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Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF-β1

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Summary

Transforming growth factor β1 (TGF-β1) is a cardinal cytokine in the pathogenesis of airway remodeling, and promotes epithelial-to-mesenchymal transition (EMT). As a molecular interaction between TGF-β1 and Jun N-terminal kinase (JNK) has been demonstrated, the goal of this study was to elucidate whether JNK plays a role in TGF-β1-induced EMT. Primary cultures of mouse tracheal epithelial cells (MTEC) from wild-type, JNK1$^{-/-}$ or JNK2$^{-/-}$ mice were comparatively evaluated for their ability to undergo EMT in response to TGF-β1. Wild-type MTEC exposed to TGF-β1 demonstrated a prominent induction of mesenchymal mediators and a loss of epithelial markers, in conjunction with a loss of trans-epithelial resistance (TER). Significantly, TGF-β1-mediated EMT was markedly blunted in epithelial cells lacking JNK1, while JNK2$^{-/-}$ MTEC

underwent EMT in response to TGF-β1 in a similar way to wild-type cells. Although Smad2/3 phosphorylation and nuclear localization of Smad4 were similar in JNK1$^{-/-}$ MTEC in response to TGF-β1, Smad DNA-binding activity was diminished. Gene expression profiling demonstrated a global suppression of TGF-β1-modulated genes, including regulators of EMT in JNK1$^{-/-}$ MTEC, in comparison with wild-type cells. In aggregate, these results illuminate the novel role of airway epithelial-dependent JNK1 activation in EMT.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/7/1036/DC1

Key words: Lung, EMT, TGF-β, JNK, SMAD, Mouse

Introduction

Epithelial-to-mesenchymal transition (EMT) has emerged as a cardinal process crucial to the promotion of tissue fibrosis and metastasis (Kalluri and Neilson, 2003), and multiple mediators and regulators of EMT have been identified (Gotzmann et al., 2004; Thiery and Sleeman, 2006). The EMT phenotype represents a striking manifestation of epithelial plasticity that encompasses dissolution of tight junctions, modulation of adherens junctions, reorganization of the actin cytoskeleton, loss of apical-basal polarity and the induction of a mesenchymal gene expression program (Ozdamar et al., 2005; Thiery, 2002; Zavadil and Bottinger, 2005). The cytokine transforming growth factor β1 (TGF-β1) has been identified as a potent inducer of EMT in numerous cell types (Lee et al., 2006). Although a role for EMT in the development of kidney fibrosis is well recognized (Iwano et al., 2002), a role for EMT in fibrogenesis of the lung was unclear until recently, when multiple studies highlighted the fact that TGF-β1 could induce EMT in rat or human alveolar type II epithelial cells (Jain et al., 2007; Kasai et al., 2005; Kim et al., 2006; Willis et al., 2005). Evidence for EMT was also recently reported in lung biopsies from patients with idiopathic pulmonary fibrosis (IPF) (Kim et al., 2006; Wang et al., 2006), and in mouse models of fibrosis (Kim et al., 2006; Wu et al., 2007).

Jun N-terminal kinase (JNK) is a member of the family of mitogen-activated protein kinases (MAPK) that is well known for its role in stress responses and regulation of apoptosis (Davis, 2000). Recent studies have identified evidence for crosstalk between JNK and TGF-β1 signaling pathways. For example, TGF-β-activated

kinase 1 (TAK-1) can mediate the activation of JNK1 (Hocevar et al., 2005; Mori et al., 2004; Verrecchia et al., 2001). TGF-β1-induced Smad2 and Smad3 phosphorylation has been shown to be JNK-dependent (Wang et al., 2006), and contributes to the transcriptional upregulation of select mesenchymal genes (Kasai et al., 2005; Mori et al., 2004; Xie et al., 2005). A recent study revealed that TGF-β1-mediated loss of caveolin-1 is a crucial regulatory event in the pathophysiology of pulmonary fibrosis, in association with the activation of JNK1 in fibroblasts (Wang et al., 2006). Furthermore, the activity of JNK was shown to be elevated in fibroblasts from patients with chronic pulmonary fibrosis, and facilitated TGF-β1-driven endothelin-1 expression (Shi-Wen et al., 2006).

These collective findings suggest that JNK signaling may be required for TGF-β1-induced fibrogenesis of the lung, via a process that may involve EMT. Therefore, the goal of the present study was to determine whether the activity of JNK in primary cultures of airway epithelial cells is required for TGF-β1-induced EMT, and to elucidate the potential mechanisms by which JNK affects TGF-β1-induced signaling.

## Results

Characterization of primary epithelial cells in culture

In order to test the impact of TGF-β1 on airway epithelial cells, primary cultures of mouse tracheal epithelial cells (MTEC) were established. MTEC expressed the epithelial marker proteins E-cadherin and cytokeratin and were negative for the mesenchymal markers, fibronectin 1 and α-smooth muscle actin (SMA) (Fig. 1A).

Fig. 1. Characterization of primary MTEC. (A) Compared to isolated lung fibroblasts, MTEC expressed cytokeratins and E-cadherin, but were negative for fibronectin and α-SMA. (B) MTEC expressed the localized tight junctional protein ZO-1, indicative of barrier function (ZO-1 is depicted by the red stain, whereas the green signal represents the DNA counterstain, Sytox Green). (C) Scanning electron micrograph of MTEC depicting well-defined cell-cell contacts, 2500× magnification. (D) Transmission electron micrograph depicting tight junction formation, desmosomes, nuclei, vacuole; 15000× magnification. (E) Primary MTEC established stable TER in culture. Shown are MTEC cultured under submerged conditions, or cultured on ALI in the presence of absence of retinoic acid (30 nM); TER was measured every other day for 18 days. D, desmosome; N, nucleus; T, tight junction; V, vacuole.
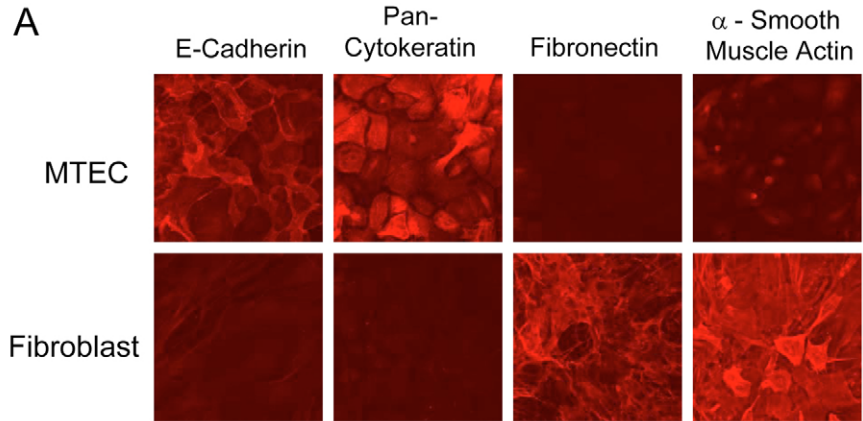

Conversely, primary lung fibroblasts were positive for mesenchymal markers and negative for epithelial markers. MTEC cultures established well-defined rings of the tight junction protein zona occludens 1 (ZO-1), indicative of tight junction formation, a predominant characteristic of polarized epithelial monolayers (Fig. 1B). Scanning and transmission electron micrographs of MTEC in culture also showed well-defined cell-cell contacts and epithelial cell morphology (Fig. 1C,D), and when grown on air-liquid interface (ALI), MTEC established tight junctions and desmosomes. (Fig. 1C,D). To confirm that MTEC cultures establish functional tight junctional complexes, trans-epithelial resistance (TER) was measured, and revealed that over time in culture, cells established a high TER (Fig. 1E). Under submerged culture conditions, MTEC displayed a higher TER than previously reported on ALI (You et al., 2002). Indeed, MTEC cultures that were grown on ALI in the presence or absence of retinoic acid displayed a reduced level of TER, consistent with previously reported values. Collectively, these data demonstrate that the primary MTEC cultures that were established here express multiple epithelial markers and form tight junctions in vitro.

TGF-β1 induces an EMT phenotype in MTEC

Although EMT has been demonstrated in alveolar epithelial cells, it is not clear whether epithelial cells derived from proximal airways can also undergo EMT. Indeed, MTEC exposed to TGF-β1 demonstrated increased expression of collagen type 1a1 and fibronectin 1, two mesenchymal protein markers. As expected, expression of the epithelial marker, E-cadherin decreased in MTEC following exposure to TGF-β1 (Fig. 2A). TGF-β1 also enhanced expression of α-SMA, and PAI-1 protein, in lysates or supernatants, respectively (Fig. 2B). These changes in protein content were accompanied by changes in mRNA expression. Results in Fig. 2C demonstrate that cells exposed to TGF-β1 increased mRNA expression of collagen type 1a1, fibronectin 1 and PAI-1, whereas they decreased mRNA content of clara cell secretory protein (CCSP), a transcript selectively expressed in airway epithelial cells.
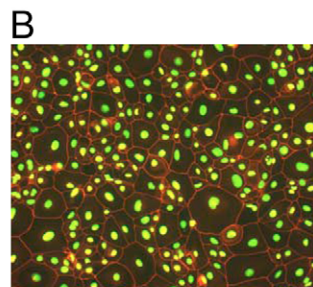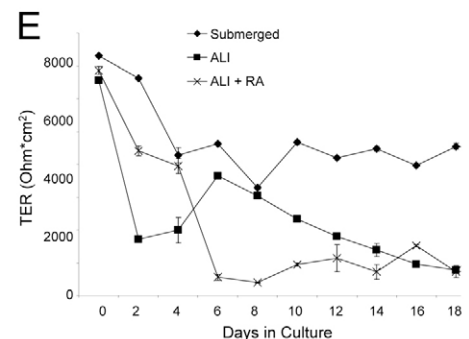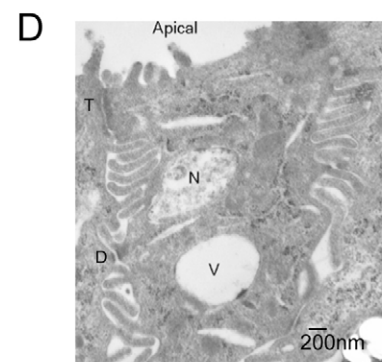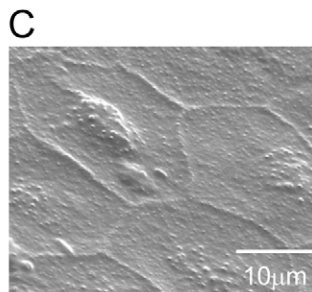
These collective shifts in expression from epithelial to mesenchymal markers that are induced by TGF-β1 in MTEC are a hallmark of EMT (Lee et al., 2006). To determine whether the observed epithelial plasticity was reversible, MTEC were grown in TGF-β1-containing medium for 10 days and were then maintained for an additional 6 days in the absence of TGF-β1. Although expression of TGF-β1-induced mesenchymal markers decreased to control levels following MTEC recovery, expression of the epithelial marker CCSP did not recover within this time frame (Fig. 2C). Nonetheless, these results suggest that TGF-β1 induced a transient EMT phenotype in MTEC.

Activation of JNK and TGF-β1 signaling in MTEC

We first assessed whether TGF-β1 activates the canonical Smad signaling pathway in MTEC. As is demonstrated in Fig. 3A,B, TGF-β1 induced Smad2 phosphorylation, Smad4 nuclear accumulation and increased binding of nuclear proteins to Smad DNA-binding elements (SBE). Evaluation of phosphorylation of JNK1 and 2 demonstrated that exposure to 5 ng/ml TGF-β1 caused increases
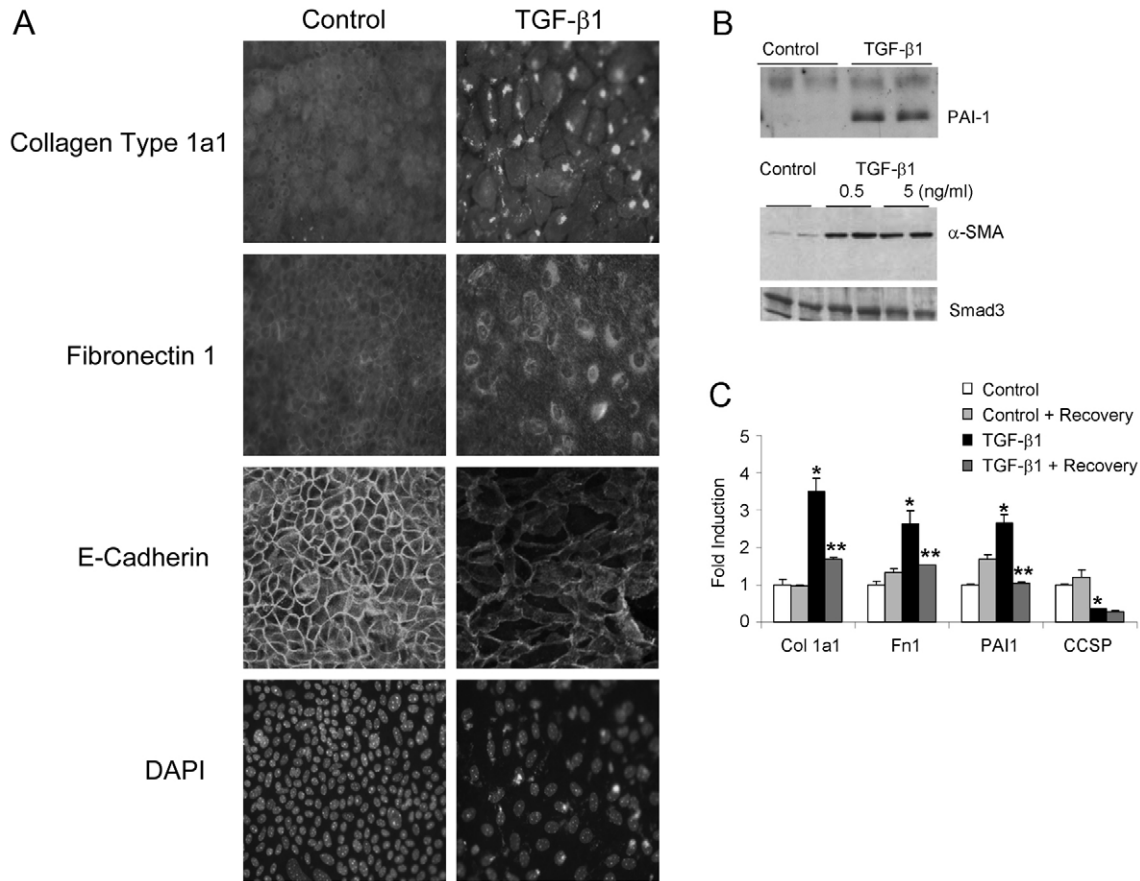
Fig. 2. Assessment of effects of TGF-β1 in MTEC on expression of mesenchymal and epithelial markers. (A) Evaluation of collagen type 1a1, fibronectin and E-cadherin via immunofluorescence. MTEC were exposed to 5 ng/ml TGF-β1 every other day for 14 days, and prepared for immunofluorescence. Magnification 400×. DAPI illustrates nuclei. (B) MTEC were exposed to 5 ng/ml TGF-β1, and expression of PAI-1 was assessed in cell culture media 24 hours later. α-SMA protein expression was determined in cell lysates following exposure to 0.5 or 5 ng/ml of TGF-β1 for 6 days. Smad3 represents loading control. Different lanes shown represent results from independent samples. (C) Evaluation of mRNA levels of mesenchymal or epithelial genes in MTEC exposed to TGF-β1 treatment (5 ng/ml, every other day for 10 days). To evaluate reversibility of TGF-β1 responses, MTEC were maintained in culture for 6 additional days in the absence of TGF-β1. mRNA expression was evaluated by real-time PCR analysis, and data normalized to the housekeeping gene HPRT. Results are expressed as fold changes from controls. * $P<0.05$, ANOVA versus control-treated, ** $P<0.05$, ANOVA versus TGF-β1-treated.

in phosphorylation of JNK1 and JNK2, which were apparent after 1 hour and further increased after 4 hours of exposure (Fig. 3C). These findings demonstrate that TGF-β1 is capable of inducing JNK and Smad pathway activation in airway epithelial cells.

JNK1 is required for TGF-β1-induced EMT in MTEC
Two isoforms of JNK, JNK1 and 2 are expressed in airway epithelial cells. To elucidate the respective roles of JNK1 or JNK2 in TGF-β1-induced EMT, MTEC were isolated from wild-type, JNK1$^{-/-}$ or JNK2$^{-/-}$ mice and evaluated comparatively for a TGF-β1-induced loss in TER (Fig. 4A), as an indication of dissolution of tight junctions and barrier function (Lee et al., 2006). As expected, TGF-β1 caused a decrease in TER in wild-type MTEC cultures. Although JNK2$^{-/-}$ cells were protected from TGF-β1-induced loss of TER between 3 and 6 days, at later time points a similar loss in TER was observed in JNK2$^{-/-}$ cells compared to wild-type cells (Fig. 4A). By contrast, JNK1$^{-/-}$ MTEC were markedly protected against the decrease in TER induced by TGF-β1 at all time points evaluated. Consistent with these findings, TGF-β1 enhanced expression of the mesenchymal protein α-SMA in wild-type and to a slightly lesser extent in JNK2$^{-/-}$ MTEC, but failed to do so in

JNK1$^{-/-}$ MTEC (Fig. 4B). Western blot analysis for total JNK confirmed that MTEC from JNK1$^{-/-}$ and JNK2$^{-/-}$ mice indeed lacked JNK1 or JNK2, respectively (Fig. 4C). These data indicate that the TGF-β1-induced EMT in MTEC is JNK1-dependent, and appears to be largely independent of JNK2.

Wild-type and JNK1$^{-/-}$ MTEC display similar phenotypes in culture
The failure of JNK1$^{-/-}$ MTEC to undergo EMT may be due to an intrinsic inability of these cells to differentiate. We therefore further characterized the phenotype of JNK1$^{-/-}$ MTEC compared to wild-type cells. Although JNK1$^{-/-}$ MTEC plated at a higher density, the rate of growth was similar between wild-type and JNK1$^{-/-}$ cells (Fig. 5A, Fig. 4B). JNK1$^{-/-}$ and wild-type MTEC appeared phenotypically similar (Fig. 5B). When cultured on ALI in the presence of retinoic acid, JNK1$^{-/-}$ and wild-type MTEC also produced similar levels of mucins, a characteristic of differentiation of airway epithelial cells (Fig. 5C) (Gray et al., 2001). These data strongly suggest that JNK1$^{-/-}$ MTEC are not intrinsically incapable of differentiation, but have a selective defect in plasticity towards EMT.
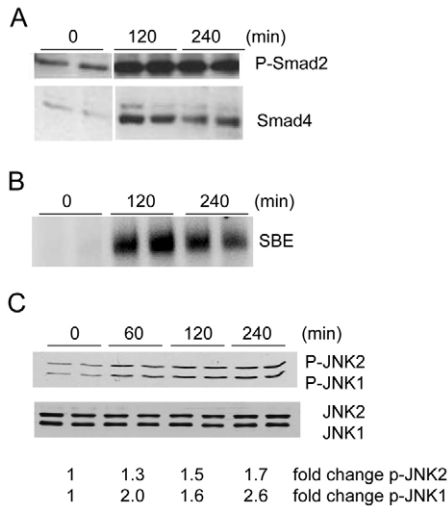
Fig. 3. Evaluation of Smad and JNK signaling in MTEC exposed to TGF-β1. (A) Assessment of Smad2 phosphorylation and Smad4 nuclear localization in MTEC in response to TGF-β1. Phospho-Smad2 and Smad4 were analyzed by western blot of nuclear extracts, after 120 or 240 minutes of exposure to 5 ng/ml TGF-β1. Note that samples were run on the same gels, but irrelevant lanes were deleted for consistency of presentation. (B) Electrophoretic mobility shift assay evaluating binding of nuclear proteins to the consensus SBE in response to TGF-β1 (10 ng/ml) for the indicated times. (C) Assessment of phosphorylation of JNK1 and JNK2 following stimulation of MTEC with TGF-β1 (5 ng/ml) for the indicated times. The upper band indicates predominantly JNK2, whereas the lower band mainly reflects JNK1. Fold change denotes differences in signal compared to sham controls based upon densitometric evaluation. The lower panel represents total JNK1 and 2 western blot as loading control. Duplicate lanes in panels A, B and C represent results from independent samples.

JNK1 promotes TGF-β1-induced gene expression

To determine whether JNK1 affected TGF-β1-induced transcriptional responses in primary lung epithelial cells, gene expression profiles were evaluated comparatively in MTEC from wild-type and JNK1$^{-/-}$ mice by microarray analyses. TGF-β1 caused marked increases in expression of mesenchymal genes and a loss of epithelial specific transcripts in wild-type cells, consistent with the induction of EMT (Table 1). By contrast, MTEC derived from JNK1$^{-/-}$ mice showed markedly blunted responses to TGF-β1 when evaluating global gene expression profiles, in addition to genes that reflect EMT (Table 1, Fig. 6A). Real-time PCR analyses of mesenchymal (collagen type 1a1, fibronectin 1, plasminogen activator inhibitor 1) and epithelial (CCSP) genes (Fig. 6B), confirmed that the presence of JNK1 is required for a maximal TGF-β1-induced EMT transcriptional program in MTEC. Several transcription factors involved in the induction of EMT have been identified (Lee et al., 2006; Thiery and Sleeman, 2006; Venkov et al., 2007). TGF-β1 induced the EMT regulators HMGA2, Ets-1 and Jagged 1 in a JNK1-dependent manner (Fig. 6C). Collectively, these data highlight a crucial requirement of JNK1 in the causation of TGF-β1-induced EMT in isolated primary airway epithelial cells. To confirm that the lack of EMT in JNK1$^{-/-}$ primary MTEC cells is due to ablation of JNK1 instead of other compensatory changes, we used a generic JNK1 inhibitor. MTEC cells treated with 10 μM SP600125 demonstrated marked protection against TGF-β1-induced Fn and PAI-1 mRNA expression (supplementary material Fig. S1). Lastly, SiRNA-mediated knockdown of JNK1 in a line of epithelial cells also diminished TGF-β1-induced mRNA expression of PAI-

Table 1. Microarray analysis of TGF-β1-modulated genes in wild-type or JNK1$^{-/-}$ MTECs

Gene name/symbol	Wild type	JNK1$^{-/-}$
Extracellular matrix and adhesion		
TNFR superfamily 11b	9.92	4.72
Fibronectin 1	6.92	2.38
Serine peptidase inhibitor clade I 1	4.56	2.35
Latent TGF-β binding protein 1	3.23	1.59
Cadherin 6	3.14	2.11
Cadherin 10	2.83	1.27
Plasminogen activator inhibitor 1	2.53	1.96
Procollagen type 5a2	2.51	1.54
Protocadherin α1	2.06	−1.01
Procollagen 2 oxoglut. 5-dioxygen. 2	2.01	1.20
Tissue inhibitor metalloproteinase 3	−2.20	−1.17
Cytoskeleton		
Lactate dehydrogenase b	2.51	1.33
Myosin light chain kinase	2.33	−1.02
α-actin 1	2.22	2.97
Palladin	2.10	1.52
Anillin	−2.00	−1.27
Myozenin 2	−2.07	−1.27
Small proline-rich protein 1b	−2.58	−1.39
Keratin 16	−3.73	−1.49
Keratin 13	−8.94	−3.94
Cell division and growth		
Colony stimulating factor 2	4.66	1.87
Vascular endothelial growth factor C	2.17	1.24
Death associated kinase 2	2.14	1.54
Colony stimulating factor 1	2.13	1.39
Cell division cycle 20	−2.10	−1.21
Cyclin D1	−2.17	−1.62
Cyclin A2	−2.45	−1.16
Topoisomerase 2a	−2.48	−1.59
Cell motility		
Secreted phosphoprotein 1	5.31	2.16
Roundabout homolog 1	3.71	1.83
EMT regulators		
Jagged 1	2.03	−1.22
Wnt5a	1.97	1.12
HMGA2	1.84	1.03

Data are grouped by gene ontology, and fold expression changes are shown (positive value for increased, – for decreased compared with sham controls). All genes listed showed a significant interaction with the presence of JNK1 ($P<0.05$) and were changed at least twofold compared with sham controls, with the exception of the EMT-regulatory genes.

1 (supplementary material Fig. S2), demonstrating that acute disruption of the JNK pathway attenuates TGF-β1-regulated EMT.

Altered TGF-β1 pathway activation in JNK1$^{-/-}$ epithelial cells

The attenuated TGF-β1-induced transcriptional responses in JNK1$^{-/-}$ cells could potentially be explained by attenuated Smad signaling in JNK1$^{-/-}$ MTEC. Results shown in Fig. 7A demonstrate that TGF-β1 caused increases in phospho-Smad2/3 and Smad4 in nuclear extracts to similar extents to those in wild-type and JNK1$^{-/-}$ MTEC (Fig. 7A), indicating that TGF-β1-receptor-driven phosphorylation of receptor Smads and recruitment of Smad4 to the nucleus were not different between wild-type and JNK1$^{-/-}$ MTEC. However, increases in the binding of Smad3/4 to the SBE induced by TGF-β1 were attenuated in JNK1$^{-/-}$ epithelial cells, demonstrating that the presence of JNK1 is essential for maximal TGF-β1-induced Smad DNA binding (Fig. 7B). As expected, TGF-β1-stimulated increases in nuclear phospho-Jun levels were diminished in JNK1$^{-/-}$ cells compared with wild-type counterparts.
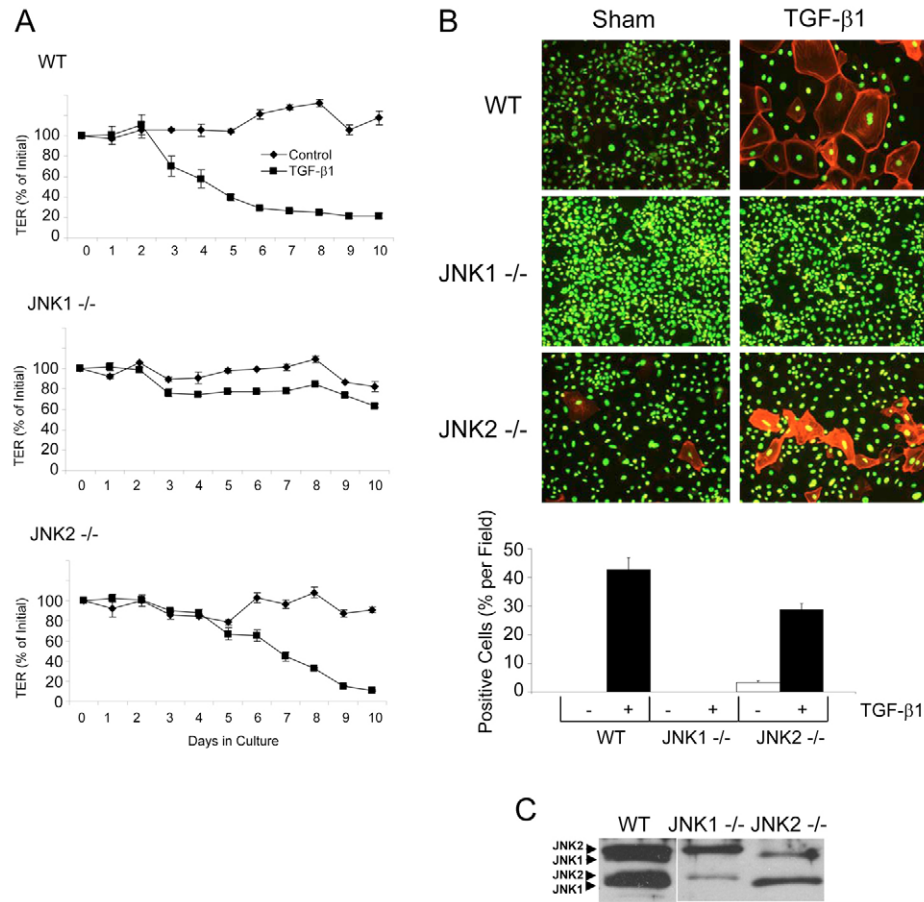
Fig. 4. TGF-β1 induces EMT in MTEC in a JNK1-dependent, but JNK2-independent, manner. (A) TGF-β1-induced loss of TER, indicative of barrier disruption, in wild-type MTEC is abrogated in MTEC lacking JNK1$^{-/-}$, but not in JNK2$^{-/-}$ MTEC. Cells were exposed to 5 ng/ml TGF-β1 every other day for the indicated days of culture. Data are graphed as percentage change in TER from baseline, as recorded over a time period of 10 days. Results represent three independent experiments. Absolute TER readings (Ohms × cm^2) before initiation of TGF-β1 exposure were: wild type 1143.3±149.2; JNK1$^{-/-}$ 3521.7±161.4; JNK2$^{-/-}$ 2956.7±150.5 (n=6/group). (B) Comparative evaluation of α-SMA expression in wild-type, JNK1$^{-/-}$ and JNK2$^{-/-}$ MTEC, exposed to TGF-β1 (10 ng/ml, every other day for 8 days). α-SMA expression (Red) was detected by immunofluorescence. Green, Nuclear counterstain, Sytox). The percentage of α-SMA expressing cells was quantified by counting the number of positive cells per field (n>50 cells). (C) Confirmation of genotypes of JNK1$^{-/-}$ or JNK2$^{-/-}$ MTEC by western blot analysis of total JNK.

These data suggest a role of JNK1 in modulating TGF-β1 signaling at the level of DNA binding and transcriptional activation.

Discussion

The lineages of the cells crucial to the pathogenesis of pulmonary fibrosis have been enigmatic. Whereas circulating fibrocytes or activation of local tissue fibroblasts have been considered as putative sources for production of mesenchymal products (Garantziotis et al., 2004; Schmidt et al., 2003), recent evidence has culminated to support the causal role of epithelial cells in the process of tissue remodeling, via the process of EMT. The findings from the present study identify the potential importance of epithelial cells in fibrogenesis through a number of observations and elucidate some key signals relevant to fibrosis. First, we demonstrated that TGF-β1 induced EMT using a well-defined model of primary lung epithelial cells (MTEC). Next, we unraveled the significance of JNK1 in EMT by demonstrating that JNK1$^{-/-}$ MTEC were markedly protected from TGF-β1-induced EMT. Then, we showed that Smad DNA-binding activity and induction of known EMT transcriptional regulators were attenuated in JNK1$^{-/-}$ MTEC compared with wild-type cells. Collectively, the present data highlight the functional importance of an epithelial TGF-β1-JNK1 signaling axis in EMT, which might be cardinal to the pathophysiology of pulmonary remodeling, including fibrosis. It is relevant to note that with the exception of one set of experiments our studies were conducted in primary epithelial cells that were isolated and propagated in culture in order to maintain their differentiated state. Our experimental design is of significance, as the process of EMT is linked to

transformation and carcinogenesis (Tse and Kalluri, 2007), and experiments conducted in transformed cell lines therefore have potential limited utility towards unraveling biochemical signals that are regulators of EMT.

The present findings are supported by a number of previous studies. Analysis of gene expression profiles in lungs of mice subjected to sensitization and challenge with ovalbumin using laser capture microdissection revealed that the most prominent induction of mesenchymal genes occurred in airway epithelial captures, with gene induction being less robust or absent in parenchymal regions or captures of smooth muscle (Kelly et al., 2005). Furthermore, lineage-tracing studies in models of kidney or pulmonary fibrosis also provided unequivocal genetic evidence to support the notion that epithelial cells provide a source for expansion of mesenchymal cells and mesenchymal products (Iwano et al., 2002; Kim et al., 2006). Lastly, fibroblastic cells that express both epithelial and mesenchymal markers can be found in biopsies of humans with IPF, highlighting the significance of EMT in vivo (Kim et al., 2006). Whereas pulmonary alveolar Type II pneumocytes were shown to undergo EMT in response to TGF-β1 in vitro and in vivo (Kasai et al., 2005; Kim et al., 2006; Willis et al., 2005), the current study clearly demonstrates the plasticity of epithelial cells in proximal airways. Additional studies will be needed to identify the locales at which EMT occurs in vivo, which are likely to be dependent on the source and nature of the fibrogenic stimulus.

The present study, which utilizes primary tracheal epithelial cells in vitro, unravels the importance of JNK1 in the process of EMT, and highlights the functional interplay of JNK1 with the TGF-β1

signaling cascade in airway epithelial cells. Although comparative evaluation of JNK2$^{-/-}$ MTEC did not demonstrate marked protection from TGF-β1-induced EMT, a role for JNK2 in this process cannot be fully excluded, based upon our data demonstrating some transient protection from TGF-β1-induced loss of TER (Fig. 4A), and the trends towards attenuated α-SMA expression in JNK2$^{-/-}$ MTEC compared with wild-type counterparts, in addition to the differences in staining patterns of α-SMA observed in JNK2$^{-/-}$ cells compared with controls (Fig. 4B). Additional studies are therefore needed to formally unravel the role of JNK2 in the process of EMT.

Our present findings are supported by a study demonstrating that a pharmacological inhibitor of JNK, CEP-1347, attenuated the phenotypic conversion of human lung fibroblasts to myofibroblasts induced by IL-4 and IL-13 (Hashimoto et al., 2001). In addition, an inhibitor of JNK or antisense RNA constructs blocked TGF-β1-induced mesenchymal gene expression in a keratinocyte cell line (Santibanez, 2006). Furthermore, a recent study demonstrated that degradation of caveolin-1 plays a crucial role in lung fibrosis, and, importantly, that the loss of caveolin-1 was crucial in the activation of JNK in response to TGF-β1 in lung fibroblasts (Wang et al., 2006). The same authors also demonstrated evidence for JNK phosphorylation in lung tissue from patients with IPF, in support of the functional significance of JNK in fibrogenesis in the lung.
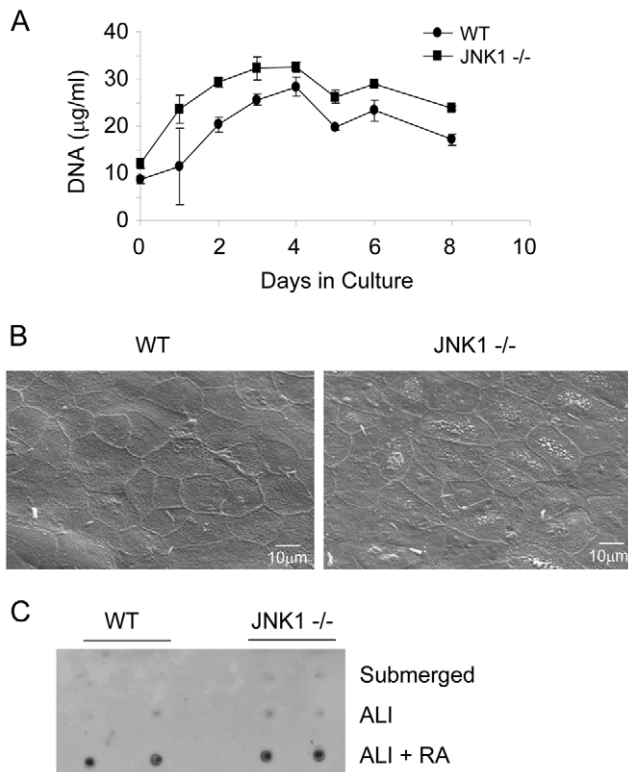
The mechanism by which JNK1 affects TGF-β1 signaling could be manyfold, based upon the complexities of the TGF-β and JNK signaling cascades. This interaction may include direct
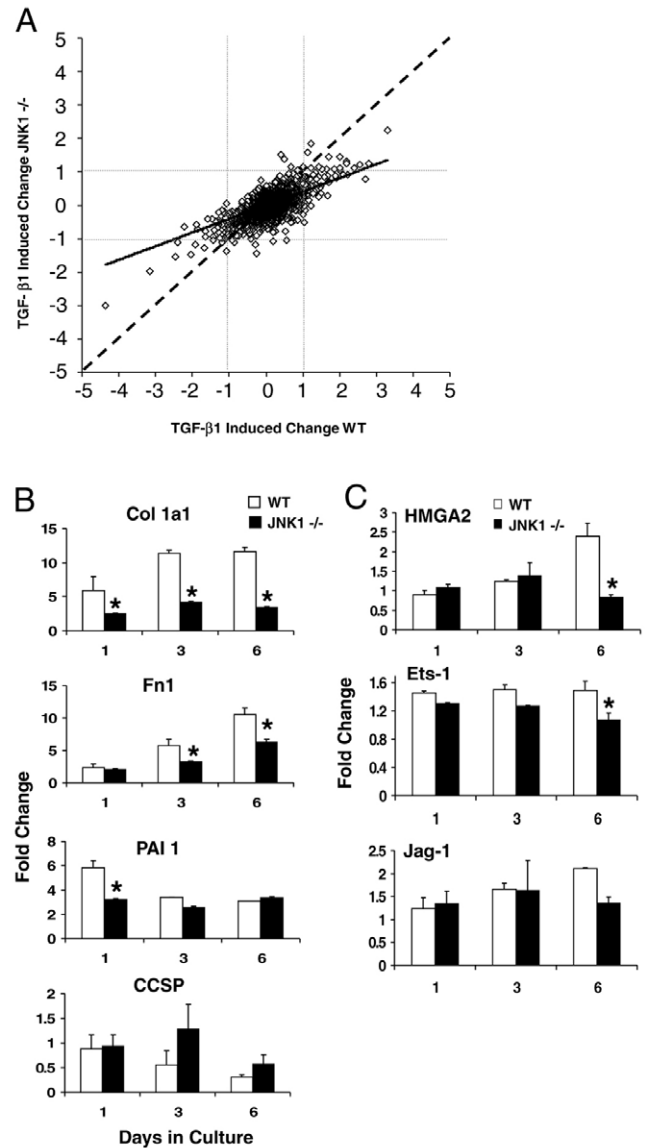

Fig. 5. Wild-type and JNK1$^{-/-}$ MTEC display similar growth, morphology and production of mucin in culture. (A) Growth curves of MTEC derived from wild type and JNK1$^{-/-}$ were conducted for the indicated times, and cell numbers were assessed by evaluation of total DNA content. (B) Wild-type and JNK1$^{-/-}$ cells established tight junctions, as shown by scanning electron microscopy. (C) Wild-type and JNK1$^{-/-}$ MTEC maintained in ALI for 10 days in the presence of 30 nM retinoic acid produced mucin. Media from two independent cultures from each condition were analyzed by slot blot analysis, using an anti-mucin antibody. Different dots shown represent results from independent samples.

Fig. 6. TGF-β1 induces EMT transcriptional responses in MTEC in a JNK1-dependent manner. (A) TGF-β1-induced gene expression changes obtained via microarray analysis are blunted in JNK1 MTEC compared with wild-type cells. Data plot shows the fold change (2^n) in gene expression induced by TGF-β1 compared to sham controls (TGF-β1-treated, sham controls) for wild-type MTEC (x-axis) and JNK1$^{-/-}$ MTEC (y-axis). The dashed line depicts the normal distribution expected if the gene expression changes were similar in wild-type and JNK1$^{-/-}$ MTEC. The solid line depicts the mean gene expression changes observed, and indicate a blunted response to TGF-β1 in JNK1$^{-/-}$ MTEC. Horizontal and vertical gray dotted lines represent the twofold change cutoffs. (B) TGF-β1-dependent modulation of mesenchymal and epithelial gene expression in MTEC depends on JNK-1. MTEC were treated with 5 ng/ml TGF-β1 for the indicated times and RNA evaluated by Taqman analysis. Data are represented as fold change in expression compared to sham controls (n=2, representative of five independent experiments). (C) TGF-β1 enhanced expression of known EMT regulatory transcription factors in a JNK1-dependent manner as assessed by Taqman analysis. * P<0.05, ANOVA versus wild-type MTEC. CCSP, clara cell secretory protein; Col1a1, collagen type 1a1; Fn1, fibronectin 1; HMGA 2, high mobility group AT-hook 2; Jag-1, jagged-1; PAI-1, plasminogen activator inhibitor 1.

phosphorylation of Smads, in addition to phosphorylation of Jun, a crucial component of the activator protein 1 (AP-1) transcription factor that can in some cases cooperate with Smads to drive TGF-β1-dependent transcription (Derynck and Zhang, 2003; Hocevar et al., 1999; Javelaud and Mauviel, 2005; Zhang et al., 1998). TGF-β1-induced phosphorylation of Smad2 and Smad3 has been shown to be JNK-dependent, and to mediate the transcriptional upregulation of connective tissue growth factor (CTGF), plasminogen activator inhibitor (PAI-1) and matrix metalloproteinase 2 (Kasai et al., 2005; Mori et al., 2004; Wang et al., 2006; Xie et al., 2005). An elegant study recently demonstrated that active JNK is directly capable of phosphorylating Smad2 and Smad3 in the linker regions at consensus MAPK phosphorylation sites. Smad2/3 linker phosphorylation by JNK was shown to promote their association with Smad4, and was crucial in the augmentation of PAI-1 gene expression (Mori et al., 2004). In direct contrast to our present observations, and the studies cited above, JNK has also been shown to abrogate the effects of TGF-β (Dennler et al., 2000; Verrecchia et al., 2001). JNK-dependent phosphorylation of Jun has been shown to inhibit Smad3-dependent transcription (Dennler et al., 2000), and to mediate the antagonistic effects of inflammatory cytokines on TGF-β signaling (Verrecchia et al., 2003). Mouse embryonic fibroblasts from JNK1 and JNK2 double null mice produced elevated levels of TGF-β in vitro, and displayed markedly altered expression patterns of many genes crucial to TGF-β signaling, demonstrating a role for JNK as a repressor of TGF-β1 transcription (Ventura et al., 2004). These disparate effects of JNK1 probably reflect the cell- and stimulus-specific context of JNK and TGF-β1 signaling outcomes, and the integration with additional signals.

The possibility exists that JNK1$^{-/-}$-deficient airway epithelial cells are resistant to TGF-β1 signals due to intrinsic differences in expression of TGF-β pathway components. However, evaluation of Smad2/3 expression levels and TGF-β1-induced Smad2/3 carboxyterminal phosphorylation revealed virtually identical responses to TGF-β1 in wild-type and JNK1$^{-/-}$ cells, demonstrating that the proximal TGF-β1 pathway is intact in JNK1$^{-/-}$ airway epithelial cells. Thus, the attenuation of EMT in JNK1$^{-/-}$ epithelial cells is probably due to dampening of downstream TGF-β1-induced transcriptional activation, results supported by gene expression profiling (Fig. 5). Our findings are in contrast with a recent observation in which JNK1$^{-/-}$ lung fibroblasts demonstrated attenuation of Smad2 phosphorylation, and nuclear accumulation of Smad2 and Smad3 in response to administration of TGF-β1, in comparison to wild-type cells (Wang et al., 2006). Furthermore, in that study, TGF-β1-induced expression of collagen 1 and fibronectin was not attenuated in JNK1$^{-/-}$ cells, compared to fibroblasts isolated from wild-type mice. Instead, the authors revealed that JNK1 appeared to play a crucial role in the caveolin-1-mediated suppression of TGF-β1-induced production of extracellular matrix. These disparate results are intriguing, and again suggest that the molecular action of JNK1 in interpreting and relaying TGF-β1-induced signals may be highly cell-type- and context-dependent.

The molecular actions of JNK1 in the causation of apoptosis are well known and have been extensively described (Davis, 2000). As TGF-β1 has been shown to cause apoptosis in epithelial cells, and apoptosis is required for pulmonary collagen deposition (Lee et al., 2004), it can be extrapolated that JNK1 could play a role in EMT as a consequence of apoptosis. However, a number of observations do not support this hypothesis. First, experiments that used the generic caspase inhibitor zVAD (10 μM) revealed that TGF-β1-induced loss of TER (supplementary material Fig. S3) or increases in mesenchymal gene expression (supplementary material Fig. S4) were not affected by the caspase inhibitor, whereas, as expected, zVAD blocked FasL-induced caspase 3 cleavage (supplementary material Fig. S5). Second, a recent study that dissected the molecular actions of TGF-β1 in cells in different stages of the cell cycle demonstrated that TGF-β1-induced EMT was prominent in cells in G1/S phases of the cell cycle, when caspase activity was low, whereas in cells in G2/M phase EMT was low, with caspase activity being high, suggesting a disparity between caspase activation and EMT (Yang et al., 2006). Third, TGF-β1-induced cell cycle arrest in epithelial cells, which precedes apoptosis, was also demonstrated to be uncoupled from induction of mesenchymal gene expression (Laiho et al., 1991). Collectively, these observations suggest that the molecular action of JNK1 in the induction of EMT in airways appears to be uncoupled from its well-known role in apoptosis.

A variety of potential mechanisms exist by which JNK1 might regulate EMT. The data presented herein suggest that JNK1 acts as a regulator of TGF-β1-induced transcriptional regulation. Smad DNA-binding and the mRNA levels of known EMT regulators Jagged 1, HMGA 2, Ets-1 and Wnt5a (Table 1, Fig. 6C) were all decreased in JNK1$^{-/-}$ MTEC versus wild-type controls. Recently in a kidney epithelial cell line, a novel transcriptional complex was described at the fibroblast transcription site FTS-1, which contained CArG box-binding factor-A (CBF-A) and KRAB-associated protein 1 (KAP-1). The authors furthermore demonstrated that CBF-A was a proximal activator of transcription in EMT, and that FTS-1 promoter regions were found in many genes functionally linked to EMT, including α-SMA, collagen type 1a1 and HMGA 2 (Venkov et al., 2007), which we demonstrated here to be modulated by TGF-β1 in a JNK1-dependent manner. It is therefore intriguing to speculate that JNK1 may be required for CBF-A induction by TGF-β1, which then controls subsequent activation of EMT-related genes. Another possible mechanism by which JNK1 could promote EMT might involve the regulation of Wnt5a production, as suggested by
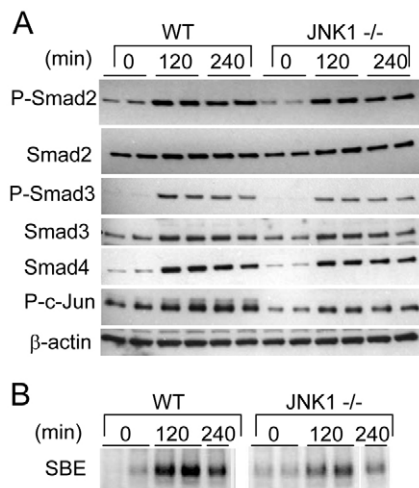

Fig. 7. Evaluation of TGF-β1 pathway activation in wild-type and JNK1$^{-/-}$-deficient MTEC. (A) Assessment of nuclear accumulation of phospho-Smad2 (P-Smad2), Smad2, P-Smad3, Smad3, Smad4 and P-Jun in wild-type or JNK1$^{-/-}$ MTEC after exposure to 5 ng/ml TGF-β1 for 120 or 240 minutes. (B) Assessment of binding of nuclear proteins to the consensus SBE via electrophoretic mobility shift analysis in wild-type or JNK1$^{-/-}$ MTEC after exposure to 5 ng/ml TGF-β1 for 120 or 240 minutes. Different lanes shown represent results from independent samples.

our gene expression analysis. Wnt5a has been shown to induce snail and vimentin, and to decrease E-cadherin levels consistent with EMT (Dissanayake et al., 2007). Lastly, JNK1 might be crucial to EMT via its role in regulating RhoA signaling via activation of JNK by ROCK and subsequent Jun phosphorylation (Marinissen et al., 2004). RhoA has been linked to loss of tight junctions and changes in cell structure and motility associated with EMT (Fan et al., 2007; Martin-Villar et al., 2006; Ozdamar et al., 2005). In addition, the RhoA-ROCK-JNK pathway has been linked to myofibroblast differentiation and increases of α-SMA expression (Smith et al., 2006). Based upon these reports, the molecular pathways by which JNK1 facilitates the process of EMT induced by TGF-β1 are likely to be complex and diverse.

The extent of fibrosis has been directly linked with poor prognosis in various human diseases (Elias et al., 1999), and the functional importance of TGF-β1 herein has been highlighted (Leask and Abraham, 2004). The present work identifies JNK1 as a crucial amplifier of TGF-β1 signaling in promoting EMT. Strategies to specifically attenuate the molecular actions of JNK1 in the TGF-β1 signaling module could have therapeutic value towards the attenuation of tissue fibrosis for which present therapeutic modalities have been proven to be ineffective. Findings from our study also suggest a functional significance of epithelial plasticity in fibrogenesis in the lung.

Materials and Methods

Animals and reagents

C57BL/6 mice, 2-4 months old, were originally purchased from Charles River Laboratories (Ste. Constant, QE, Canada) and were maintained within the colony as control animals. JNK1^{−/−} and JNK2^{−/−} mice were backcrossed more than ten generations into the C57Bl/6 background and have been previously described (Dong et al., 1998; Yang et al., 1998). Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Vermont. All chemicals utilized were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Smad2, Smad3 and phospho-JNK antibodies were obtained from Cell Signaling Technology (Danvers, MA); antibodies to JNK1, PAI-1 and ZO-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-cytokeratin and E-cadherin antibodies were purchased from Zymed (San Francisco, CA), Fibronectin-1 antibody was from Chemicon (Temecula, CA), and Collagen type 1a1 antibody was purchased from Fitzgerald Industries International (Concord, MA). The mucin antibody utilized was a gift from Samuel B. Ho (University of Minnesota).

Primary MTEC and fibroblast culture

MTEC cultures were isolated according to previously published methods (Wu and Smith, 1982). Briefly, tracheas were isolated, filled with 0.1% protease Type 14 in minimum essential media and incubated overnight at 37°C. The following day, tracheas were flushed, and MTEC were propagated on rat tail collagen I gel (BD Biosciences, San Jose, CA) coated tissue culture flasks in DMEM/F12 media containing 20 ng/ml cholera toxin (List Biologicals Laboratories, Campbell, CA), 4 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA), 10 ng/ml epidermal growth factor (EMD Biosciences, San Diego, CA), 100 nM dexamethasone, 2 mM L-glutamine and 50 U/50 μg/ml of Pen/Strep (Invitrogen). For experiments, MTEC were plated on collagen-I-coated culture dishes (BD Biosciences) or Transwell plates (12 mm well diameter, 0.4 μm pore size) (Corning Inc., Corning, NY) that were coated with rat tail collagen (50 μg/ml) and grown to confluence or $1000\Omega\times cm^2$ TER before initiation of experiments. Lung fibroblast cultures were prepared by digestion of lung pieces using 0.2% trypsin (Invitrogen, Carlsbad, CA), 0.1% collagenase type IV (Invitrogen) and 400 μg/ml DNAse (Worthington) for 30 minutes. The digest was then filtered with a 70 μm filter and released cells were cultured in DMEM with 10% fetal bovine serum (Invitrogen).

ALI culture

After growing cells to $1000\Omega\times cm^2$ on transwells, the media in the upper chamber was removed. Cells were then fed every other day in the lower chamber and TER was measured by washing the upper chamber with media then removing it. Trans-retinoic acid (30 nM; Sigma-Aldrich) was added to the lower chamber as indicated.

MTEC growth curve

Cells were plated and grown as above until the indicated days of harvest. To measure the total DNA, cells were washed twice with HBSS and then fixed for 10 minutes with ice-cold 70% ethanol plus 500 μl 5 M sodium hydroxide. Samples were then read at 260 nm.

Immunocytochemistry

At the indicated times for staining, cells were washed twice with PBS then fixed for 20 minutes with 4% paraformaldehyde in PBS. Following fixation, cells were permeablized for 10 minutes with 100% methanol, washed twice with PBS, and then blocked with PBS plus 1% BSA. Primary antibodies were then incubated as indicated by the manufacturer, and AlexaFluor (Molecular Probes, Eugene, OR) secondary antibodies were used for imaging. Images were collected by fluorescence microscopy.

Measurement of gene expression

Total RNA was isolated from MTEC using the RNeasy Mini Kit (Qiagen, Valencia, CA), subjected to reverse transcription and DNase treatment to produce cDNA for Taqman gene analysis using Assays on Demand for the individual target genes (Applied Biosystems, Foster City, CA).

Microarray analysis of gene expression

cDNA from primary MTEC isolated as outlined above was subjected to gene array analysis using a two-factor Affymetrix GeneChip experiment, using 430A 2.0 Array chips. GCOS (Affymetrix, Santa Clara, CA) was used to calculate the signal intensity for each probe and sample. Bioconductor software was used to calculate the RNA summary expression statistic for each probe set and sample (Bolstad et al., 2003; Irizarry et al., 2003). R was used to implement linear models that support estimation of expression statistics for each probe set and treatment group and to test hypotheses expressed as linear combination of the resulting values. Biological processes, cellular components and molecular functions associated with sets of differentially regulated probe sets were identified based on Gene Ontology (GO) annotation (Camon et al., 2003).

Evaluation and manipulation of JNK

MTEC cells were treated with TGF-β1 (5 ng/ml) for 1, 2 or 4 hours. Lysates were prepared for evaluation of phosphorylation of JNK1 and 2 by western blotting. Blots were evaluated by densitometry and fold changes in JNK1 or JNK2 phosphorylation were compared to control samples calculated after normalization to total JNK1 and 2 expression levels. JNK was inhibited pharmacologically using SP600125 (Calbiochem; La Jolla CA). MTEC were incubated with 10 μM SP600125 for 30 minutes before exposure to TGF-β1. A line of mouse alveolar type II epithelial cells (C10) (Malkinson et al., 1997) were incubated with Dharmacon SMARTpool control non-targeting siRNA (100 nM) or Dharmacon SMARTpool siRNA specific against JNK1 (100 nM) (Lafayette; CO) and subsequently exposed to TGF-β1 for 2 days for evaluation of mRNA expression using Taqman analysis.

Determination of TER

MTEC were grown on transwell culture plates (BD Biosciences, Bedford, MA). After culture to confluence, TER was measured in the media using an EVOM Epithelial Voltohmeter following the manufacturer's instructions (World Precision Instruments, Sarasota, FL).

Protein nuclear extraction and EMSA

Nuclear extracts from MTEC were prepared after lysing cells in hypotonic buffer, followed by incubation with 10% NP-40. Nuclei were pelleted, and suspended in hypertonic buffer to extract nuclear proteins (Janssen et al., 1997). EMSA was then performed with oligonucleotides containing SBE DNA-binding motifs (Promega, Madison, WI).

Scanning electron microscopy analyses

Cells were grown on transwell dishes as described above. Cells were fixed in 2% glutaraldehyde in PBS at 4°C for 1 hour and were post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.2 for 1 hour at room temperature. Next cells were immersed in 1% tannic acid in cacodylate buffer for 1 hour at room temperature and then immersed in 0.5% uranyl acetate in dH_2O for 1 hour at room temperature. Following fixation, cells were dehydrated in ethanol series using 10 minutes each in 25, 50, 75 and 85% ethanol. Then two times 10 minutes in 95%, and three times 15 minutes in anhydrous 100% ethanol. Then, 100-150 ml of hexamethyldisilazine (Sigma-Aldrich) was added into each insert and allowed to evaporate overnight in a fume hood. The Transwell membranes were removed using a scalpel, attached to 12 mm diameter aluminum specimen holders using colloidal graphite (Ladd Research Industries, Inc) and allowed to dry overnight. The next day, specimens were sputter coated with gold/palladium in Polaron sputter coater (Model 5100). Specimens were imaged on a JSM-6060 scanning electron microscope from JEOL USA, Inc. (Peabody, MA).

Transmission electron microscopy analyses

Cells were washed with 0.1 M Millonig's phosphate buffer (pH 7.2), then fixed in 1:1 H_2O dilution of Karnovsky's fixative (2.5% glutaraldehyde, 1% paraformaldehyde) at 4°C for 45 minutes. Samples were then washed with Millonig's phosphate buffer (pH 7.2), and post-fixed in 1% osmium tetroxide (OsO_4) at 4°C for 30-45 minutes.

Samples were then dehydrated in graded ethanols, from 35% to 100%. Cells were then infiltrated with Spurr's resin according to the following schedule: (100% ethanol:Spurr's resin) 3:1 for 30 minutes; 1:1 for 30 minutes; 1:3 for 30 minutes; and 100% Spurr's resin for 30 minutes. Flat embedding molds were filled with Spurr's resin, and cells were placed onto the surface of the resin, cell side down. Resin was then polymerized overnight at 70°C. Semi-thin sections (1 μm) were cut using glass knives on a Reichert Ultracut microtome, stained with Methylene Blue-Azure II and evaluated for areas of cells. Ultra-thin sections (60-80 nm) were cut with a diamond knife, retrieved onto 150 mesh copper grids, contrasted with uranyl acetate (2% in 50% ethanol) and lead citrate, and examined with a JEM 1210 transmission electron microscope (JEOL USA, Inc.) operating at 60 kV.

Statistical analyses

Data were analyzed by one-way ANOVA, using the Fisher's least significant difference test to adjust for multiple comparisons. Where appropriate, data were analyzed by unpaired two-tailed t-tests. Analyses with resultant $P<0.05$ were accepted as statistically significant. All statistics were performed using the Microsoft Excel software package (Redmont, WA). Data from individual experiments are presented as mean value ± standard error of the mean, and all experiments, with the exception of microarray analysis, were repeated at least three times.

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