fig. 3D). It has been shown that suppression of E-cadherin is regulated by transcriptional repressor Snail (Peinado et al., 2003). Figure 3C shows that Smad2 or Smad3 downregulation only slightly affected Snail expression, which suggests Smad independent Snail signaling and might explain the lack of effect on E-cadherin. These data suggest that fibronectin, E-cadherin, and the morphologic changes induced by TGF- β 1, are Smad independent, while collagen expression is regulated through Smads.

Involvement of p38 MAPK in EMT

The Smad independent changes in gene expression, protein expression and cell morphology suggested that TGF- β 1 uses alternate signal pathways to induce these changes. We hypothesized that TGF- β 1 may signal through an alternative pathway involving MAPKs. TGF- β 1 activated p38 MAPK within 30 min, as indicated by dual phosphorylation at Thr180 and Tyr182 (Fig. 4A). To assess the role of p38 in EMT-related changes, we inhibited p38 MAPK activity using the p38 MAPK-specific small molecule inhibitor SB203580. p38 MAPK inhibition significantly reduced TGF- β 1-dependent gene expression for the extracellular matrix-related genes fibronectin and collagen I over 50% and 80% respectively ($p < 0.02$) (Fig. 4B), reduced fibronectin protein expression, reduced TGF- β 1-induced epithelial monolayer disruption and cell elongation (Fig. 4E), and diminished formation of actin stress fibers (Fig. 4F). Surprisingly, SB203580 inhibition of p38 MAPK had no effect on TGF- β 1 mediated loss of E-cadherin protein expression (Fig. 4C) despite of the fact that Snail expression was significantly inhibited (Fig. 4D). Thus TGF- β 1 mediates some of its effects on EMT through non-Smad dependent pathways that include p38 MAPK.

DISCUSSION

One theory of the development of fibrotic lung diseases, such as IPF, is dysregulated fibroblast and epithelial cell function with abnormal epithelial-mesenchymal interactions and little or no inflammatory component leading to EMT (Gross et al., 2001; Selman M et al., 2001). TGF- β 1 appears to be a key regulator of this process. Recently, considerable progress has been made in understanding intracellular signaling downstream of the TGF- β 1 receptors. However, little is known regarding the cellular mechanisms of TGF- β 1-induced EMT in pulmonary epithelium. TGF- β 1 has been postulated to be a key factor in the induction of EMT in several tissues, including the lung, and in alveolar type II cells (Konigshoff et al., 2010; Konigshoff et al., 2009). In human idiopathic pulmonary fibrosis (IPF), TGF- β 1 gene expression is increased in fibroblasts and TGF- β 1 levels are elevated in bronchoalveolar lavage fluid compared to patients without IPF (Ramos et al., 2001). TGF- β 1 is broadly expressed in these patients by alveolar epithelial cells, alveolar macrophages,

bronchial epithelium and associated with the extracellular matrix (Meloni et al., 2004). These findings, together with the animal data point to an important role of TGF- β 1 in pulmonary fibrosis. Most TGF- β 1-induced responses require Smad-mediated signaling. Presently, p38 MAPK activation is being considered as an alternative/additional signal pathway to mediate TGF- β 1 effects. To investigate the importance of these pathways in TGF- β 1-induced EMT we utilized both siRNA and a chemical inhibition approach assessing cell phenotypic changes as indicators of EMT. Phenotypic changes characteristic of EMT stimulated by TGF- β 1 include disassembly of cell junctions, cytoskeletal reorganization, loss of epithelial polarity, and simultaneous degradation and *de novo* synthesis of ECM (Zavadil et al., 2001). We demonstrated that 1HAEo⁻ cells respond to TGF- β 1 stimulation with typical signs of EMT: loss of the adherence junction protein E-cadherin, cell elongation, actin stress fiber formation, and synthesis of ECM proteins fibronectin and collagen I (Fig. 1).

The TGF- β receptor signals through the Smad transcription factors. However, it is not clear if Smad2 or Smad3 or both proteins are responsible for TGF- β 1-induced EMT. To understand the role of these two Smad proteins in TGF- β 1-induced EMT we depleted Smad2 or Smad3 using a siRNA approach. Depletion of either Smad2 or Smad3 decreased collagen I mRNA levels in TGF- β 1-stimulated 1HAEo⁻ cells, however it did not affect other signs of EMT; changes in E-cadherin, fibronectin expression, or cell shape (Fig. 3). Thus, collagen I gene expression appears Smad2 and Smad3 dependent, while the other EMT changes appear to be Smad independent. Several studies have shown that tissue deposition of type I collagen during the fibrotic process is largely due to an increase in the rate of transcription of the corresponding genes (Ghosh et al., 2002). A number of collagen genes including COL1A1 and COL1A2 are dependent upon Smad3 (Verrecchia et al., 2001). Using a transgenic approach Smad3 activation has been shown to occur downstream of TGF- β 1 in the pathogenesis of pulmonary fibrosis. Smad3-deficient mice had diminished type I procollagen mRNA expression in the lungs compared with wild-type mice after bleomycin injury (Zhao et al., 2002). Further, adenovirus-mediated overexpression of TGF- β 1 in the lungs of wild-type mice resulted in progressive pulmonary fibrosis but had no effect on *Smad*^{-/-} mice (Bonniaud et al., 2004). The involvement of Smad3 in fibrotic EMT has also been demonstrated *in vitro* and *in vivo* in other tissues such as the eye and kidney (Sato et al., 2003; Saika et al., 2004b). However, the function of Smad2 in fibrosis and in response to TGF- β 1 is not clear. Our current studies have shown Smad2 is important in collagen I gene expression in pulmonary epithelial cells. Our data implicate both Smad2 and Smad3 as important in the pulmonary fibrotic response, since collagen I gene upregulation is dependent on both Smads.

Fibronectin is another component of ECM that enhances the profibrotic effects of TGF- β 1 (Leask et al., 2004), possibly by effecting the formation of collagen fibrils (Kadler et al., 2008). Our data showed that fibronectin induction was independent of either Smad2 or 3. This is in agreement with the previously published observation that TGF- β 1-induced fibronectin synthesis was not altered in Smad2 or Smad3-deficient fibroblasts (Piek et al., 2001). The loss of the epithelial marker E-cadherin is a distinguishing characteristic of EMT. We failed to observe any effect of Smad2/3 depletion on either E-cadherin or its transcriptional regulator Snail (Fig. 3), again supporting Smad independent effects of TGF- β 1. It has been reported previously that TGF- β 1 promotes transcription of Snail via Smad-independent signaling pathways (Peinado et al., 2003). Studies evaluating TGF- β 1-induced E-cadherin regulation are controversial. TGF- β 1-induced E-cadherin downregulation was inhibited in Smad3, but not in Smad2-knockout hepatocytes (Ju et al., 2006). Similarly, in human proximal tubular epithelial cells, decreased E-cadherin was Smad3-dependent, but Smad2-independent (Phanish et al., 2006). However, in the transformed pulmonary epithelial cell line A549, TGF- β 1-induced E-cadherin downregulation was Smad2-

dependent (Kasai et al., 2005). Therefore, Smad effects on E-cadherin may be tissue and transformation dependent.

There is a limited amount of data regarding the significance of TGF- β 1-mediated p38 MAPK signaling in EMT. We show that inhibiting p38 MAPK signaling decreased both collagen I and fibronectin levels in TGF- β 1-stimulated 1HAEo⁻ cells (Fig. 4). The effect of p38 MAPK inhibition on collagen I gene expression was stronger than Smad2/3 depletion. Therefore, both p38 MAPK and Smad signaling independently regulate collagen I gene expression by transcriptional activation as has been seen in hepatic stellate cell (Tsukada et al., 2005) and dermal fibroblasts (Sato et al., 2002). Type I collagen expression induced by TGF- β 1 can be suppressed by the specific p38 MAPK inhibitors in normal fibroblasts (Ihn et al., 2005). Extending these data *in vivo*, a specific inhibitor of p38 MAPK, FR-167653, ameliorated pulmonary fibrosis induced by bleomycin in mice (Matsuoka et al., 2002). FR167653 treatment also decreased α 1(I) collagen mRNA expression in a unilateral ureteral obstruction model of renal fibrosis in mice (Nishida et al., 2008), and blockade of p38 MAPK ameliorates renal fibrinogenesis (Stambe et al., 2004).

Our results show that inhibition of p38 does not affect TGF- β 1-induced E-cadherin downregulation, while strongly inhibiting Snail expression (Fig. 4). It is possible, that other transcription factors such as Slug, ZEB1, or SIP1 (Zavadil et al., 2005) are involved in E-cadherin downregulation. However, in accordance with previously published observations (Bakin et al., 2002) SB202190 reduced TGF- β 1-mediated changes in epithelial cell shape and reorganization of the actin cytoskeleton (Fig. 4). This suggests that dissociation of intercellular junctions and remodeling of the actin cytoskeleton do not depend exclusively on E-cadherin downregulation.

EMT manifests itself in inter-related phenotypic changes, resulting in the expression of a mesenchymal phenotype. These changes or EMT “modules” (Zavadil et al., 2005), including loss of epithelial markers and synthesis of mesenchymal markers together with morphological and cytoskeletal changes are thought to occur in a coordinated manner. However, it is not clear which of these EMT “modules” are regulated by the same signaling pathways. Differential regulation of epithelial and mesenchymal markers during EMT has been reported (Shirakihara et al., 2007). In this work we show that in cultured 1HAEo⁻ pulmonary epithelial cells undergoing TGF- β 1-induced EMT specific inhibition of Smad or p38 MAPK pathways “uncouples” different EMT modules from the full phenotype. In summary, E-cadherin repression appears Smad2/3 and p38 MAPK independent, both Smad2/3 and p38 MAPK signaling regulate collagen I gene expression, while and fibronectin expression depend only on p38 MAPK. Inhibiting these signals may represent a therapeutic pathway for treatment of pulmonary fibrosis.

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Figure 1. TGF-β1 induces EMT-related changes

A. Real-time PCR data shows that TGF-β1 up-regulates fibronectin and collagen I and down-regulates E-cadherin mRNA. mRNA levels are presented as target gene/GAPDH ratio and normalized to control. Means $\pm$ SE of four experiments are shown. * $p < 0.005$. B. Western blot analysis shows that TGF-β1 up-regulates fibronectin and down-regulates E-cadherin protein expression. C. TGF-β1 induces a mesenchymal morphology. Phase contrast microscopy of cells treated with TGF-β1 over a 4-day period. D. TGFβ1 induces reorganization of actin cytoskeleton. Cells were treated with TGF-β1 for 4 days, then stained with Texas-Red Phalloidin.

Figure 2. TGF-β1 activates Smad2/3

A. TGF-β1 causes time-dependent phosphorylation of Smad2 and Smad3. **B.** Smad2 and Smad3 siRNA treatment for 48 hours results in specific knockdown of respective Smad proteins.

Figure 3. Role of Smad2/3 signaling in TGF- β 1-induced EMT

A. Smad2 and Smad3 siRNA affects TGF- β 1 induced mRNA expression of EMT markers. Cells were pre-treated with siRNA for 48 hours prior to TGF- β 1 stimulation for an additional 48 hours. Relative gene expression was evaluated by real-time PCR. Collagen IA, but not fibronectin mRNA expressions was Smad2 and Smad3-dependent. Means $\pm$ SE of three experiments are shown; * $p=0.01$. **B.** Western blot shows that Smad2 and Smad3 depletion does not affect fibronectin and E-cadherin protein expression. Cells were pre-treated with siRNA for 48 hours prior to TGF- β 1 stimulation for an additional 96 hours. **C.** Effect of Smad2 and Smad3 depletion on TGF- β 1-induced Snail expression. **D.** Smad depletion does not affect TGF- β 1 induced morphological changes. Cells were pre-treated with siRNA for 48 hours prior to TGF- β 1 stimulation for an additional 96 hours.

Figure 4. Involvement of p38 kinase in EMT-related responses

A. TGF- β 1 causes phosphorylation/activation of p38 MAPK. **B.** SB203580 inhibits up-regulation of fibronectin and collagen 1A mRNA. Cells were treated with 5 μ M SB203580 for 40 min, then were stimulated with TGF- β for 48 hours prior to RNA isolation. Relative gene expression was evaluated by real-time PCR. Means $\pm$ SE of three experiments are shown; * $p=0.02$; ** $p=0.006$. **C.** Effect of SB203580 on E-cadherin and fibronectin protein expression. Cells were treated with 5 μ M SB203580 for 40 min, then were stimulated with TGF- β for 96 hours prior to immunoblotting. **D.** Effect of SB203580 on TGF- β 1-induced Snail expression. Pharmacological inhibition of p38 with SB203580 prevents TGF- β 1-induced changes in cell morphology (**E**) and diminishes formation of actin stress fibers (**F**).