Gremlin-mediated Decrease in Bone Morphogenetic Protein Signaling Promotes Pulmonary Fibrosis

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***Rationale*: Members of the transforming growth factor (TGF)-β superfamily, including TGF-βs and bone morphogenetic proteins (BMPs), are essential for the maintenance of tissue homeostasis and regeneration after injury. We have observed that the BMP antagonist, gremlin, is highly up-regulated in idiopathic pulmonary fibrosis (IPF).**

***Objectives*: To investigate the role of gremlin in the regulation of BMP signaling in pulmonary fibrosis.**

***Methods*: Progressive asbestos-induced fibrosis in the mouse was used as a model of human IPF. TGF-β and BMP expression and signaling activities were measured from murine and human fibrotic lungs. The mechanism of gremlin induction was analyzed in cultured lung epithelial cells. In addition, the possible therapeutic role of gremlin inhibition was tested by administration of BMP-7 to mice after asbestos exposure.**

***Measurements and Main Results*: Gremlin mRNA levels were up-regulated in the asbestos-exposed mouse lungs, which is in agreement with the human IPF biopsy data. Down-regulation of BMP signaling was demonstrated by reduced levels of Smad1/5/8 and enhanced Smad2 phosphorylation in asbestos-treated lungs. Accordingly, analyses of cultured human bronchial epithelial cells indicated that asbestos-induced gremlin expression could be prevented by inhibitors of the TGF-β receptor and also by inhibitors of the mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase pathways. BMP-7 treatment significantly reduced hydroxyproline contents in the asbestos-treated mice.**

***Conclusions*: The TGF-β and BMP signaling balance is important for lung regenerative events and is significantly perturbed in pulmonary fibrosis. Rescue of BMP signaling activity may represent a potential beneficial strategy for treating human pulmonary fibrosis.**

Keywords: gremlin; pulmonary fibrosis; bone morphogenetic protein; transforming growth factor-β

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Gremlin is up-regulated in the lungs of patients with idiopathic pulmonary fibrosis. The role of gremlin in the regulation of bone morphogenetic protein (BMP) signaling in association with lung fibrosis had not been studied previously.

What This Study Adds to the Field

Gremlin induces lung fibrosis in the mouse and human lung via decreased BMP-signaling and increased transforming growth factor-β signaling. This effect can be inhibited by BMP-7 administration to the mouse.

Aberrant expression of transforming growth factor (TGF)-β superfamily ligands is associated with the development of several chronic diseases, such as cancer, fibrosis, and autoimmune disease (1, 2). Sustained TGF-β activation is a key element in promoting the progression of idiopathic pulmonary fibrosis (IPF) (3). In the adult lung, TGF-β is believed to maintain the mesenchymal cell number and phenotype, as well as to regulate extracellular matrix synthesis and degradation (4). Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily of proteins. BMP-4 is an essential molecule in lung development (5), but BMPs also appear to play a role in the adult lung (6, 7). The biological responses to BMPs are negatively regulated by BMP antagonists that can directly associate with BMPs and inhibit receptor binding. Gremlin belongs to the DAN family of BMP antagonists, whereas noggin and chordin form their own subgroup of antagonists (for review, *see* Reference 8).

TGF-βs and BMPs signal through a heteromeric cell surface serine/threonine kinase complex consisting of type I and type II receptors (9). Ligand binding leads to receptor-mediated phosphorylation of Smad2/3 (TGF-βs) or Smad1/5/8 (BMPs) proteins, which are then transported to the nucleus and alter gene transcription. The receptor and ligand expression profiles determine the target cells for these growth factors. In addition, the biological responses are regulated at the level of ligand activation and growth factor inhibitor expression, as well as by a cross-talk between other signal transduction pathways.

IPF is the most common form of the idiopathic diffuse lung disorders. It has a poor prognosis, with a mean survival of only 3 years (10, 11). IPF lesions present a histological pattern, termed usual interstitial pneumonia (UIP). Asbestosis is a fibrotic interstitial lung disease that displays many similarities with IPF, including the occurrence of UIP histopathology (12). Although many interstitial pneumonias respond to corticosteroid therapy, antiinflammatory therapy has little or no effect on UIP lesions, and it has been recommended that efforts to combat IPF/UIP should be directed toward developing antifibrotic treatment modalities (13).

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We hypothesized that changes in the TGF-β/BMP balance might play an important role in the development of fibrosis. This hypothesis was tested by first comparing the BMP signaling pathway in lung fibrosis to normal lungs, and then by altering this balance in experimentally induced lung fibrosis using BMP-7 treatment. We report that, in the mouse model of asbestos-induced pulmonary fibrosis (14–16), BMP signaling was impaired due to up-regulation of the BMP antagonist, gremlin. In cultured human bronchial epithelial cells, the induction of gremlin could be blocked by exposure to a type I TGF-β receptor (TGF-βRI) inhibitor. Furthermore, BMP-7 treatment significantly reduced fibrosis *in vivo* in asbestos-exposed mice.

METHODS

Additional details on reagents and methods are provided in the online supplement.

Mouse Asbestos-induced Pulmonary Fibrosis

Progressive pulmonary fibrosis was induced in C57BL/6 mice with a single 0.1-mg dose of intratracheally instilled crocidolite asbestos (National Institute of Environmental Health Sciences, Research Triangle Park, NC) or using titanium dioxide (Sigma, St. Louis, MO) as an inert control, as previously described (16). The mice were killed on Day 3 or 14 (five in each group). BMP-7 was administered to asbestos-treated mice intraperitoneally at a dose of 300 μg/kg/injection from Day 7 to 14. The BMP-7–treated mice were killed at Day 14.

Hydroxyproline Assay

The analyses were performed as previously described (15). Briefly, the right lungs were dried for 48 hours and acid hydrolyzed in sealed, oxygen-free glass ampoules, containing 2 ml of 6 N HCl, at 110°C for 24 hours. Hydroxyproline was quantified using chloramine T.

Patient Material

The use of patient biopsies was approved by the ethics committee of the Helsinki University Central Hospital, Helsinki, Finland, and registered online at www.hus.fi/clinicaltrials. All patients involved had biopsy-proven IPF/UIP or asbestosis, and provided informed consent. Biopsies for immunohistochemistry and RNA isolation were obtained either during pulmonary transplantation from the explanted lung or from diagnostic biopsies taken by thoracoscopy. The patient with asbestos-induced pulmonary fibrosis underwent surgical lobectomy due to a malignant tumor. The control biopsies were obtained from healthy lung tissue from transplantation donors if only single-lung transplantation was performed, or from patients that had undergone lobectomy because of benign pulmonary tumors.

RNA Isolation and Quantitative Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA). The levels of gene expression were determined using TaqMan Assays-on-Demand gene expression products (Applied Biosystems, Foster City, CA) and GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems).

Immunohistochemistry

Immunohistochemical stainings were performed from paraffin-embedded tissue sections, as previously described (7), using Zymed ABC Histostain-Plus kit (Zymed, South San Francisco, CA) or Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Before staining, antigens were retrieved by heating the sections in citrate buffer.

Cell Culture

A549 lung adenocarcinoma cells (American Type Culture Collection, Manassas, VA) were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD), 100 IU/ml penicillin, and 50 μg/ml streptomycin. Normal human bronchial epithelial (NHBE) cells (Cambrex, East Rutherford, NJ) were cultured in BEGM medium containing retinoic acid (Cambrex), according to the manufacturer's instructions. The first three passages after subculture of the primary NHBE cells were used for experiments.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

Lung tissues were homogenized in RIPA buffer using Lysin Matrix D (Q-BIOgene, Irvine, CA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were performed as described (7).

Statistical Analysis

In the experimental animal studies, statistical calculations were performed using the GraphPad Prism statistical program (GraphPad Software, Inc., San Diego, CA). Comparisons were performed with one-way analysis of variance followed by Tukey's post test. Otherwise, statistical significance was determined using the Student's *t* test. *P* values of less than 0.05 were considered significant.

RESULTS

The BMP Antagonist, Gremlin, Is Induced in Asbestos-exposed Mouse Lungs

The elevated expression of gremlin in association with human IPF has previously been described (7). We used a mouse model of asbestos-induced pulmonary fibrosis, in which the histopathology and progressive fibrosis resembles human IPF/UIP, to functionally characterize the role of BMP antagonists in fibrosis. A single dose of intratracheally instilled crocidolite asbestos is known to cause a neutrophilic inflammatory response in the first week, with progressive fibrosis then ensuing (14–16). The mRNA expression levels of the BMP antagonists, gremlin, noggin, and chordin, were analyzed from asbestos-treated mouse lungs at 3 and 14 days using titanium dioxide (TiO_2) as inert particulate control. Gremlin mRNA levels were clearly increased in asbestos-exposed mice at 14 days (Figure 1A). This is consistent with the finding of elevated gremlin expression in human IPF (7). Noggin and chordin mRNA levels remained unaltered after asbestos exposure (Figure 1A). The increase in gremlin protein levels was confirmed by immunohistochemical staining of mouse lung tissue. Gremlin protein was almost undetectable in TiO_2-treated lungs, but clearly visible in the asbestos-treated lungs (Figure 1B). In asbestos-induced fibrosis, gremlin immunoreactivity was localized to the thickened interstitium, especially at the epithelium adjacent to the fibroblastic lesions (Figure 1B).

Down-Regulation of BMP Signaling and Target Gene Expression in the Fibrotic Mouse Lung

The mRNA expression levels of BMP-2, -4, and -7, as well as BMP target genes, inhibitor of differentiation (Id) 1 and Id2, were analyzed to characterize further alterations in BMP signaling in asbestos-exposed mice. As expected, BMP-4 mRNA was abundantly expressed in the mouse lung (Figure 2A) (17). Exposure to asbestos had no effects on the mRNA expression levels of any of the analyzed BMPs at 14 days. However, the expression of Id1 was significantly reduced (Figure 2B), suggesting that up-regulation of BMP antagonists can lead to down-regulation of BMP target gene expression. To analyze BMP signaling more directly, the phosphorylation of the BMP-specific regulatory Smads (Smad1/5/8), was analyzed by immunohistochemistry. In the lungs from TiO_2 particulate–treated control animals, the bronchiolar epithelial cells stained positive

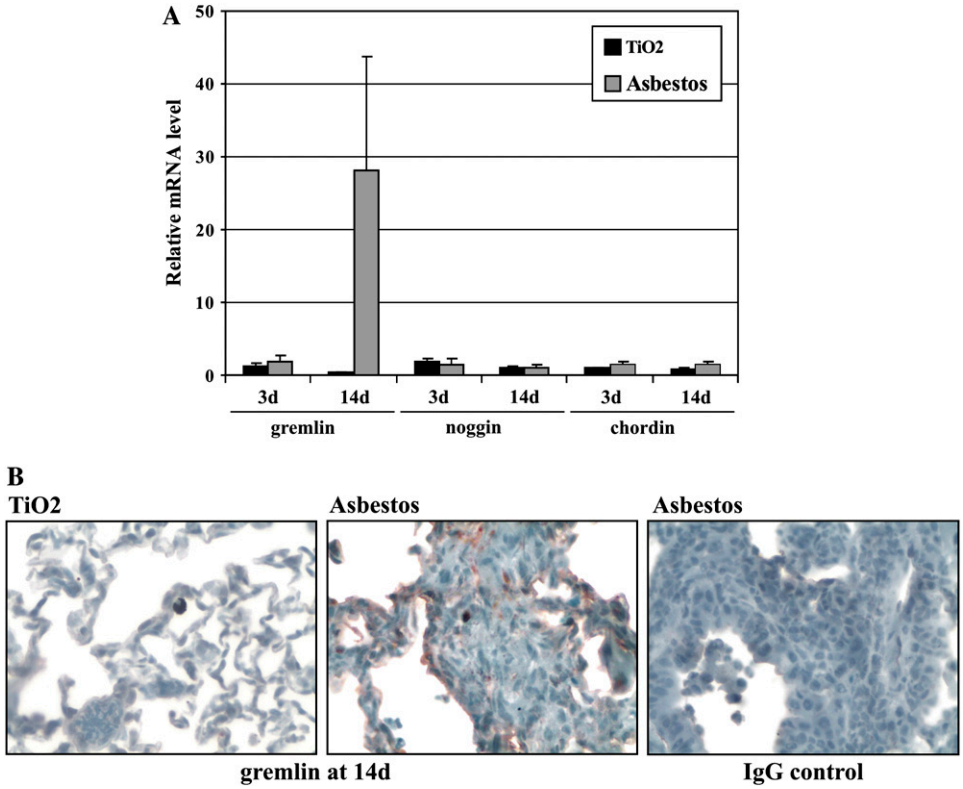


Figure 1. Induction of gremlin expression in asbestos-exposed mouse lungs. (*A*) Relative mRNA expression levels of gremlin, noggin and chordin in lung tissues exposed to particulate control (titanium dioxide [TiO_2]) or asbestos at 3 and 14 days as analyzed by quantitative real-time reverse transcription–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of a control gene (TATA-binding protein [TBP]), and are expressed relative to control-1 (set to 1). *Error bars* represent SEM of the samples (n = 5). (*B*) Paraffin sections from particulate control (TiO_2) and asbestos-treated lungs at 14 days were stained with a gremlin-specific antibody. The control section was treated with goat IgG isotype control. Positive staining is *reddish brown*. Original magnification = ×400.

for phosphorylated Smad1/5/8 (P-Smad1/5/8), which is indicative of BMP signaling activity (Figure 2C). In asbestos-exposed lungs, the P-Smad1/5/8 immunoreactivity was almost undetectable (i.e., evidence of reduced BMP signaling) (Figure 2C).

Up-Regulation of Gremlin in Biopsies of Human Asbestosis and Patients with IPF

Gremlin is up-regulated in the lung of patients with IPF (Figure 3A) (7). Analysis of the expression levels of BMP target genes in this patient material indicated that, although Id2 levels were not altered, the levels of Id1 mRNA were significantly reduced in IPF lungs (Figure 3B). This is in agreement with the finding of reduced Id1 levels in asbestos-exposed mouse lungs. Gremlin localization was then analyzed by immunohistochemistry on biopsies from patients with asbestosis and IPF (Figure 3C). In the normal human lung, traces of gremlin immunoreactivity were localized at alveolar epithelial lining and in macrophages. In contrast, intense gremlin immunoreactivity was detected in the interstitium of human IPF biopsies, as well as in a specimen from one patient with asbestos-induced pulmonary fibrosis.

Gremlin Expression Is Induced in Human Epithelial Cells *In Vitro* by Asbestos

Induction of epithelial-to-mesenchymal transition (EMT) in lung epithelial cells by TGF-β is augmented by gremlin overexpression (7). Because gremlin expression was mainly found in the epithelium of asbestos-exposed mouse lungs, the mRNA induction mechanisms of gremlin were analyzed in cultured human lung epithelial cells. Exposure of NHBE cells to increasing concentrations of asbestos, but not the particulate control (TiO_2), induced gremlin expression at 20 hours (Figure 4A). Asbestos at 20 μg/cm^2 concentration induced an approximately fourfold increase in gremlin mRNA levels. In addition, gremlin expression levels were increased in A549 alveolar epithelial adenocarcinoma cells after asbestos exposure (Figure 4A). These results indicate that the alterations detected *in vivo* could be confirmed in cultured epithelial cells. Subsequently, the functional consequence of this increased expression of the BMP antagonist was tested in NHBE cells transiently transfected with a BMP-responsive promoter construct ($[Bre]_2$-luciferase; *see* METHODS). BMP signaling activity was measured after asbestos treatment for 24 hours. There was a nearly 60% reduction of BMP signaling activity in asbestos-treated cells (Figure 4B).

Induction of Gremlin mRNA Expression Can Be Prevented with an Inhibitor of TGF-βRI

Because TGF-β can be activated by exposure to asbestos fibers (18), and it has been found to regulate gremlin expression (19, 20), we explored whether TGF-β activity was involved in the regulation of gremlin expression in NHBE cells. TGF-β signaling activity was analyzed by transiently transfecting cells with the TGF-β responsive promoter, $(CAGA)_{12}$-luciferase, followed by exposure to asbestos, and then assay for luciferase activity. Asbestos was found to increase TGF-β signaling activity by more than 10-fold at 24 hours (Figure 5A). Next, we evaluated whether the inhibition of TGF-β signaling could prevent asbestos-induced gremlin mRNA induction by treating the NHBE cells with SB431542 (10 μM) together with asbestos. SB431542 is a chemical inhibitor of TGF-βRIs (ALK-4, -5, and -7), which does not interfere with type I BMP receptor signaling (21). Blockade of TGF-β signaling with SB431542 reduced the basal expression levels of gremlin, and completely prevented asbestos-induced gremlin mRNA induction (Figure 5B). In addition, treatment with exogenous TGF-β1 (500 pg/ml) induced the expression of gremlin. As expected, SB431542 prevented gremlin induction by TGF-β1. Thus, it seems likely that asbestos-induced TGF-β signaling plays an important role in the induction of gremlin expression.

TGF-β Activity Is Induced by Asbestos in Mouse Lungs

Induction of TGF-β expression and activation in patients with IPF/UIP and in mouse models of pulmonary fibrosis are well

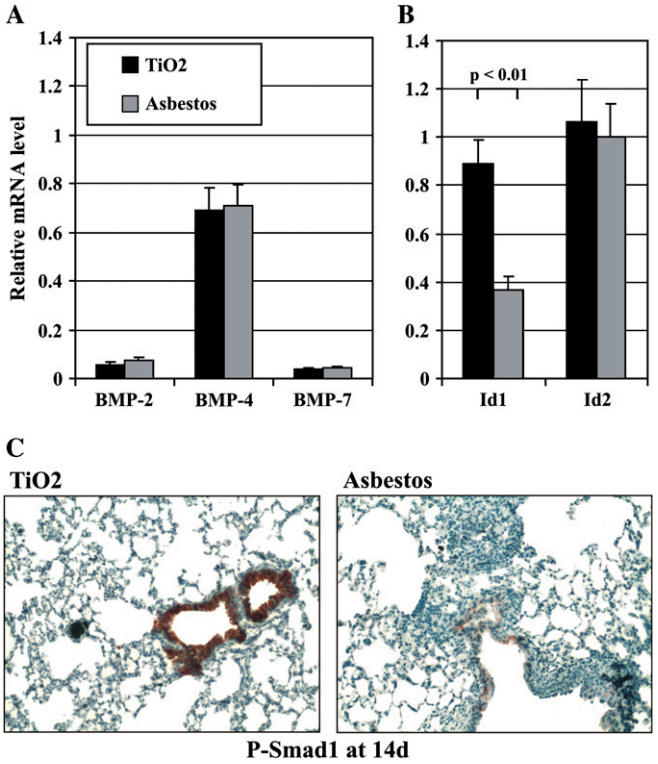


Figure 2. Down-regulation of bone morphogenetic protein (BMP) signaling in asbestos-exposed mouse lungs. Relative mRNA expression levels of BMPs (*A*) and the BMP target inhibitor of differentiation (Id) genes (*B*) in lung tissues exposed to particulate control (TiO_2) or asbestos at 14 days, as analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP (TATA-binding protein) and are expressed relative to BMP-2 control-1 (*A*) or Id1 control-1 (*B*). *Error bars* represent SEM of the samples (n = 5). (*C*) Paraffin sections from particulate control (TiO_2) and asbestos-treated lungs at 14 days were stained for phosphorylated Smad (P-Smad) 1/5/8. Positive staining is *reddish brown*. Original magnification = ×100.

documented (22, 23). We observe here that asbestos treatment did not significantly alter the mRNA expression profiles of TGF-βs in the mouse lung at 14 days (Figure 6A). However, the expression of the TGF-β target gene, plasminogen activator inhibitor (PAI)-1, was notably increased (Figure 6B). Accordingly, the expression of connective tissue growth factor (CTGF), another TGF-β target gene, was also slightly but not statistically significantly elevated. This suggested that TGF-β activation might be increased in asbestos treated mouse lungs. TGF-β signaling activity was analyzed next by staining the lung tissues with an antibody against P-Smad2. In TiO_2-treated lungs, P-Smad2 immunoreactivity was detected only in occasional cells in the alveolar/bronchiolar wall. As expected, asbestos-exposure increased P-Smad2 immunoreactivity at 14 days (Figure 6C) and the P-Smad2 immunoreactivity co-localized with the asbestos fibers and fibrotic lesions. Immunoblotting analyses of lung tissue lysates indicated that P-Smad2 levels were increased appoximately 1.5-fold in asbestos-exposed mice (Figure 6D).

Inhibition of Mitogen-activated Protein Kinase Kinase Activity Prevents Induction of Gremlin

TGF-β mediates its effects through the activation of Smad proteins, but other signal transduction pathways may be activated in response to TGF-β (for review, *see* Reference 24). Mitogen-activated protein kinase (MAPK) cascades, specifically the MAPK kinase (MEK)/extracellular signal-regulated protein kinase (ERK) pathway, is known to cooperate with Smad2/3 in the induction of p21, collagen, and CTGF by TGF-β (25–27). In addition, c-Jun N-terminal kinase activity can regulate TGF-β target gene expression (28), and futhermore, asbestos fibers can activate MAPK signaling cascades in the lung (29); therefore, we analyzed the possible role of the activity of the MEK/ERK pathway in the asbestos-induced mRNA expression of gremlin. The NHBE cells were treated with specific inhibitors of MEK enzyme, PD98059 (30) or U0126 (31), in conjunction with asbestos, and the mRNA expression levels of gremlin were analyzed at 20 hours. Inhibition of MEK activity blocked the induction of gremlin expression evoked by asbestos, highlighting the crucial role of the MEK/ERK pathway in the

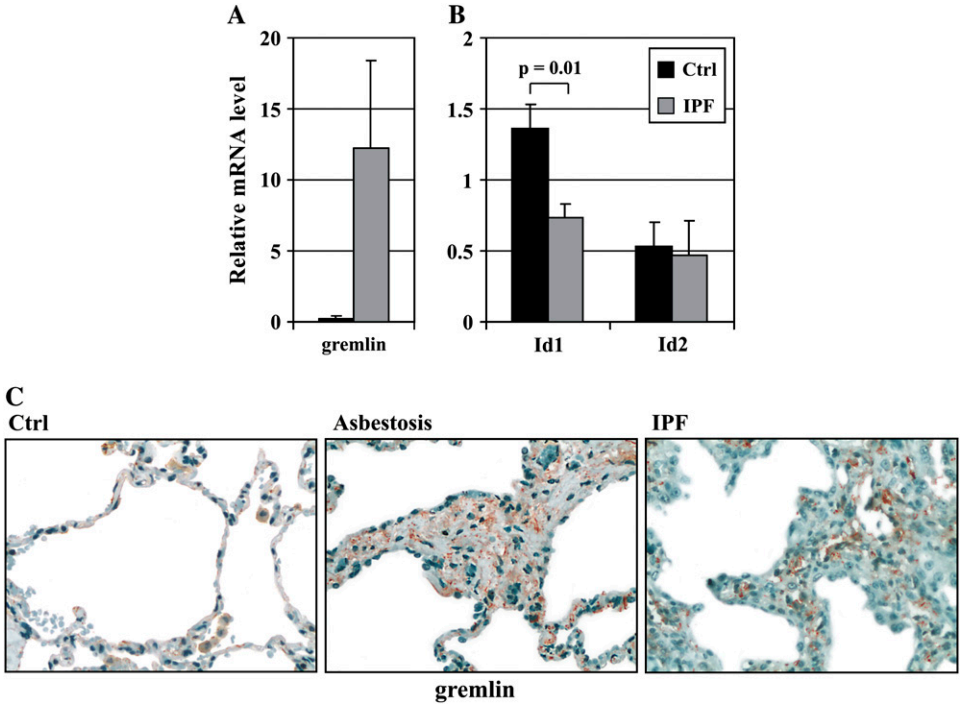
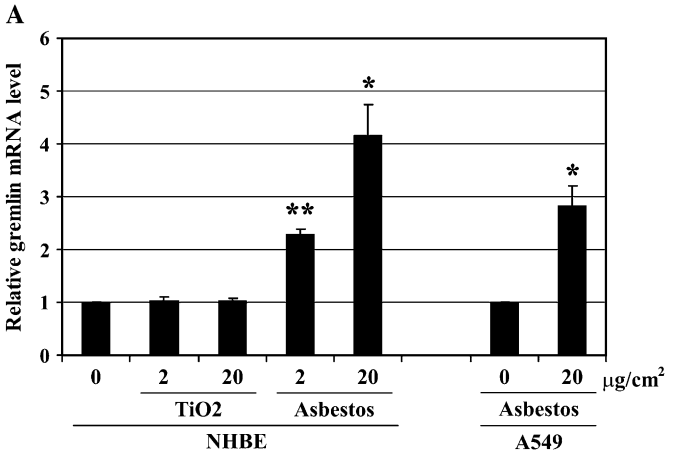


Figure 3. Expression of gremlin and Id1 in human asbestosis and idiopathic pulmonary fibrosis (IPF) lung biopsies. Total cellular RNA was isolated from control lung tissue (Ctrl) or IPF lung tissue (IPF) from six subjects, and the expressions of gremlin (*A*) or Id1 and Id2 (*B*) were analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP (TATA-binding protein) and are expressed relative to control-1 (*A*) or Id1 control-1 (*B*). *Error bars* represent SEM of the samples. (*C*) Paraffin sections from normal adult lung and lungs of patients with IPF or asbestosis were stained for gremlin. Positive staining is *reddish brown*. Original magnification = ×200.



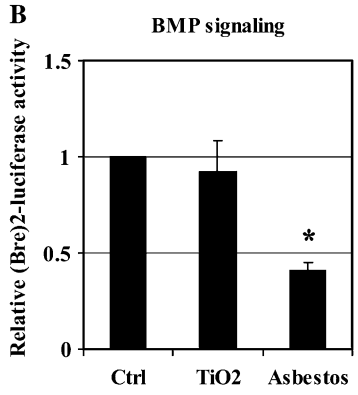


Figure 4. Induction of gremlin by asbestos in cultured epithelial cells *in vitro*. (*A*) Normal human bronchial epithelial (NHBE) or A549 cells were treated with the indicated concentrations of TiO_2 or asbestos for 20 hours and gremlin expression levels were analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP (TATA-binding protein) and expressed relative to untreated control. (*B*) NHBE cells were transiently transfected with a bone morphogenetic protein (BMP) responsive $[Bre]_2$-luciferase promoter construct and exposed to asbestos for 24 hours. Luciferase activities were measured and normalized by comparing them with the activities of cotransfected *Renilla* luciferase activities. The results are expressed as relative luciferase activities. *Error bars* represent SEM of the samples (n = 3). *$P \leq 0.05$; **$P \leq 0.01$.

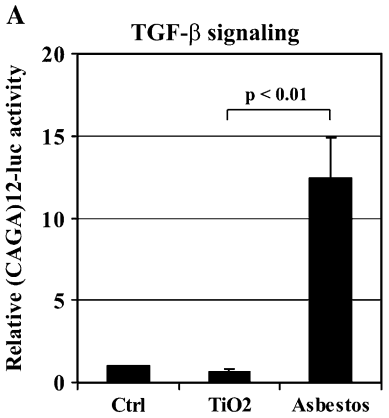
regulation of gremlin (Figure 7A). Gremlin induction by TGF-β1 was not affected by MEK inhibitors (Figure 7B). These results suggest that the MEK pathway is involved in the early response to asbestos.

BMP-7 Treatment Reduces Asbestos-induced Fibrosis in Mice

BMP-7 can reverse TGF-β–induced EMT as well as fibrosis in mouse models of kidney and liver injury (32–34). BMP-7 therapy was used to determine if a restoration of BMP signaling in asbestos-exposed animals would be able to inhibit fibrosis. Because gremlin overexpression was observed at Day 14 (not at Day 3), administration of BMP-7 was started when the initial inflammatory reaction was declining and a rapid, ongoing fibrotic response was in progress. Fibrosis was thus allowed to develop for 7 days, after which daily intraperitoneal injections of BMP-7 (300 μg/kg/injection) or vehicle were started. Mice were killed at Day 14 followed by measurement of total hydroxyproline. The asbestos-induced increase in the hydroxyproline content, which is an indicator of collagen deposition in the lungs, was reduced by approximately 50% in the BMP-7–treated animals (Figure 8A). These results suggest that BMP-7 can reduce asbestos-induced fibrotic alterations in the lung. The role of BMP-7 treatment in the inflammatory response to asbestos was analyzed by bronchoalveolar lavage (BAL) fluid cell counts. As expected, asbestos induced an increase in total BAL fluid cell counts, especially in the number of neutrophils (Figure 8B). In BMP-7–treated mouse lungs, there was a tendency toward diminished cellular response in the BAL fluid; however, this was not statistically significant (Figure 8B).

DISCUSSION

IPF/UIP is a progressive, fatal disorder that presents a major challenge for clinicians, as there is currently no effective treatment for this disease. The pathogenesis of IPF/UIP is not well understood, but one hallmark of the clinical course is its unresponsiveness to antiinflammatory therapy. In our recent study, we detected significant up-regulation of the BMP antagonist, gremlin, in patients with IPF/UIP (7), and speculated that this might contribute to fibrosis by preventing antifibrotic BMP signaling. Many mouse models of pulmonary fibrosis have been developed for mechanistic and therapeutic studies, with bleomycin-induced fibrosis being the most commonly used model. We found no evidence in microarray data that would point to gremlin up-regulation or impaired BMP-signaling in the mouse bleomycin model of pulmonary fibrosis (data accessible at http://www.ncbi.nlm.nih.gov/geo/; National Center for Biotechnology Information, Gene Expression Omnibus database [accession



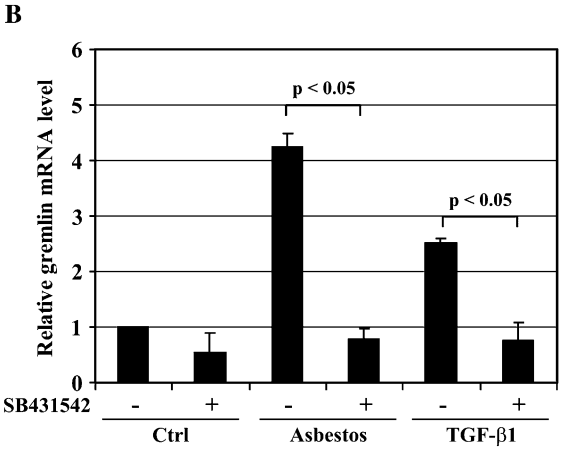


Figure 5. Transforming growth factor (TGF)-β activation is involved in gremlin mRNA induction in normal human bronchial epithelial (NHBE) cells. (*A*) NHBE cells were transiently transfected with a TGF-β–responsive $(CAGA)_{12}$-luciferase promoter construct and exposed to asbestos for 24 hours (compare with Figure 4B). The results are expressed as relative luciferase activities. (*B*) NHBE cells were treated with asbestos (20 μg/cm²) or TGF-β1 (500 pg/ml) in the presence or absence of the type I TGF-β receptor inhibitor, SB431542 (10 μM), for 20 hours. Gremlin expression levels were analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP (TATA-binding protein) and are expressed relative to untreated control. *Error bars* represent SEM of the samples (n = 3).

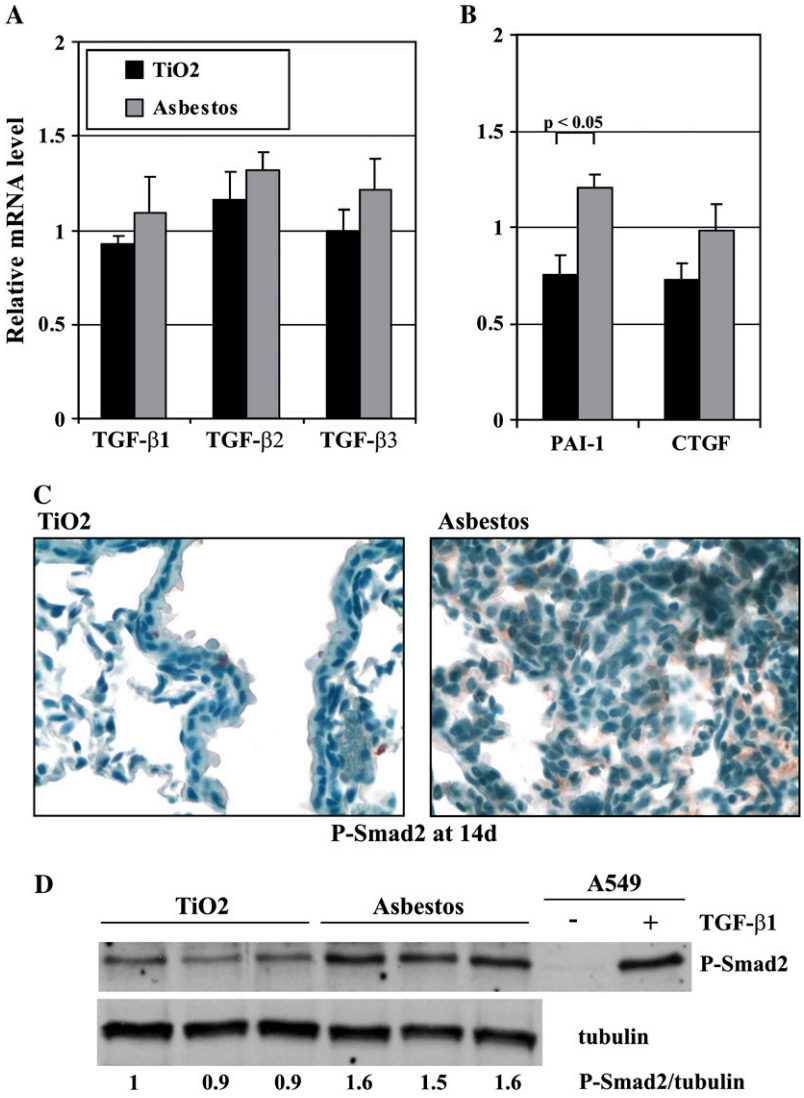


Figure 6. Transforming growth factor (TGF)-β activation and target gene expression in asbestos-exposed mouse lungs. Relative mRNA expression levels of TGF-βs (*A*) and TGF-β target genes, plasminogen activator inhibitor (PAI)-1 and connective tissue growth factor (CTGF) (*B*), in particulate control (TiO_2) and asbestos-exposed lungs at 14 days analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP (TATA-binding protein) and expressed relative to control-1. *Error bars* represent SEM of the samples (n = 5). (*C*) Paraffin sections from particulate control (TiO_2) and asbestos-treated lungs at 14 days were stained for P-Smad2. Positive staining is *reddish brown*. Original magnification = ×400. (*D*) Equal amounts of lung tissue lysates were analyzed for P-Smad2 and tubulin (control) protein levels by immunoblotting. Lysate of A549 cells treated with TGF-β1 (0.5 ng/ml) for 45 minutes was used as a positive control. Relative P-Smad2 protein levels are indicated.

number GSE485]). The crocidolite asbestos–induced model of fibrosis is progressive, and it can be considered as having similar temporal characteristics to human IPF/UIP lesions. We observed up-regulation of gremlin in asbestos-induced fibrosis in mice, suggesting that the mouse asbestos-induced fibrosis exhibits a similar gremlin response as human IPF/UIP. Notably, we found evidence that impaired BMP signaling was involved in promoting fibrosis, suggesting that gremlin overexpression may directly contribute to the pathogenesis of pulmonary fibrosis.

Immunohistochemical analyses of mouse lungs indicated that gremlin was mainly localized to the epithelial cells adjacent to fibroblast proliferative areas at 14 days after asbestos

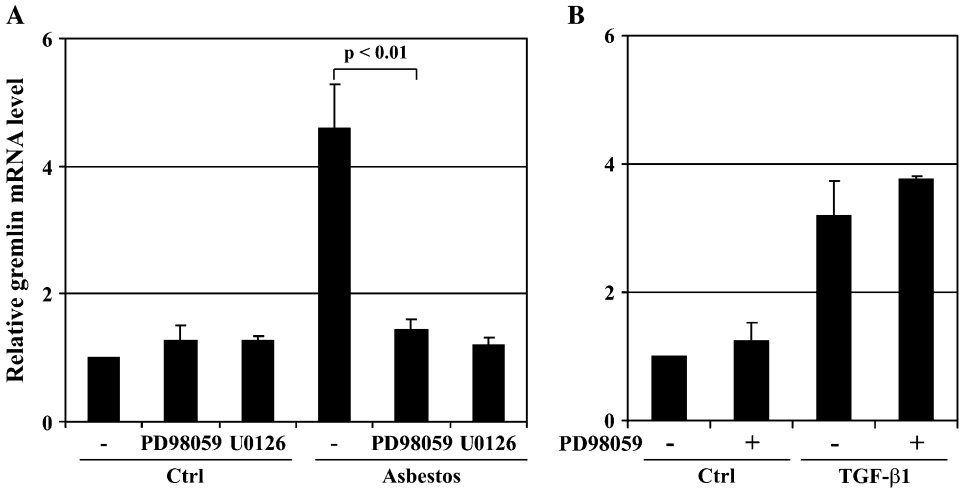


Figure 7. Mitogen-activated protein kinase kinase (MEK) inhibitors block gremlin mRNA induction by asbestos *in vitro*. Normal human bronchial epithelial (NHBE) cells were treated with inhibitors of MEK (20 μM PD98059; 10 μM U0126) in the presence or absence of asbestos (*A*) or transforming growth factor (TGF)-β1 (*B*) for 20 hours. Gremlin expression levels were analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction. The mRNA expression levels were normalized to the expression levels of TBP and are expressed relative to untreated control. *Error bars* represent SEM of the samples (n = 3).

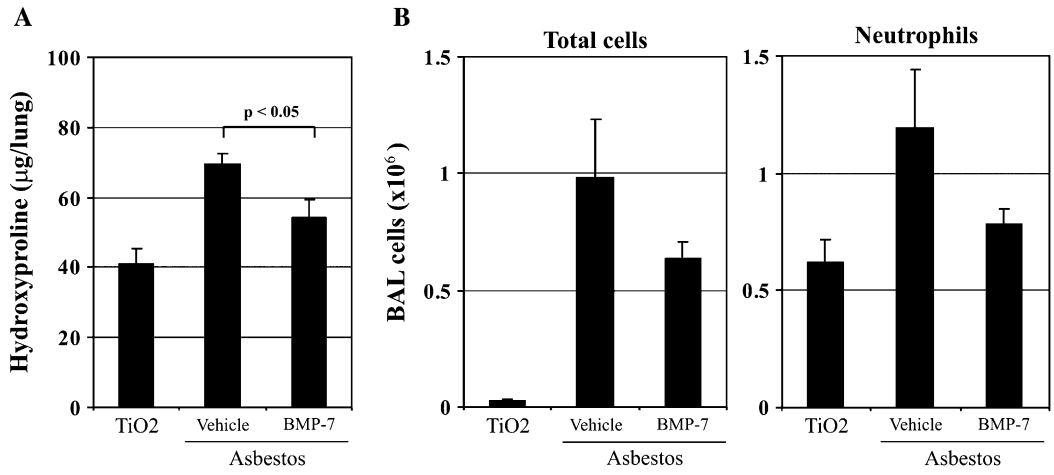


Figure 8. Bone morphogenetic protein (BMP)-7 treatment reduces fibrosis in asbestos-exposed mouse lungs. (*A*) Mice were exposed to TiO_2 or asbestos at Day 0. The asbestos-treated animals then received daily injections of BMP-7 or vehicle (phosphate-buffered saline) from Days 7–14. All mice were killed at Day 14 and the lung hydroxyproline contents were analyzed. (*B*) Bronchoalveolar lavage (BAL) fluid cell counts. Statistical analyses were performed by one-way analysis of variance and Tukey's post test (n = 5).

exposure. In contrast, gremlin was expressed mostly in the interstitium in human IPF lungs. Our previous results have shown very high gremlin levels in human lungs at advanced stages of the disease (patients that had undergone lung transplantation), suggesting that gremlin is a marker of advanced disease, possibly contributing to disease progression (7). In a biopsy from a patient with advanced asbestos-induced fibrosis, the parenchymal fibroblasts exhibited gremlin positivity similar to that in the IPF lungs. The observed mouse lung histopathology suggests that enhanced gremlin expression may also contribute to early fibrogenesis. Characterization of early changes in the lung fibrogenesis in man is, however, challenging.

Gremlin can inhibit the actions of BMP-2 and -4 and, to some extent, also BMP-7. The levels of chordin and noggin, which can inhibit the very same BMPs, did not change, providing evidence for specificity for the gremlin induction after exposure to asbestos. The levels of noggin and chordin were similarly unchanged in human IPF (unpublished observations). The levels of P-Smad1/5/8, an indicator of BMP signaling, exhibited a dramatic decrease in asbestos-exposed mouse lungs. The expression of the BMP target gene, Id1, was down-regulated in the fibrotic mouse lungs, which is consistent with the known biological actions of gremlin. Accordingly, we detected down-regulation of Id1 expression in biopsies from patients with IPF, further emphasizing the similarities between human IPF and the model of asbestos-induced mouse lung fibrosis.

The role of TGF-β in fibrotic diseases is well known, and it is also an important regulator of fibroblast accumulation and matrix deposition in asbestos-induced pulmonary fibrosis. Recent studies have indicated that EMT is an ongoing process in the fibrotic lung *in vivo* and a potential mechanism leading toward the accumulation of fibroblasts (35). TGF-β–induced EMT can be reversed by BMP-7, and the signaling balance between BMPs and TGF-β seems to be crucial to evoke these phenotypic changes. We have observed that overexpression of gremlin can sensitize cultured epithelial cells to TGF-β–induced EMT (7). Notably, we found that TGF-β is involved in the regulation of BMP antagonist expression, which will further promote a fibrosis phenotype in response to TGF-β. In cultured primary lung bronchial epithelial cells, we observed that blockade of TGF-β signaling inhibited gremlin mRNA induction by asbestos. TGF-β signaling activity was markedly increased in asbestos-treated cells *in vitro*, which probably resulted in increased expression of gremlin, as well as decreased BMP-signaling. Previous studies have indicated that the release of active TGF-β by alveolar epithelial cells *in vivo* can induce fibrosis (36). The current studies provided evidence of increased TGF-β signaling, as measured by Smad2 phosphorylation, in epithelial cells of asbestos-exposed mouse lungs. The similar localization pattern of TGF-β activity and gremlin protein in mouse lungs indicates that TGF-β may play a role in regulating gremlin expression and BMP-signaling *in vivo* as well. Interestingly, similar alterations in TGF-β/BMP signaling have recently been found in a hyperoxia mouse model of bronchopulmonary dysplasia (37), a condition in which fibrosis is also a hallmark of the pathology.

The MEK/ERK signaling pathway is involved in mediating some of the profibrotic activities of TGF-β, including induction of CTGF and collagen expression (27, 38). We observed that blockade of the MEK/ERK cascade by specific MEK inhibitors could prevent asbestos-induced up-regulation of gremlin mRNA in cultured epithelial cells. Asbestos exposure is known to evoke induction of ERK1/2 phosphorylation through the epidermal growth factor receptor (39, 40), and thus to contribute to the expression of gremlin, as well as other TGF-β–regulated genes. In our cell culture models, asbestos exposure also induced ERK1/2 phosphorylation (data not shown). Gremlin has BMP-independent functions and, interestingly, it was recently suggested that cell surface binding of gremlin can induce ERK activation in endothelial cells (41). Recent experimental data suggest that part of the profibrotic effects of TGF-β in mesenchymal cells are Smad independent and mediated by the c-Abl tyrosine kinase (42). We find here that the involvement of the MEK/ERK pathway in BMP antagonist expression represents another mechanism that should be considered, when the inhibition of TGF-β–triggered profibrotic signals are evaluated for the treatment of IPF. In support of this hypothesis, Liu and colleagues (43) have proposed that cAMP-induced down-regulation of ERK1/2 phosphorylation can reduce the profibrogenic effects of TGF-β in cardiac fibroblasts.

The *in vivo* role of reduced BMP signaling in the development of fibrosis was assessed by treatment with BMP-7. We observed that BMP-7 treatment inhibited asbestos-induced fibrotic changes, when the treatment was started 7 days after asbestos exposure. Hydroxyproline levels, which reflect collagen deposition in the lung, were reduced by about 50%. A tendency toward a diminished neutrophilic cellular response was also observed in the BMP-7–treated mouse lungs, implying that inflammatory responses might also be targets of the BMP therapy. These results are in full agreement with the role of BMP-7 in reversing EMT and fibrosis in the models of kidney and liver injury (32–34).

A common new mechanism related to fibrotic diseases (i.e., down-regulation of BMP signaling) is emerging from our studies, as well as from work by other groups. Up-regulation of gremlin

expression has been reported in fibrotic diseases of the lung, kidney, and liver (7, 44, 45). Endogenous BMP-7 appears to be involved in the regeneration of normal tissue after kidney injury (32, 33), and perhaps BMPs have a similar function in the lung. If BMP activity is blocked by overproduction of BMP antagonists, the development of fibrosis is enhanced. In experimental kidney injury models, the lack of BMP inhibitor, uterine sensitization–associated gene-1, or administration of recombinant BMP-7 can rescue the normal architecture of the kidneys (32, 33, 46). Furthermore, mice lacking the BMP signaling enhancer kielin/chordin-like protein (KCP) are hypersensitive to developing renal interstitial fibrosis (47). The close reciprocal regulation between TGF-β and BMP signaling pathways is further strengthened by the observation that, in addition to enhancing BMP signaling, KCP can suppress TGF-β signaling (48). CTGF, which also has a chordin-like domain, has been reported to bind directly to BMP-4 and TGF-β and to regulate their activities in a similar fashion as KCP (49). The current results indicate that the balance between TGF-β and BMP signaling activities is an important regulator for the development of fibrotic diseases. Novel therapeutic treatment strategies may be aimed at inhibiting TGF-β and/or enhancing BMP signaling activities.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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