Smad3 Deficiency Ameliorates Experimental Obliterative Bronchiolitis in a Heterotopic Tracheal Transplantation Model

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Chronic allograft rejection manifested as obliterative bronchiolitis (OB) remains the single greatest impediment to long-term survival after lung transplantation. Transforming growth factor- β 1 (TGF- β 1) has been implicated in the tissue remodeling response associated with OB. Therefore, its intracellular signal transducer, Smad3, is a prime target of investigation. Herein, we examine the role of TGF- β 1, through Smad3, in the development of OB using heterotopic tracheal transplantation in wild-type and Smad3-null mice. TGF- β 1 was detectable within infiltrating mononuclear cells early after transplantation. Later it was detected in fibroblasts and in the connective tissue accumulating within the lumen and the airway wall of the transplanted allografts. Connective tissue growth factor had a similar time and tissue distribution. Nuclear detection of Smad3 and phosphorylated Smads within intraluminal fibroblasts coincided with increased intraluminal deposition of fibronectin and collagen. When transplanted into Smad3-null mice, allografts failed to organize the intraluminal exudates despite fibroblast accumulation and showed reduced fibronectin and collagen deposition. In culture, Smad3-deficient fibroblasts expressed reduced fibronectin in response to TGF- β 1 compared to wild-type cells. Together, these studies suggest that the TGF- β signal transducer, Smad3, is required for the development of experimental OB in transplanted tracheas. (*Am J Pathol* 2004, 165:1223–1232)

Throughout the last 20 years, lung transplantation has evolved into an accepted treatment option for patients with end-stage lung disease because of emphysema, pulmonary fibrosis, pulmonary hypertension, and cystic fibrosis.¹ However, chronic allograft rejection manifested as obliterative bronchiolitis (OB) remains the major obstacle to long-term survival after lung transplantation.² Clinically, OB is characterized by airflow limitation, defined by a 20% decline in forced expiratory volume in 1 second (FEV₁), and is often complicated by recurrent lower respiratory tract infections. The histological hallmark of OB is the presence of obstructing intraluminal polyps comprised of fibromyxoid granulation tissue and plaques of dense submucosal eosinophilic scar on lung biopsy. OB can complicate up to 60% of lung allografts and becomes increasingly prevalent the further removed from the transplant operation.³ Progressive OB ultimately results in worsening hypoxemia and death.

Many studies on the pathogenesis of OB have concentrated on the role of cellular allogeneic immune responses during OB development. However, fibroproliferation and tissue remodeling clearly play an important role in its pathogenesis because OB is characterized by the accumulation of fibroblasts and the excessive deposition of connective tissue matrix within the lumen of the affected bronchioles. The factors that initiate and maintain fibroproliferation and tissue remodeling within the airway lumen in OB have not been fully elucidated. One candidate factor is transforming growth factor (TGF)- β , a pleiotropic cytokine with potent profibrotic activities.⁴

Extensively studied in solid organ transplantation, TGF- β has been found to have beneficial effects on alloimmunity. For instance, overexpression of a TGF- β transgene leads to marked reductions in acute rejection and prolongation of graft survival in experimental heart and

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lung transplantation.^{5,6} Moreover, tolerance has been induced in an orthotopic lung transplant model in association with elevated serum levels of TGF- β , although allo-immune responses were restored with the administration of neutralizing antibodies to TGF- β .⁷

Despite its potent anti-inflammatory properties that would otherwise seem desirable in transplantation, TGF- β has been implicated in the pathogenesis of allograft rejection, and is markedly overexpressed in patients with chronic liver, kidney, and heart rejection.^{8–10} Similarly, emerging results indicate a role for TGF- β in lung allograft rejection and the development of OB. For example, TGF- β protein expression by immunohistochemistry is increased in OB patients, with intensity of staining correlating with severity of luminal fibrosis.¹¹ Detection of TGF- β in allografts has even been shown to be an early marker for OB.^{11,12} As a proof of concept, studies in an animal model of OB that interrupt TGF- β binding to its receptor have shown reduced intraluminal airway matrix deposition.¹³ The difficulty in fully understanding the biology of TGF- β in organ transplantation lies in reconciling these seeming dichotomous actions: its protective early up-regulation in acute rejection versus its prolonged overexpression in chronic rejection. We are investigating the latter aspects of TGF- β 1 and its downstream effectors in their involvement in the development of OB after lung transplantation.

In the present study, a heterotopic tracheal transplant model as described by Hertz and co-workers¹⁴ was used to investigate the involvement of Smad3 in the development of OB. Smad3 is a member of the highly conserved Smad family of intracellular signaling proteins that mediate many of the effects of TGF- β 1. Smad3 is directly phosphorylated by the ligand-activated TGF- β type I receptor (T β RI),¹⁵ After partnering with a common mediator Smad (Smad4), the heteromeric complex translocates into the nucleus where it regulates gene transcription. Specifically, Smad3 has been linked to the up-regulation of a number of genes involved in connective tissue remodeling including the profibrotic growth factors platelet-derived growth factor¹⁶ and connective tissue growth factor (CTGF),¹⁷ the matrix molecules fibronectin and collagen,^{18,19} matrix-binding integrin receptors such as the fibronectin receptor α 5 β 1,²⁰ and matrix metalloproteinases,²¹ among others.

Our studies show that in allograft specimens with intraluminal fibroproliferation, there is increased expression of TGF- β 1 and CTGF, and increased deposition of fibronectin and collagen. In Smad3 knockouts, there was marked inhibition of intraluminal fibroproliferation with decreased expression of these molecules. These studies strongly suggest that Smad3 mediates many of the TGF- β 1 effects that promote OB after transplantation.

Materials and Methods

Animal Model of Tracheal Transplantation

Six- to ten-week old BALB/c, C3H/HeJ (Harlan, Indianapolis, IN), and Black Swiss mice (generous gift from C. Deng, National Institutes of Health, Bethesda, MD) were

Figure 1. Histopathology (H&E) of heterotopically transplanted tracheas. **A:** Tracheal allografts 14 days after transplantation are thickened and show marked lymphocytic infiltration and epithelial loss. **B:** The 28-day allograft is characterized by the fibrous obliteration of the airway, neovascularization, and the presence of fibroblastic cells. **C:** Syngeneic grafts maintain normal tracheal architecture even after 28 days. Original magnifications, $\times 100$.

housed in flat-bottomed cages, provided food and water *ad libitum*, and used in accordance with the rules and regulations of the Animal Care Committee of the University of Illinois at Chicago. Heterotopic tracheal transplantation was performed as previously described.¹⁴ Briefly, allografts and isografts were obtained by transplanting BALB/c tracheas subcutaneously into C3H/HeJ and BALB/c recipient hosts or Smad3^{ex8/ex8} wild-type and null Black Swiss mice.²² Grafts were harvested at indicated

Figure 2. Immunostaining for TGF- β 1 and CTGF in day 14 tracheal allografts. TGF- β 1 is expressed within infiltrating inflammatory cells and extracellularly within the luminal space (A) whereas CTGF staining is seen intracellularly in fewer inflammatory cells in the airway (C). Isotype controls for TGF- β 1 (B) and CTGF (D) are also depicted. Original magnifications: $\times 1000$ (A); $\times 400$ (B–D).

time points, formalin-fixed, and paraffin-embedded, and cut into 6- μ m-thick sections (Leica, Deerfield, IL). Sections from allografts and isografts were stained with hematoxylin and eosin, or Masson's trichrome, and examined in parallel by a blinded observer (M.S.).

Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated with graded alcohol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Antigen retrieval was performed by heating sections in target retrieval solution (DAKO, Carpinteria, CA) with a 95°C water bath. Slides were washed in Tris-buffered saline with 0.05% Tween-20 (Sigma, St. Louis, MO), blocked with serum-free protein block (DAKO), and incubated with an optimized dilution of primary antibody overnight at 4°C and then for 60 minutes with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by horseradish peroxidase-streptavidin (Vector). Primary antibodies against CTGF/*fisp-12* (generous gift from L.F. Lau, University of Illinois at Chicago), TGF- β 1 and phospho-Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Smad2 (Cell Signaling Technology, Inc.,

Beverly, MA), Smad3 (Zymed Laboratories, San Francisco, CA), and fibronectin (Abcam, Cambridge, MA) were used. Color was developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (Zymed), counterstained with Mayer's hematoxylin, and coverslipped with mounting medium. For each primary antibody, an appropriate irrelevant IgG was used as negative control to ensure that effects of nonspecific binding were recognized.

Image Analysis

Image analysis was performed as previously described²³ with multiple digital photomicrographs (Olympus, Melville, NY) of sections taken under high-power view. Photomicrographs were imported into Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA). A color sampler tool was used to gate representative shades of the color of interest. To determine the stain density of each field, the area of the field containing the selected color stain was selected, the remaining background was removed, and the resulting image was loaded into Scion Image Beta 4.0.2 (Scion Corp., Frederick, MD). An average density of staining was calculated for each section.

Figure 3. Immunostaining for TGF- β 1 and CTGF in day 28 tracheal allografts. By 28 days after transplantation, TGF- β 1 (**A**) and CTGF (**C**) both localize within fibroblastic cells (**arrows**), tracheal wall, subepithelium and basement membrane, and fibrous tissue (**inset**). Isotype controls for TGF- β 1 (**B**) and CTGF (**D**) are stained negative. Original magnifications: $\times 1000$ (**A**, **C**); $\times 400$ (**B**, **D**).

Cell Culture and Western Blot Analysis of Fibronectin

Embryonic fibroblasts were established from 14-day post-coitum Smad3-null mice or wild-type littermates, as described previously.²⁴ Cells were grown at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum and 1% antibiotic-anti-mycotic (100 U/ml penicillin G sodium, 100 U/ml streptomycin sulfate, and 0.25 g/ml amphotericin B), and studied between passages 4 to 10. Fibroblasts were grown to near confluence, serum starved for 24 hours, and incubated with 5 ng/ml of rTGF- β 1 (R&D Systems, Minneapolis, MN). Culture supernatants were collected after 72 hours. Protein concentration was determined by the Bradford method. Aliquots (30 μ g of protein) were fractionated by electrophoresis in 5% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose paper. The nitrocellulose paper was blocked with 5% milk in Tris-buffered saline-0.1% Tween-20 buffer, incubated with a 1:1000 dilution of anti-fibronectin antibody (Sigma), and then a horseradish peroxidase-conjugated secondary antibody (Sigma). Immunoreactivity was visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ). To

verify equal loading of protein, gels were stained after the transfer procedure with 0.25% Coomassie Blue in methanol (50%) and glacial acetic acid (10%) for 4 hours and destained (50% methanol and 1% glacial acetic acid) overnight.

Statistical Analysis

Data are expressed as means $\pm$ SD. Analysis of variance was applied to determine the significance of differences between data, with a *P* value of less than 0.05 considered significant.

Results

Localization of TGF- β 1, CTGF, and Smad3 in Tracheal Allografts

Transplanted tracheas were explanted from five C3H/HeJ and five BALB/c recipients at 14 and 28 days after transplantation, with findings consistent with previous reports.^{14,25} At 14 days after transplantation (Figure 1A), tracheal walls were thickened, characterized by predominantly submucosal mononuclear cell infiltration with a

Figure 4. Immunostaining of tracheal allografts for Smad proteins. Fibroblasts are found to express Smad3 (inset in A). A: Both nuclear (arrowheads) and cytoplasmic (large arrows) localization of Smad3 is present. Other fibroblasts (large arrow) are negative for Smad3 staining. C: Grafts stained for phosphorylated Smads 2 and 3 demonstrate positively stained nuclei of fibroblastic cells. Negative controls for Smad3 (B) and phospho-Smad 2/3 (D) are shown. Original magnifications: $\times 400$ (inset in A, and C); $\times 1000$ (A–D).

modest degree of intraluminal influx. These cells, presumably of monocytic and lymphocytic origin, were distributed throughout the luminal exudates. Grafts appeared to be largely denuded of respiratory epithelium. At 28 days (Figure 1B), the intraluminal exudates appeared to be increasingly organized with increasing extracellular matrix deposition. Lymphoplasmacytic infiltrates were noted along with spindle-shaped fibroblastic cells. In contrast, syngeneic tracheal grafts at 14 and 28 days were indistinguishable from normal tracheas (Figure 1C).

Immunohistochemical analysis showed that at 14 days, TGF- β 1 was detected within many, but not all, infiltrating mononuclear cells. TGF- β 1 was also detected extracellularly within the luminal space (Figure 2A). CTGF was also detected intracellularly in similar infiltrating cells at day 14, although in fewer cells within the airway lumen (Figure 2C). By 28 days after transplantation, intracellular TGF- β 1 (Figure 3A) and CTGF (Figure 3C) were detected in inflammatory cells and fibroblasts in addition to the fibrous tissue and tracheal wall. Syngeneic tracheal transplants appeared normal and thus showed no luminal staining for TGF- β 1 and CTGF; similar observations were made in normal tracheas (not shown).

Staining for the TGF- β 1 intracellular signal transducer, Smad3, was detected at 28 days in fibroblasts distributed throughout the fibrous tissue within the tracheal lumen (Figure 4A). Higher magnification revealed predominantly nuclear compartmentalization of Smad3 in many fibroblasts (Figure 4A, arrowheads), suggesting activation of the Smad pathway, whereas others showed no staining (Figure 4A, large arrows). In confirming Smad activation, tracheal grafts were immunostained for phosphorylated Smads 2 and 3 (Figure 4C). As with Smad3, phospho-Smad2/3 was identified in most, but not all, nuclei of lesional fibroblastic cells.

Tracheal Transplantation in Smad3-Null Mice

To examine the role of Smad3 in the development of airway obliteration in the heterotopic tracheal transplant model, tracheas from HLA-mismatched mice were transplanted into each of four Smad3-null and wild-type littermate recipient mice. Smad3-null mice appeared healthy with minor skeletal abnormality and slightly reduced size compared to wild-type littermates.²² To diminish problems related to the potential variability in fibrosis in this

Figure 5. Histopathology (H&E) of 14 day tracheal allografts in Smad3-null and wild-type littermates. Allografts explanted at 14 days from the wild-type (A) and knockout (B) recipients demonstrate comparable cellular influx and epithelial attenuation. C: Tissue section images of the nuclei of infiltrating cells were subjected to digital analysis, revealing no significant differences between the two groups. Data are expressed as mean $\pm$ SD. Original magnifications, $\times 200$.

outbred background of Smad3 knockout, airway obliteration was allowed to progress for 42 days. Tracheas at 14 days showed greatly attenuated airway epithelium in both groups, demonstrating varying degrees of denudation and/or small foci of cuboidal or squamous metaplasia [Figure 5, A (WT) and B (KO)]. Using image analysis software, the degree of cellular influx into tracheas was quantified by determining the average density of the nuclei of infiltrating cells from three random high-power fields per section. No significant difference in cellular infiltration was found in the allografts from Smad3-null and wild-type mice (Figure 5C).

Figure 6. Histopathology (H&E) and morphometry of luminal fibrosis of 28 day tracheal allografts in Smad3-null and wild-type littermates. A: There is dense organization of the matrix within the airway lumen in the wild-type animal at 42 days. B: In contrast, the trachea shown from a Smad3-deficient recipient remained free of luminal fibrosis. Tissue sections from Smad3 wild-type (C) and null (D) mice were trichrome stained to identify collagen fibers as blue. All shades of blue were averaged and displayed in black; noncollagen area was subtracted (insets). The transformed images were imported into an imaging program, and the area in black was calculated (E). Collagen staining from wild-type recipients was greater in comparison to knockout recipients ($P < 0.005$). Data are expressed as mean $\pm$ SD. Original magnifications: $\times 200$ (A, B); $\times 1000$ (C, D).

By 42 days, tracheas from Smad3-deficient mice demonstrated variable but overall dramatic reductions in airway obliteration and poor organization of intraluminal tissue (Figure 6, A and B). This attenuation of luminal fibrosis from wild-type and Smad3-null mice was compared quantitatively using morphometric image analysis. At least five photomicrographs of trichrome-stained tracheas encompassing the airway lumen were taken, with average staining density per high-power field calculated for each section. As demonstrated in Figure 6, C to E, the amount of fibrosis in tracheas from Smad3-null mice, indicated by blue-stained collagen, was reduced by greater than 50% compared to wild-type littermates ($P < 0.005$).

Immunohistochemistry was used to determine how Smad3 deficiency affects expression of TGF- $\beta 1$ and CTGF in heterotopic tracheal transplantation. Localization of these profibrotic growth factors in recipients lacking Smad3 was noted to be analogous to that seen in wild-type recipients, with staining detectable in the airway extracellular matrix and subepithelium, and additionally in the cellular infiltrate (Figure 7, A and B). To ascertain if other non-Smad3, TGF- $\beta 1$ signal intermediates are also activated in heterotopic tracheal transplantation, phospho-Smad2 was examined in Smad3-null mice. Im-

Figure 7. Immunostaining for TGF- β 1, CTGF, and phospho-Smad2 of tracheal allografts in Smad3-deficient recipients. TGF- β 1 (**A**) and CTGF (**B**) were found predominantly in the extracellular matrix as well as in fibroblastic and inflammatory cells in the airway. **C**: Phospho-Smad2 was detectable in the nuclei of luminal fibroblasts. Original magnifications: $\times 400$ (**A**, **B**); $\times 1000$ (**C**).

munostaining for phospho-Smad2 (Figure 7C) was again notable, like Smad3 and phospho-Smad2/3 in wild-type recipients, within most nuclei of lesional fibroblastic cells.

Role of Smad3 in the in Vivo and in Vitro Expression of Fibronectin

Smad3 and the induction of the matrix glycoprotein, fibronectin, were studied in experimental OB and in cell culture. In contrast to the strong fibronectin immunoreac-

tivity observed within the intraluminal connective tissue of wild-type recipients, the intensity of fibronectin staining was markedly reduced throughout the fibrous stroma of tracheal allografts in Smad3-null mice (Figure 8, A and B). Because Smad3 plays a key role in mediating the stimulatory effects of TGF- β 1 on connective tissue gene expression by fibroblasts, we examined the TGF- β 1 response directly. Wild-type and Smad3-deficient embryonic fibroblasts were incubated in the presence or absence of TGF- β 1 for 72 hours, and supernatants were processed to evaluate fibronectin expression by immunoblot. As demonstrated in Figure 8D, wild-type fibroblasts expressed high levels of fibronectin protein at baseline, and this was further increased by incubation with TGF- β 1. In contrast, Smad3-null fibroblasts expressed significantly less fibronectin at baseline, and most importantly, produced less fibronectin in response to TGF- β 1 when compared to treated wild-type controls.

Discussion

This study describes the importance of TGF- β 1 and Smad3 in the development of experimental OB induced by heterotopic tracheal transplantation. Early after transplantation, TGF- β 1 was detected within mononuclear cells infiltrating the lumen. At later time points, TGF- β 1 was also found in fibroblasts and in the connective tissue within the lumen and the wall of the transplanted allografts. CTGF had a similar time and tissue distribution pattern. Smad3 and phosphorylated Smad3 were detected in intraluminal fibroblasts suggesting their responsiveness to TGF- β 1. During the fibroproliferative phase, the organized tissue within the tracheal allografts showed increased deposition of fibronectin and collagen protein. When they were transplanted into Smad3-null mice, tracheal allografts failed to organize the intraluminal exudates and revealed less fibronectin and collagen deposition, despite comparable degrees of earlier inflammation. Cultured Smad3-deficient embryonic fibroblasts expressed reduced amounts of fibronectin at baseline and in response to TGF- β 1 when compared to wild-type control cells.

The increased expression of TGF- β 1 in the tracheal allografts predated the development of luminal fibroproliferation. Together with the inhibition of experimental OB in Smad3-deficient animals, this suggests an important role for TGF- β 1 in the exaggerated tissue-remodeling response that is characteristic of transplant-associated OB. Early after tracheal transplantation (14 days), TGF- β 1 was detected in mononuclear cells among the intraluminal exudates implicating these cells as the cellular origin for this profibrotic factor. During the fibroproliferative phase, cells found to express TGF- β 1 were fibroblasts and lymphoplasmacytic cells. In addition, TGF- β 1 was detected within the extracellular matrix consistent with the idea that connective tissue matrices may serve as reservoirs for growth factors.²⁶ It should be highlighted that continued expression of TGF- β 1 was observed in allografts of Smad3 knockout recipients. Although recent studies have revealed that TGF- β 1 autoinduction can be suppressed in Smad3-deficient fibroblasts,²⁷ it is not

Figure 8. Fibronectin (FN) expression in tracheal allografts and in TGF- β 1-treated fibroblasts from Smad3-deficient and wild-type mice. **A:** Staining for FN was strongly positive in the extracellular matrix of transplanted airways of wild-type recipients. **B:** FN staining in Smad3-null mice demonstrated diminished intensity in the obliterated trachea in comparison to that of wild-type littermates. **C:** An isotype control for FN is shown. **D:** Supernatants of control and Smad3-deficient fibroblasts with and without treatment with TGF- β 1 were analyzed for FN. After 72 hours, induction of FN in wild-type fibroblasts was observed in response to TGF- β 1. Densitometric analysis revealed less FN expression in untreated Smad3-null fibroblasts when compared with unstimulated wild-type cells. Incubation of Smad3-null fibroblasts with TGF- β 1 resulted in a significant increase in fibronectin expression but not to the levels observed in treated wild-type fibroblasts. A representative gel stained with Coomassie Blue is shown as a control. Original magnifications: $\times 400$ (A–C).

completely eliminated, and TGF- β 1 is known to induce its own gene expression through the Ras/MAPK signaling pathway and AP-1 complex.²⁸ Thus, it is likely through this Smad3-independent autoinduction that TGF- β 1 can remain available within the OB lesion.

It is unclear if TGF- β 1 alone is sufficient to promote fibroproliferation after lung transplantation or if other profibrotic growth factors are also required. CTGF, named for its mitogenic and chemotactic effects on fibroblasts, is implicated in multiple fibrotic disorders including scleroderma, cirrhosis, idiopathic pulmonary fibrosis, and even chronic renal allograft rejection.²⁹ Depending on the tissue type, endothelial cells, vascular smooth muscle cells, epithelial cells, and chondrocytes can serve as a source of CTGF.³⁰ Among the chief regulators of CTGF in mesenchymal and epithelial cells is TGF- β 1. The TGF- β 1-induced stimulation of CTGF appears to be mediated via Smad3, and Smad3-binding elements have been identified in the CTGF gene promoter.¹⁷

In the allografts, CTGF was detected in fibroblasts as well as within the extracellular matrix. The appearance of CTGF during the fibroproliferative phase underscores the close correlation between CTGF and tissue remodeling as opposed to inflammation. Interestingly, the bulk of

CTGF did not appear to be derived from airway epithelium nor from infiltrating inflammatory cells, unlike TGF- β 1. Rather, the principal source of CTGF may be fibroblasts already recruited to the airway lumen. There, CTGF can stimulate fibroblast proliferation in an autocrine and paracrine manner as has been suggested with other models.³¹ As with TGF- β 1, CTGF was further noted in allografts from Smad3-deficient recipients. Together with others' findings in cultured Smad3-deficient fibroblasts showing no increased production of CTGF after treatment with TGF- β 1,¹⁴ we conclude that this up-regulation in experimental OB is independent not only of Smad3, but of TGF- β 1 itself. This is consistent with the concept put forth by Holmes and colleagues¹⁷ of a Smad3-independent basal control element (BCE-1) in the CTGF gene promoter, which is responsible for its constitutive secretion; yet, it remains to be determined what factors interact with this region to up-regulate CTGF promoter basal output.

The most important finding of this work is the demonstration that Smad3 is required to fully establish the histological features of experimental OB in the heterotopic tracheal transplantation model, in particular, those related to the fibroproliferative response. Of note, antecedent inflammation in experimental OB, as reflected by the

influx of mononuclear cells, continued uninterrupted in Smad3-deficient mice, suggesting that Smad3, or factors dependent on Smad3 for their biological effects (eg, TGF- β 1), are not required for this process. Our results are consistent with studies showing that the abrogation of bleomycin-induced pulmonary fibrosis in mice with impaired Smad3 signaling occurs regardless of macrophage chemotaxis³² and inflammatory cell composition.³³ The loss of Smad3, however, did not completely prevent extracellular matrix expression in our model. This is highlighted by the detection of fibronectin in allografts of knockout recipients and corroborated *in vitro* by data demonstrating decreased constitutive fibronectin expression in Smad3-null fibroblasts that is further induced with exposure to TGF- β 1, albeit to levels far less than TGF- β 1-treated wild-type fibroblasts. This is similar to results from other models of fibrosis, likely owing to extensive redundancy of the wound-healing machinery.³² Smad3-independent mechanisms are undoubtedly at play, and perhaps our finding of Smad2 activation accounts for incomplete suppression of TGF- β 1 signal transduction in the absence of Smad3. Additional non-Smad factors can also be implicated in the TGF- β -driven fibrogenic response. For one, other TGF- β 1 signaling intermediates such as mitogen-activated protein kinases have been found to induce collagen and fibronectin gene expression.^{34,35} Moreover, a wide array of profibrotic cytokines in addition to TGF- β 1, such as platelet-derived growth factor, has been linked to the pathogenesis of OB.³⁶

In summary, our observations establish that Smad3 is an essential, novel component of the fibroproliferative response in experimental OB wherein TGF- β 1 and CTGF are abundantly expressed. Disruption of Smad3 gene expression ameliorates obliterative airway disease, in part, through a reduction in collagen and fibronectin deposition. Pathways independent of Smad3 signal transduction appear to contribute to the development of airway obliteration. In all, these findings suggest that strategies targeting downstream mediators of TGF- β 1, in particular the Smad3 pathway, may provide useful therapeutic options in the treatment and/or prevention of OB.

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