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Smad3 Null Mice Develop Airspace Enlargement and Are Resistant to TGF-β-Mediated Pulmonary Fibrosis

Philippe Bonniaud,‡ Martin Kolb,‡ Tom Galt, Jennifer Robertson, Clinton Robbins, Martin Stampfl,* Carol Lavery, Peter J. Margetts, Anita B. Roberts, § and Jack Gauldie 2 *

Transforming growth factor-β1 plays a key role in the pathogenesis of pulmonary fibrosis, mediating extracellular matrix (ECM) gene expression through a series of intracellular signaling molecules, including Smad2 and Smad3. We show that Smad3 null mice (knockout (KO)) develop progressive age-related increases in the size of alveolar spaces, associated with high spontaneous presence of matrix metalloproteinases (MMP-9 and MMP-12) in the lung. Moreover, transient overexpression of active TGF-β1 in lungs, using adenoviral vector-mediated gene transfer, resulted in progressive pulmonary fibrosis in wild-type mice, whereas no fibrosis was seen in the lungs of Smad3 KO mice up to 28 days. Significantly higher levels of matrix components (procollagen 3A1, connective tissue growth factor) and antiproteinases (plasminogen activator inhibitor-1, tissue inhibitor of metalloproteinase-1) were detected in wild-type lungs 4 days after TGF-β1 administration, while no such changes were seen in KO lungs. These data suggest a pivotal role of the Smad3 pathway in ECM metabolism. Basal activity of the pathway is required to maintain alveolar integrity and ECM homeostasis, but excessive signaling through the pathway results in fibrosis characterized by inhibited degradation and enhanced ECM deposition. The Smad3 pathway is involved in pathogenic mechanisms mediating tissue destruction (lack of repair) and fibrogenesis (excessive repair). The Journal of Immunology, 2004, 173: 2099–2108.

The integrity and metabolism of extracellular matrix (ECM) components are central processes in lung development and homeostasis of lung function and morphology. Collagen accounts for ~15% of the dry lung mass and is actively metabolized, with one-tenth of the total lung collagen being degraded and newly synthesized each day (1). Two different lung diseases can be considered to result from an imbalance of ECM homeostasis. Emphysema associated with the adult lung is thought to result from the progressive proteolytic destruction of ECM without adequate repair, occurring through an imbalance in proteinase-antiproteinase activity (2). In contrast, pulmonary fibrosis is characterized by excessive interstitial deposition of ECM (3), possibly also resulting from an imbalance in ECM metabolism, with a more inhibitory proteolytic microenvironment with decreased matrix removal (4). For both conditions, no treatment is known to affect the natural history of the disease.

TGF-β1 is a key cytokine involved in the process of fibrogenesis (4), and has been demonstrated to play a pivotal role in fibrosis of many different organ systems (5). We have previously shown that transient adenoviral vector-mediated gene transfer of active TGF-β1 (AdTGF-β1223/225) to rat (6) or mouse (7) lungs leads to progressive and severe fibrosis. TGF-β causes myofibroblast differentiation, induces expression of connective tissue growth factor (CTGF), and increases the synthesis of ECM components such as collagen and fibronectin (3). Moreover, the role of TGF-β1 in inhibition of ECM degradation by up-regulation of proteinase inhibitors, such as tissue inhibitor of metalloproteinases (TIMPs) or plasminogen activator inhibitor-1 (PAI-1), has been demonstrated (8). In contrast, TGF-β1 has both positive and negative effects on synthesis of specific matrix metalloproteinases (MMP). MMPs are a large group of zinc-dependent enzymes that play a central role in the metabolism of ECM and basement membrane components (9). Among the metalloproteinases, MMP-2, MMP-9 (gelatinase A and B, respectively), and MMP-12 (macrophage elastase) have been implicated as having significant activity in the pathogenesis of emphysema (2). Recently, Morris et al. (10) showed that loss of activation of latent TGF-β in α5β1 null mice causes a MMP-12-dependent emphysema. In addition, MMP-2 and MMP-9 have been shown to be present in fibrotic tissues (11).

TGF-β signaling pathways are complex, and the Smad, MAPK, PI3K, and JNK pathways have been implicated by various studies (12). Smads are a family of cytoplasmic signal transducer proteins. Among these, Smad2 and Smad3 predominantly mediate signals from activated TGF-β receptors. Once phosphorylated, they bind to Smad4, translocate to the nucleus, and activate numerous TGF-β-responsive promoters (5, 13, 14). Smad3 appears to be a crucial element in the signal transduction pathways involved in wound healing and fibrosis (15–17). Smad3 null mice are protected against radiation-induced fibrosis of the skin (18), and a second Smad3 null line demonstrates attenuated lung fibrosis induced by bleomycin (19). Overexpression of Smad7, an inhibitor of the Smad pathway that prevents the phosphorylation and activation of...
Smad2 and Smad3, has also been shown to protect against fibrosis in a bleomycin model (20).

In this study, we exposed Smad3 null (knockout (KO)) or wild-type (WT) mice (21) to high local concentrations of TGF-β1 in the lungs by transient overexpression of the active TGF-β1 gene using adenoviral vector gene transfer, AdTGF-β1223/225 (6). We show in this study that loss of the Smad3 signaling pathway prevents specific TGF-β1-induced ECM gene regulation and blocks the development of progressive lung fibrosis. Moreover, we report for the first time that Smad3-deficient mice spontaneously develop increasing airspace enlargement with age, and demonstrate a marked presence of MMPs in the lung, with high acquired expression of MMP-9 and MMP-12. These data suggest that impaired regulation of both MMPs and TIMPs and other inhibitors, either under pathologic or homeostatic conditions, mediated through the Smad3 signaling pathway, may be causally related to the development of emphysema as well as pulmonary fibrosis.

Materials and Methods

Recombinant adenovirus

A replication-deficient adenovirus carrying a mutated TGF-β1 gene was constructed, as previously described (22). The TGF-β1 gene is mutated at positions 223 and 225 (cysteine to serine), which prevents binding to its latency-associated protein and thus yields a biologically active TGF-β1. The resulting replication-defective virus (AdTGF-β1223/225) was amplified and purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography, and finally plated on 293 cells (6). The control vector (adenovirus null vector control) with no insert in the E1 region was produced in the same way (22).

Animal treatment

Exon 8 of the Smad3 gene was disrupted in mice of background 129Sv/EV × C57BL/6 by Yang et al. (21). Smad3 heterozygous mice were bred under special pathogen-free conditions. The genotypes of both WT and Smad3 KO mice were determined by PCR analysis on tail DNA obtained from 3-wk-old animals; all experiments were performed with littermates to ascertain identical genetic backgrounds. Rodent laboratory food and water were provided ad libitum. The animals were treated in accordance with the guidelines of the Canadian Council of Animal Care. All animal procedures were performed under inhalation anesthesia with intratracheal instillation of 10% neutral buffered formalin for 70 °C until further use. The left lung was fixed by intratracheal instillation of 10% neutral buffered formalin for 24 h, longitudinal sections of 20 µl of PBS to 8-wk-old mice. Mice were sacrificed without any treatment, 4 and 28 days after adenoviral treatment. Five to seven animals were studied at each time point. After washing with PBS, the right main bronchus was tied; the right lung was removed, rinsed in PBS again, and frozen for 24 h, supernatants were removed and cells were harvested with TRIzol. Isolation and culture of primary lung fibroblasts from Smad3 KO and WT mice were grown to confluence in 25-cm² flasks (BD Biosciences, Sandy, UT) using DMEM with 10% FCS. Cells were then rested in DMEM with 1% FCS for 12 h and exposed to medium containing 1 ng/ml human rTGF-β1 (R&D Systems, Minneapolis, MN). At four time points between 0 and 24 h, supernatants were removed and cells were harvested with TRIzol (Invitrogen Life Technologies, Burlington, Canada)

Hydroxyproline assay

Lungs of untreated 6-wk-old mice were removed under sterile conditions after exsanguination of the animals. The pulmonary vasculature was perfused blood free with 10 ml of PBS through the right ventricular cavity. Large bronchi and vessels were removed; the lung parenchyma was cut into 2- to 3-mm² pieces, plated onto dry culture dishes, and incubated at 37% for 30 min to ensure attachment. Eagle’s MEM containing 10% FCS, 1% d-glutamine, and 1% penicillin/streptomycin was then added, and the tissue was left for 7 days without further handling. After 7 days, fibroblasts were seen to be growing out of the tissue. Cells were used between passages 2 and 4. Primary pulmonary fibroblasts from Smad3 KO and WT mice were grown to confluence in 25-cm² flasks (BD Biosciences, Sandy, UT) using DMEM with 10% FCS. Cells were then rested in DMEM with 1% FCS for 12 h and exposed to medium containing 1 ng/ml human rTGF-β1 (R&D Systems, Minneapolis, MN). At four time points between 0 and 24 h, supernatants were removed and cells were harvested with TRIzol (Invitrogen Life Technologies, Burlington, Canada)

Quantitative PCR

Cells lysed in TRIzol (Invitrogen Life Technologies) were processed for RNA and protein, according to the manufacturer’s instructions. Frozen lung samples were homogenized in 7 ml of TRIzol (Invitrogen Life Technologies), and RNA was extracted. RNA integrity and concentration were determined with a microgel bioanalyzer (Agilent 2100; Agilent, Waldbronn, Germany). RNA (1 µg) was DNase treated, then reverse transcribed using a standard protocol (Invitrogen Life Technologies). Quantitative real-time PCR was conducted using an ABI Prism 7700 Sequence Detector. Negative control samples (no template or no reverse transcriptase) were run concurrently. Primers (Mobix, Hamilton, Canada) and probes (Applied Biosystems, Foster City, CA) are shown in Table I. Results were normalized to GAPDH, which was measured using previously optimized probe and primers (Applied Biosystems).

Statistical analysis

Data are shown as mean ± SEM. For evaluation of group differences, we used Student’s t test. A p value <0.05 was considered significant.

Results

Smad3 KO mice show age-dependent airspace enlargement

Histology and quantification of alveolar airspace. As we found that lung tissue from untreated adult (4-mo-old) Smad3 KO mice showed histological evidence of marked airspace enlargement, we analyzed them at different ages. At 3 wk of age, there were no
major histologic differences between lungs of KO and WT mice. In both genotypes, there was minor evidence of uncompleted alveologenesis with some enlarged terminal bronchioles. However, even at this early time point, histologic examination suggested that there already was a minor increase in alveolar airspace in Smad3 KO mice (Fig. 1A). We confirmed this small, but nonsignificant difference in alveolar size using quantitative histomorphology (Fig. 1B). This increase became obvious in all KO animals from 8 wk of age onward, and at 4 mo of age, the airspace enlargement in the peripheral airspace in lungs of Smad3 KO mice was very evident. This enlargement was widespread throughout the parenchyma, but was not homogeneous, because enlarged alveoli were interspersed with normal-size alveoli (Fig. 1A). The differences in airspace in 8-wk-old and 4-mo-old mice were confirmed with quantitative histomorphology, which demonstrated, respectively, a 22 and 36% increase in mean cord length of the airspace in Smad3 KO lungs compared with WT ($p < 0.001$) (Fig. 1B).

**MMPs in lungs of Smad3 KO mice**

Untreated adult 8-wk-old KO mice showed a significant 35% increase in total cell count in BAL fluid compared with WT littermates (Table II). However, there was no significant difference in the differential cell count, except for a slight increase in neutrophils in KO animals compared with WT (respectively 1.6 and 2.2-fold, $p < 0.05$) as was MMP-12 expression not significantly different between KO and WT mice (1397 ± 352 and 1685 ± 458 pg/ml, respectively). Four days after AdTGF-β1 administration, there was no significant difference between WT and KO in either the total or the differential cell count (Table II) in BAL fluid.

**Fibrosis assessment**

At day 28, Smad3 KO or WT animals treated with control adenovector were not distinguishable from age-matched naive animals (data not shown), as we have previously described in other animal models (6, 25). However, following treatment with the adenovector expressing active TGF-β1 (AdTGF-β1), histology of lungs from WT mice showed a fibrotic pattern characterized by marked deposition of abnormal amounts of ECM at day 28, predominantly in peribronchial tissue with patchy areas within the parenchyma (Fig. 3A). In contrast, lungs from similarly treated Smad3 KO mice did not show collagen accumulation nor ECM deposition (Fig. 3A) and, in fact, were not different from lungs of Smad3 KO mice after treatment with the control virus.

To confirm and validate the histological findings, tissue fibrosis was quantified by analysis of hydroxyproline content (Fig. 3B). WT mice treated with AdTGF-β1 had a significantly higher hydroxyproline content in the lung (65% increase, $p < 0.05$) at day 28 compared with mice treated with control vector. In contrast, hydroxyproline concentration in the lung of Smad3 KO mice was not significantly different at 28 days after AdTGF-β1 administration compared with lungs of animals treated with null adenovirus.

**Gene expression in pulmonary fibroblasts after TGF-β1 stimulation in vitro**

To determine whether the lack of fibrotic response was evident at the level of the fibroblast, we used primary cultures of lung fibroblasts from Smad3 WT and KO mice to investigate differences in gene expression of fibrosis-related genes in response to exposure to rTGF-β1. CTGF mRNA expression at basal level was 2-fold lower in Smad3 KO lung fibroblasts than in WT. After treatment with rTGF-β1, there was a significantly increased expression of CTGF in WT fibroblasts at 3, 6, and 24 h (3.6-, 4-, and 3.3-fold increase, respectively), which was significantly higher at each time point than in Smad3 KO fibroblasts. PAI-1 mRNA expression was also lower in untreated Smad3 KO lung fibroblasts ($p < 0.05$) compared with WT. At 6 h after rTGF-β1, PAI-1 expression was still 2.2-fold lower in Smad3 KO fibroblasts (Fig. 4B). TIMP-1

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**Table 1. Mouse primers and probes used in quantitative real-time PCR reactions**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>TCCGGAAAGTCAAGCTCTAGCT</td>
<td>CCGTGGAAAGTCAAGCTCTAGCT</td>
</tr>
<tr>
<td>PAI-1</td>
<td>TAGCTGGCTGGGGTTTTACGTTTTATAAGGT</td>
<td>CACCTGGTGGGCTGGGGTTTTACGTTTTATAAGGT</td>
</tr>
<tr>
<td>Procollagen 3A1</td>
<td>GTTGGAAATGCAAGCTCTAGCT</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
<tr>
<td>Elastin</td>
<td>TGTTGCAATGCTCTCTCTCTTT</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>GTCGTTGCTCTCTCTCTTT</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
<tr>
<td>MMP-2</td>
<td>AGGCGGGAAGGGGATTCAGTTT</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
<tr>
<td>MMP-9</td>
<td>GCAAGCAGCATCAGCATACAA</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
<tr>
<td>MMP-12</td>
<td>TGGTGGCTGGGGTTTTATTAAGGT</td>
<td>CCGTGGAAATGCAAGCTCTAGCT</td>
</tr>
</tbody>
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*The Journal of Immunology*
gene expression (Fig. 4C) was not significantly different at basal level in either cell type. However, at 6 h after treatment with rTGF-β1, there was a significant (1.7-fold) increased expression (p < 0.03) in WT fibroblasts that was not seen in KO fibroblasts. TGF-β1 mRNA was never expressed at enhanced levels after rTGF-β1 exposure in Smad3 null fibroblasts (Fig. 4D), whereas there was a significant increase in TGF-β1 gene expression in WT fibroblasts at 6 and 24 h (p < 0.03).

**Discussion**

We have observed two distinct, but likely related processes occurring in the pulmonary matrix of Smad3 KO mice: 1) untreated Smad3 KO mice, kept in conventional animal housing, develop an age-related increase in alveolar spaces, and by 4 mo of age, show clear evidence of progressive lung airspace enlargement; 2) in contrast to WT littermates, exposure of Smad3 KO mice to enhanced levels of active TGF-β1, by adeno virus gene transfer to the lung, fails to induce matrix deposition and progressive pulmonary fibrosis. We suggest that both these processes are caused by modulation of TGF-β signaling through the Smad3 pathway, and the outcome is determined by imbalances in ECM metabolism induced by changes in spontaneous or pathologic gene expression.

**Smad3 null mice develop emphysematous alveolar enlargement associated with dysregulated expression of MMPs**

In this study, we observed that naive Smad3 KO mice appear to develop age-related severe lung airspace enlargement. Smad3-deficient mice show increased mortality between 1 and 8 mo due to primary defects in immune function with inflammatory lesions (21). However, tissues such as lung, brain, heart, and kidneys are not commonly involved, and these mice do not normally die from respiratory failure. To our knowledge, no descriptions have been reported of airspace enlargement in aging Smad3 KO mice. At 4 mo, emphysematous lesions were extensive, morphologically typical, and constant between animals (Fig. 1A), with the mean cord length of the airspace in KO mice being 36% greater than that of WT.

TGF-β is an important growth factor, among others, in lung development, having a pronounced inhibitory impact on branching morphogenesis (26, 27). Smad3 null mice, however, do not display these developmental defects at birth. Explanations include a postulated compensatory role of Smad2- or other Smad3-independent signaling pathways downstream of TGF-β1 in development of the lung (27). We observed only mild, nonsignificant airspace enlargement at 3 wk in Smad3 KO mice (Fig. 1B). However, emphysematous changes increase dramatically with time, reaching significant alveolar enlargement by 8–9 wk and easily evident enlargement by 4 mo of age.

During development of emphysema in the human, there is a decrease in total ECM components of the lung parenchyma (2), which could result from either a decrease in ECM production, an increase in ECM degradation, or both. The steady state level of procollagen 3A1 expression, which is at least in part dependent on the Smad3 pathway and TGF-β1 stimulation (16), was not significantly lower in the Smad3 KO mice than in the control group at birth or at adult age. Moreover, we did not find any significant difference in elastin expression at birth or at 8 wk between KO and WT mice, despite the fact that the Smad3 pathway has been implicated in the expression of elastin dependent on TGF-β1 stimulation.
Fibronectin expression has been described both as being dependent (29) or not dependent on Smad3 pathway signaling (17). The balance between proteinases, especially MMPs, and their inhibitors, TIMPs, is also thought to be critical in the process of emphysema. MMP-2, MMP-9, and MMP-12 have been implicated in this ECM-destructive disease (30–32). Both MMP-2 and MMP-9 are capable of elastolysis as well as collagenolysis (33), while MMP-12 is elevated in mice exposed to cigarette smoke and MMP-12 KO mice are protected from cigarette smoke-induced emphysema (30), and alveolar macrophages release more MMP-9 in chronic obstructive pulmonary disease patients and cigarette smokers (34, 35). Osteopetrotic mice, deficient in macrophage CSF, develop emphysema and have higher levels of MMP-2, MMP-9, and MMP-12 in BAL fluid and alveolar macrophages than in controls (36), while lung surfactant protein D gene-inactivated mice have airspace enlargement associated with an increase in the same three MMPs (37). Finally, the balance of MMPs to TIMPs within the local tissue microenvironment is important in the process of emphysema, as indicated in TIMP-3 KO mice, which develop progressive airspace enlargement (38).

Smad3 is required for the TGF-β-mediated inhibition of MMP-12 expression in alveolar macrophages (39, 40), and monocytes exposed to TGF-β show inhibited release of MMP-9 (41).

Table II. Differential counts in BAL fluid from WT and KO mice, untreated or 4 days after AdTGF-β1 (intranasal injection, mean ± SEM, *p < 0.05)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Total × 10⁴</th>
<th>% Macrophage</th>
<th>% Lymphocyte</th>
<th>% Neutrophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>WT</td>
<td>21 ± 2.9</td>
<td>96.7 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>28.5 ± 1.2*</td>
<td>94.6 ± 2.7</td>
<td>3.8 ± 3.2</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>WT</td>
<td>83 ± 6.2</td>
<td>74.9 ± 4.9</td>
<td>21.9 ± 4.2</td>
<td>3.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>86 ± 6.7</td>
<td>79.4 ± 9.8</td>
<td>16.7 ± 7.9</td>
<td>3.9 ± 2.6</td>
</tr>
</tbody>
</table>

FIGURE 2. Adult KO mice have an acquired and increased expression of MMP-9 and MMP-12. A, MMP-2 and MMP-9 activities in BAL fluid. An equal amount (25 μl) of BAL from 8-wk-old untreated Smad3 WT or KO animals was loaded for zymography analysis. Total protein amount in each well was 2.2, 2.1, 2.9, 3.1, 2.5, 2.5, and 3.9 μg, respectively, without significant difference. Molecular weights are shown on the left, and MMP-2 and MMP-9 standards on the right. There was marked MMP-9 activity in KO mice BAL with no detectable activity in WT. There was no difference in MMP-2 activity in KO or WT mouse BAL. B, Quantitative RT-PCR. MMP-12 mRNA expression is significantly higher in BAL cells from adult (8- to 10-wk-old) KO mice compared with same age WT. n = 6 animals per group. *, p < 0.05. C, Quantitative RT-PCR: elastin, procollagen 1a3, MMP-2, MMP-9, and MMP-12 mRNA expression in total lung RNA from 8-wk-old untreated animals; WT, n = 6; KO, n = 7. D, Quantitative RT-PCR: elastin, procollagen 1a3, MMP-2, MMP-9, and MMP-12 mRNA expression in lung from 1 day old. n = 7 animals per group.
Smad3 KO mice are resistant to TGF-β-induced fibrosis. A. Representative sections of Smad3 KO lungs show no evidence of fibrosis or ECM accumulation 28 days after AdTGF-β1 administration (b, H&E stain ×50; d, trichrome stain ×200) compared with WT mice (a, H&E stain ×50; c, trichrome stain ×200). B. Lung hydroxyproline concentration (five animals in each group). WT animals treated with AdTGF-β1 show a 65% increase of lung hydroxyproline concentration by day 28 compared with animals treated with control virus. There is no significant difference in the Smad3 KO mice group between AdTGF-β1 and control virus. *, p < 0.05.
Moreover, both MMP-2 and MMP-9 can activate TGF-β from its inactive latent proform (42), identifying a positive feedback (homeostatic) mechanism designed to closely regulate the levels of these two highly active enzymes. Our data now demonstrate a regulatory role for the Smad3 pathway in the spontaneous development of emphysema. We have shown an age-dependent increase in MMP-9 and MMP-12 activity in BAL from untreated Smad3 KO mice, compared with WT littermates. These changes in enzymatic activity correlate with up-regulation of MMP-12 mRNA in BAL cells (95% macrophages), and with elevated levels of MMP-9 and MMP-12 mRNA in total lung RNA. Whereas TGF-β1-induced MMP-9 production has been suggested to be more dependent on Ras/MAPK pathways (43, 44), our findings suggest that Smad3 is also important in TGF-β1 inhibition of MMP-9 expression. Of note, MMP-2 has been shown to be dependent on Smad2, and not Smad3, for stimulated expression (45), consistent with our observation that levels of MMP2 are not changed in Smad3 KO mice. Our data suggest that this intrinsic acquired difference in expression of MMP-9 and MMP-12, which is maintained even after TGF-β stimulation (data not shown), may be, at least in part, responsible for the progressive ECM destruction.

Together, these data suggest that the homeostatic balance of matrix synthesis and degradation in lungs of WT mice is perturbed in Smad3 KO mice to favor degradation of matrix (Fig. 1B). As these mice are housed in conventional conditions, repeated exposure to commensal agents or cage dust may result in tissue damage and inflammation at the microlevel in the lung. In the absence of Smad3, physiologic repair by TGF-β is blunted, resulting in progressive alveolar damage and airspace enlargement.

**An imbalance in matrix protein metabolism favoring degradation in Smad3 null mice confers resistance to pulmonary fibrosis**

TGF-β1 is a key cytokine in the fibrosis of various tissues, including lung. We have already shown the dramatic profibrotic activity of TGF-β1 in rat (6) and mouse lung (25) by adenoviral gene transfer of active TGF-β1. Involvement of the Smad 3 signaling pathway, known to be activated by TGF-β and also by activin, is suspected to be a major contributing factor in fibrogenesis (15) and other aspects of tissue remodeling.

In this study, we have demonstrated that transient overexpression of active TGF-β1 does not induce fibrosis at any time point examined out to day 28 in Smad3 KO mice in contrast to the accumulation of ECM and progressive fibrosis seen in WT mice.
expression of TIMPs, creating a non-fibrolytic environment, has been associated with matrix accumulation, and the importance of inhibition of MMP and impaired collagenolysis in fibrosis has been confirmed in animal and human studies (50–55). We have previously demonstrated this in mice prone to fibrosis, in which significant up-regulation of TIMP-1 could explain the propensity to develop fibrosis after TGF-β1 exposure (25). In this current study, we found that induction of expression of TIMP-1 mRNA, in response to TGF-β1 stimulation, was significantly impaired in Smad3 KO mice. The role of Smad3 in induction of TIMP-1 gene expression by TGF-β1 has also been demonstrated in human dermal fibroblasts and confirmed in Smad3 KO mouse embryo fibroblasts (16). Likewise, we found that induction of the antiproteinase PAI-1 by TGF-β1 is very dependent on Smad3, as suggested from previous work (14, 56, 57).

**Fibrosis and emphysema represent opposite ends of the spectrum of matrix metabolism, both dependent on Smad3**

Interestingly, our data are in accordance with reports on the function of lefty, a member of the TGF-β family and a potent inhibitor of TGF-β signaling. In a fibroblastic tumor model in vivo (58, 59), it was shown that lefty decreased collagen type I mRNA expression and simultaneously increased collagenolytic, gelatinolytic, elastolytic, and caseinolytic activities, similar changes as seen in the current studies with Smad3 KO mice. Moreover, in a recent study, Morris demonstrated that the loss of αvβ6 integrin in mice induces a MMP-12-dependent emphysema, related to the lack of activation of latent TGF-β (10). The importance of Smad3 in the pathogenesis of fibrosis is evident from the lack of fibrogenesis seen when the KO mice are exposed to exogenous levels of active TGF-β (Fig. 1), while the WT littermate progresses to widespread fibrosis. Similar findings were previously reported for modulation of bleomycin-induced fibrosis in Smad3 KO mice (60). However, equally important is the fact that providing excess levels of active TGF-β did not prevent the progressive alveolar space enlargement (data not shown), indicating that Smad3 signaling pathways are involved either directly or indirectly in the process of both fibrosis and emphysema.

In summary, we have provided substantial data highlighting the importance of TGF-β1 and signaling through the Smad3 pathway in the maintenance of lung ECM. We have shown that two diverse and apparently opposite disease processes, fibrosis and emphysema, are each associated, at a fundamental level, with modulation of ECM metabolism. Fibrosis and emphysema represent two extremes of matrix metabolism, with fibrosis resulting from decreased (inhibited) ECM metabolism and deposition, and emphysema from increased matrix metabolism. More specifically, TGF-β appears to play a pivotal role in both processes, with signaling through the Smad3 pathway key to both disease processes. The loss of Smad3 protects against fibrosis resulting from pathologic expression of TGF-β, but confers susceptibility to emphysema by interfering with the normal physiologic (homeostatic) action of TGF-β in maintaining alveolar structure. This also suggests that in addition to the recognized association of susceptibility for emphysema to polymorphism in the MMP-12 gene, we should suspect a similar association might exist to polymorphism in the Smad3 gene, already known to be associated with the incidence of ovarian cancer (61, 62).

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