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# Bone Morphogenetic Protein-4 Inhibitor Gremlin Is Overexpressed in Idiopathic Pulmonary Fibrosis

Katri Koli,* Marjukka Myllärniemi,† Kirsi Vuorinen,† Kaisa Salmenkivi,* Merja J. Ryynänen,* Vuokko L. Kinnula,† and Jorma Keski-Oja*

*From the Departments of Virology and Pathology,* Haartman Institute, and the Department of Medicine,† Division of Pulmonary Medicine, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland*

**Idiopathic pulmonary fibrosis (IPF), ie, usual interstitial pneumonia in histopathology, is a disease characterized by tissue destruction and active areas of fibroproliferation in the lung. Gremlin (Drm), a member of the cysteine knot family of bone morphogenetic protein (BMP) inhibitors, functions to antagonize BMP-4-mediated signals during lung development. We describe here consistent overexpression of gremlin in the lung interstitium of IPF patients. Quantitative real-time reverse transcriptase-polymerase chain reaction analyses revealed considerably higher levels of gremlin mRNA in lung biopsies from IPF patients, the highest level being 35-fold higher compared to controls. Lung fibroblasts isolated from IPF patients also expressed elevated levels of gremlin, which was associated with impaired responsiveness to endogenous and exogenous BMP-4. Transforming growth factor-β-induced epithelial-to-mesenchymal transition of A549 lung epithelial cells in culture was also associated with induction of gremlin mRNA expression. In addition, A549 cells transfected to overexpress gremlin were more susceptible to transforming growth factor-β-induced epithelial-to-mesenchymal transition. Gremlin-mediated inhibition of BMP-4 signaling pathways is likely to enhance the fibrotic response and reduce epithelial regeneration in the lung. The overexpression of this developmental gene in IPF may be a key event in the persistence of myofibroblasts in the lung interstitium and provides a potential target for therapeutic intervention.** ***(Am J Pathol 2006, 169:61–71; DOI: 10.2353/ajpath.2006.051263)***

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal disease characterized by fibroproliferation and destruction of the lung parenchyme.[1,2] IPF represents the clinical manifestation of usual interstitial pneumonia histopathology in the lung.[3] The etiology of the disease is primarily unknown, and the current treatment options are by far limited to corticosteroids and cytotoxic drugs without significant effect on patient survival. The development of novel treatment strategies targeting the inhibition of fibrosis, epithelial apoptosis, fibroblast proliferation, or the induction of alveolar repair is actively ongoing.[4,5]

Characterization of the molecular mechanisms in the development of IPF is of crucial importance for the development of new diagnostic and therapeutic strategies. IPF is characterized by patchy fibroblast expansion, tissue remodeling, and excessive accumulation of the extracellular matrix (ECM). The areas of immature fibrosis are characterized by actively proliferating myofibroblasts and fibroblasts (fibroblastic foci). It has been suggested that IPF could result from epithelial injury, after which the normal architecture of the lung is not properly restored.[2] The overexpression of profibrotic activities leads to myofibroblast accumulation, with little involvement of inflammation. Injured epithelial and mesenchymal cells secrete many profibrotic molecules including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF).[6] TGF-β is thought to play a central role in the progression of IPF because it induces the recruitment and differentiation of myofibroblasts as well as ECM accumulation.[7,8] TGF-β can cause persistent induction of fibronectin and collagen expression by mRNA stabilization.[9] In addition, TGF-β regulates growth factor pathways and proteolytic systems.[10] Recent observations suggest that the inhibition of the profibrotic activities of TGF-β in parallel with the inhibition of PDGF signaling significantly decreases fibrosis in mouse models.[4]

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Address reprint requests to Katri Koli, University of Helsinki, Biomedicum/A506, P.O. Box 63, Haartmaninkatu 8, 00014 Helsinki, Finland. E-mail: katri.koli@helsinki.fi.

TGF-βs are secreted as inactive, latent complexes that are targeted to the ECM for storage and/or for activation.[11] Physiologically relevant activation mechanisms include integrin- and proteolysis-mediated mechanisms as well as activation by the matricellular protein thrombospondin-1. Regulation of the activation process plays an important role in the biological functions of the three mammalian TGF-β isoforms. Bone morphogenetic protein (BMP)-4, a member of the TGF-β superfamily, is one of the key morphogens during embryonic lung development.[12] The balance between TGF-β and BMP signaling activities in the lung are of crucial importance during developmental and regenerative processes. BMP-4 inhibits the proliferation of human pulmonary fibroblasts and during embryogenesis induces the proliferation and dedifferentiation of pulmonary epithelial cells.[13] Gremlin/Drm is a member of the CAN family of binding proteins that antagonize the functions of BMPs.[14] Gremlin can associate with BMP-2, -4, and -7 and inhibit their binding to cell surface receptors.[14] Gremlin plays an important role in regulating proximal-distal patterning of the lung by inhibiting BMP-4 signaling.[15] In addition, BMP-independent functions for gremlin have been described.[16]

The role of BMP-4 signaling in IPF has not been clarified so far. Using established cell lines isolated from patients with IPF, we find here consistent overexpression of gremlin mRNA. This resulted in impaired responsiveness of the IPF fibroblasts to endogenous and exogenous BMP-4. Clinical relevance of these results was supported by data from freshly isolated IPF fibroblasts derived from an IPF pulmonary transplant recipient. Immunohistochemical comparison of healthy and IPF patient lungs showed increased expression of gremlin protein in the mesenchymal cells of the IPF lung. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analyses further verified the enhanced expression of the gremlin gene in IPF patients. To explore the possible significance of gremlin induction for epithelial-to-mesenchymal transition (EMT) we analyzed gremlin levels of cultured lung epithelial cells after exposure to TGF-β1. Gremlin mRNA levels were found to be induced in association with EMT. Lung epithelial cells expressing recombinant gremlin were found to be more susceptible to TGF-β1-induced EMT. Our results suggest that overexpression of the BMP inhibitor gremlin plays a role in the pathogenesis of IPF and may function to enhance the fibrotic response by modulating BMP-4 signaling in the lung.

## Materials and Methods

### Antibodies and Growth Factors

α-SMA antibody (CBL171, mouse monoclonal) was from Chemicon (Temecula, CA), Smad1 antibody (sc-7965, mouse monoclonal) from Santa Cruz Biotechnology (Santa Cruz, CA), gremlin antibody (sc-28873, rabbit polyclonal) from Santa Cruz Biotechnology, and E-cadherin antibody (HECD-1, mouse monoclonal) from Zymed (San Francisco, CA). Rabbit polyclonal P-Smad1 antibody[17] was a kind gift from Dr. Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Recombinant BMP-4 was from R&D Systems (Minneapolis, MN), and TGF-β1 was a kind gift from Dr. P. Puolakkainen (Oncogen).

### Tissues and Cells

This study was approved by the Ethics Committee of the Helsinki University Hospital (permit numbers 23/E5/04, 281/E6/03, and 3296/32/300/05), Helsinki, Finland, and is registered at *www.hus.fi/clinicaltrials*. All patients involved gave informed consent. They had biopsy-proven usual interstitial pneumonia. Biopsies for histology, immunohistochemistry, and RNA isolation were obtained either during pulmonary transplantation from the explanted lung (five patients) or from diagnostic biopsies taken using thoracoscopy (two patients). The control biopsies were obtained from healthy lung tissue from transplantation donors if only one lung transplantation was performed, or from patients that underwent lobectomy because of benign pulmonary tumors.

Primary cells from a patient with IPF (female, 42 years old) were isolated from the explanted right lung during lung transplantation. The pulmonary tissue was dispersed mechanically and suspended in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. The cells were allowed to attach overnight, and the adherent cells were washed and grown to confluency. These IPF fibroblasts were coded as IPF-U.N. The immunophenotype of the IPF-U.N. cells was characterized after two passages using immunohistochemistry. The cells expressed the myofibroblast marker α-SMA and were negative for CD45 common leukocyte antigen. Only sporadic cells stained positive with muscle cell marker desmin and epithelial cell marker cytokeratin. Antibodies were from NeoMarkers, Inc. (Fremont, CA). Normal adult human lung fibroblasts (CCL-190) and fibroblasts isolated from the lungs of IPF patients (CCL-191 and CCL-134) were obtained from American Type Culture Collection (Rockville, MD). Lung fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Life Technologies, Gaithersburg, MD) and cells up to passage 10 were used for the current experiments. Locally established normal human dermal fibroblasts (4′-strain) and dermal fibroblasts from a patient with localized scleroderma (morphea) were provided by Dr. Olli Saksela (Department of Dermatology, Helsinki University Hospital, Helsinki, Finland). Human dermal fibroblasts from a systemic sclerosis patient (CRL-1108) were from the American Type Culture Collection. Dermal fibroblasts and A549 lung epithelial adenocarcinoma cells (American Type Culture Collection) were cultured in minimal essential medium supplemented with 10% fetal calf serum. All media contained 100 IU/ml of penicillin and 50 μg/ml of streptomycin.

### Transfection of Cells

Cells to be transiently transfected were plated in six-well plates. The next day the cells were co-transfected with a total of 2 μg of $(BRE)_2$-luciferase promoter construct[18] together with pRL-TK (*Renilla* luciferase control; Promega, Madison, WI) plasmid using FuGENE 6 transfec-

tion reagent (Roche, Basel, Switzerland). Subsequently, 48 hours after transfection, the cells were lysed and subjected to luciferase activity measurements by a dual luciferase Kit (Promega) and Digene DCR-1 luminometer (MGM Instruments, Hamden, CT).

For stable transfection, the A549 cells were cultured in P60 culture dishes overnight. The transfection was performed the next day using 3 $\mu$g of gremlin/pEF-IRES construct[19] and 9 $\mu$l of FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. After transfection the cells were washed and incubated in serum-containing growth medium overnight. The antibiotic selection was started a day after transfection with 1 $\mu$g/ml of puromycin (Sigma, St. Louis, MO). The puromycin concentration was gradually increased up to 10 $\mu$g/ml.

### *Immunofluorescence Microscopy*

Equal number of cells were grown on glass coverslips and treated as indicated in Results. Coverslips were then washed three times with phosphate-buffered saline (PBS), and the cells fixed in methanol at −20°C. After washing three times with PBS, the cells were incubated in Dulbecco's PBS containing 3% bovine serum albumin to prevent nonspecific binding of the antibodies. The cells were then incubated with the primary antibody in Dulbecco's PBS containing 0.5% bovine serum albumin for 1 hour. The bound antibodies were detected using Alexa Fluor-594 secondary antibodies (Molecular Probes). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and examined under the Axioplan 2 imaging microscope (Zeiss, Gottingen, Germany) using ×20 to ×40 objectives. Images were acquired with AxioCam-HRc camera (Zeiss) and AxioVision3.1 software (Zeiss).

### *TGF-β Activity Assay*

Conditioned medium was collected and cleared by centrifugation. Medium from the same number of cells was analyzed for TGF-$\beta$ activity directly (active TGF-$\beta$) or after heat treatment (total TGF-$\beta$). Heat-treatment activates latent forms of TGF-$\beta$.[20] Mink lung epithelial cells stably transfected with a fragment of PAI-1 promoter fused to luciferase gene (TMLC) were a kind gift from Dr. Daniel B. Rifkin (New York University School of Medicine, New York, NY). These cells produce luciferase activity in response to TGF-$\beta$. TGF-$\beta$ standards and medium samples were analyzed as described.[21] All assays were performed three to five times with comparable results. The results have been presented as relative values of TGF-$\beta$ activity. The specificity was confirmed by neutralizing pan-specific TGF-$\beta$ antibodies (AB-100-NA, R&D Systems).

### *RNA Isolation and Northern Blotting*

Total cellular RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and the RNA concentrations and purities were determined spectrophotometrically (BioPhotometer 6131; Eppendorf, Hamburg, Germany). Ten $\mu$g of total cellular RNA was fractionated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-N nylon membranes (Amersham, Uppsala, Sweden) by capillary transfer. Prehybridization and hybridization were performed at 68°C in ExpressHyb hybridization solution (Clontech, Mountain View, CA). cDNA probes for genes of interest and the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with [$^{32}$P]-dCTP (>3000 Ci/mmol, Amersham) using a Ready-To-Go DNA labeling kit (Amersham). High stringency washing was followed by detection by fluorography.

### *Real-Time RT-PCR*

Total RNA was extracted from snap-frozen lung biopsies by mechanical homogenization and the Phase Lock Gel Heavy kit according to the manufacturer's (Eppendorf) instructions. Reverse transcription was performed with random hexamer primers (Invitrogen, Carlsbad, CA) and Superscript II reverse transcriptase (Life Technologies) using 1.0 $\mu$g of total RNA according to the manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems, Foster City, CA) and GeneAmp 5700 sequence detector thermal cycler (Applied Biosystems). Control amplifications directly from RNA were performed to rule out DNA contamination. Levels of gene expression were determined using the Ct method, and the results have been expressed as mRNA expression levels normalized to the levels of a gene with a constant expression (actin).

### *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting*

Concentrated (10×) cell-conditioned medium (Microcon 10 concentrators; Millipore, Bedford, MA) or cell lysates were analyzed by 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Cambrex). Electrophoretically separated proteins were transferred to nitrocellulose membranes using a semidry blotting system (Bio-Rad, Hercules, CA). Immunodetection was performed as described previously.[22] Densitometric analyses were performed using Scion Image $\beta$ 4.03 program (Scion Corporation, Frederick, MD).

### *Immunohistochemistry*

Paraffin-embedded tissue sections from normal/healthy and IPF lung biopsies were deparaffinized in xylene and rehydrated in graded alcohol. Antigens were retrieved by heating the sections in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase. For immunostaining, either the Zymed ABC Histostain-Plus kit (Zymed) or the Vectastain Elite ABC kit (Vector Laboratories) were used according to the manufacturer's protocol. The primary antibodies were mouse monoclonal anti-human BMP-4 (mAb 1049, Chemicon) and goat polyclonal anti-human gremlin (sc-

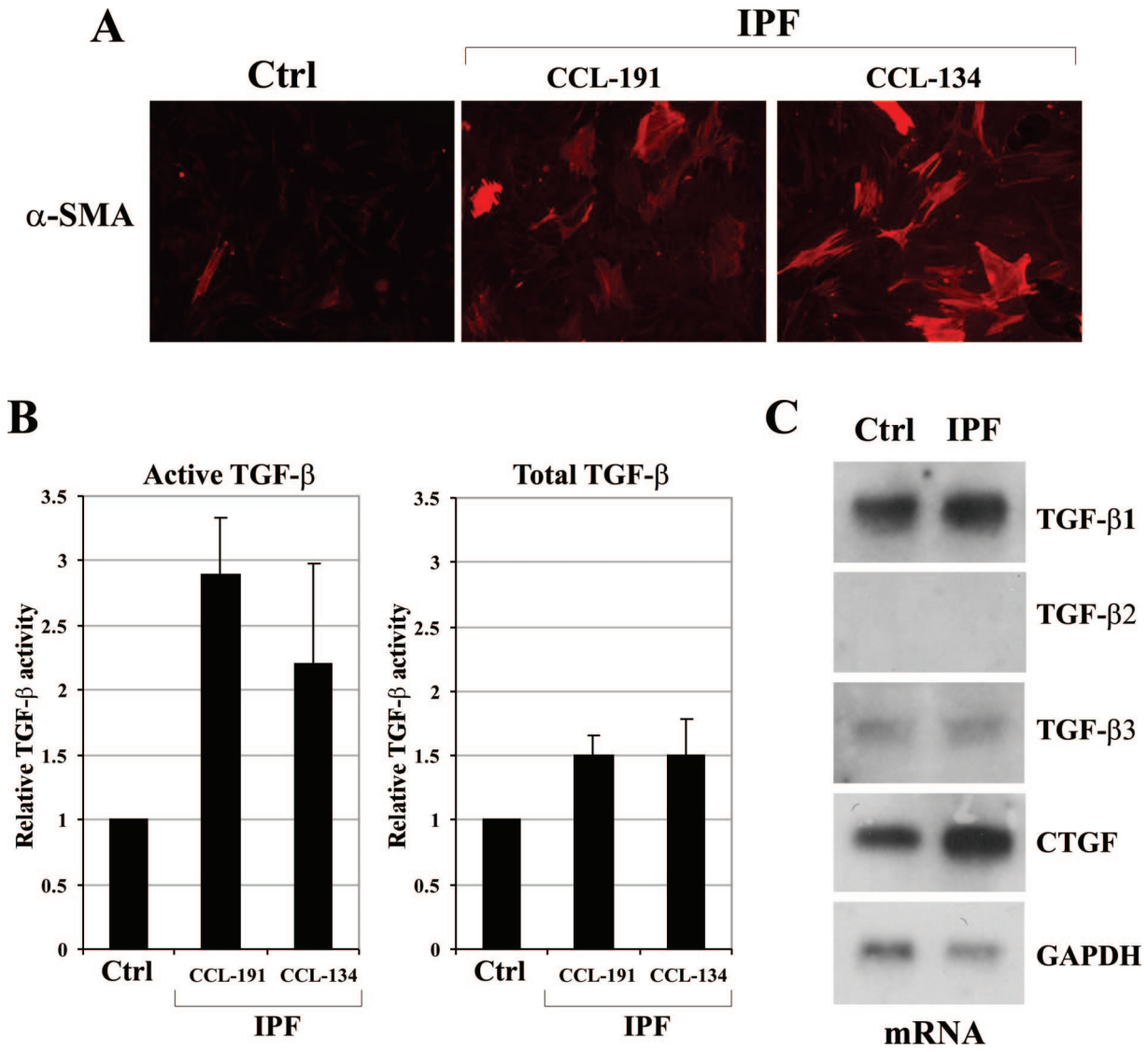


**Figure 1.** Increased secretion of TGF-$\beta$ activity in lung fibroblasts from IPF patients. **A:** Fibroblasts isolated from control adult lung (CCL-190) or IPF patient lung (CCL-191 and -134) were cultured on glass coverslips and stained for the myofibroblast marker $\alpha$-SMA. Red color indicates positive staining. **B:** Conditioned serum-free medium (24-hour collection) was harvested from fibroblasts, and aliquots of the media from the same number of cells were either analyzed directly for TGF-$\beta$ activity (active TGF-$\beta$) or after heat treatment (total TGF-$\beta$). Heat treatment activates latent forms of TGF-$\beta$. The results are expressed as relative TGF-$\beta$ activity (the activity in control medium is set to 1). The error bars represent the SEM of the samples. **C:** Total cellular RNA was isolated from cultured control (CCL-190) or IPF (CCL-134) fibroblasts and mRNA expression levels of TGF-$\beta$s 1 to 3 and CTGF were analyzed by Northern hybridization. The expression of a constant gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to control loading.

18274, Santa Cruz). Detection was performed with biotinylated anti-mouse or anti-goat secondary antibody, horseradish peroxidase complex, and AEC chromogen (Zymed). The sections were counterstained with Mayer's hematoxylin and mounted on glass slides. Control sections were treated with mouse isotype control (Zymed) or goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) to determine the specificity of the staining.

## *Results*

### *Increased Secretion of Active TGF-β in Lung Fibroblasts from IPF Patients*

TGF-$\beta$1 plays a major role in the induction of the lung myofibroblast phenotype and extracellular matrix deposition, and its overexpression has been well documented in IPF.[23] However, TGF-$\beta$s are secreted from cells as large latent complexes which need to be activated for TGF-$\beta$ to be biologically functional. The proportions of active versus latent TGF-$\beta$ forms in fibroblastic cells are, in general, not well known. We analyzed therefore the secretion of active and latent TGF-$\beta$ forms by lung fibroblasts isolated from IPF patients. Two fibroblast lines from IPF patients (CCL-191 and CCL-134) were compared with fibroblasts isolated from control human adult lung (CCL-190). The IPF fibroblasts expressed higher levels of $\alpha$-SMA, which is produced by the increased myofibroblast population characteristic of IPF (Figure 1A).[24] TGF-$\beta$ activity was quantified from cell-conditioned medium using TGF-$\beta$-responsive reporter cells.[21] The amount of TGF-$\beta$ in fibroblast-conditioned medium, har-

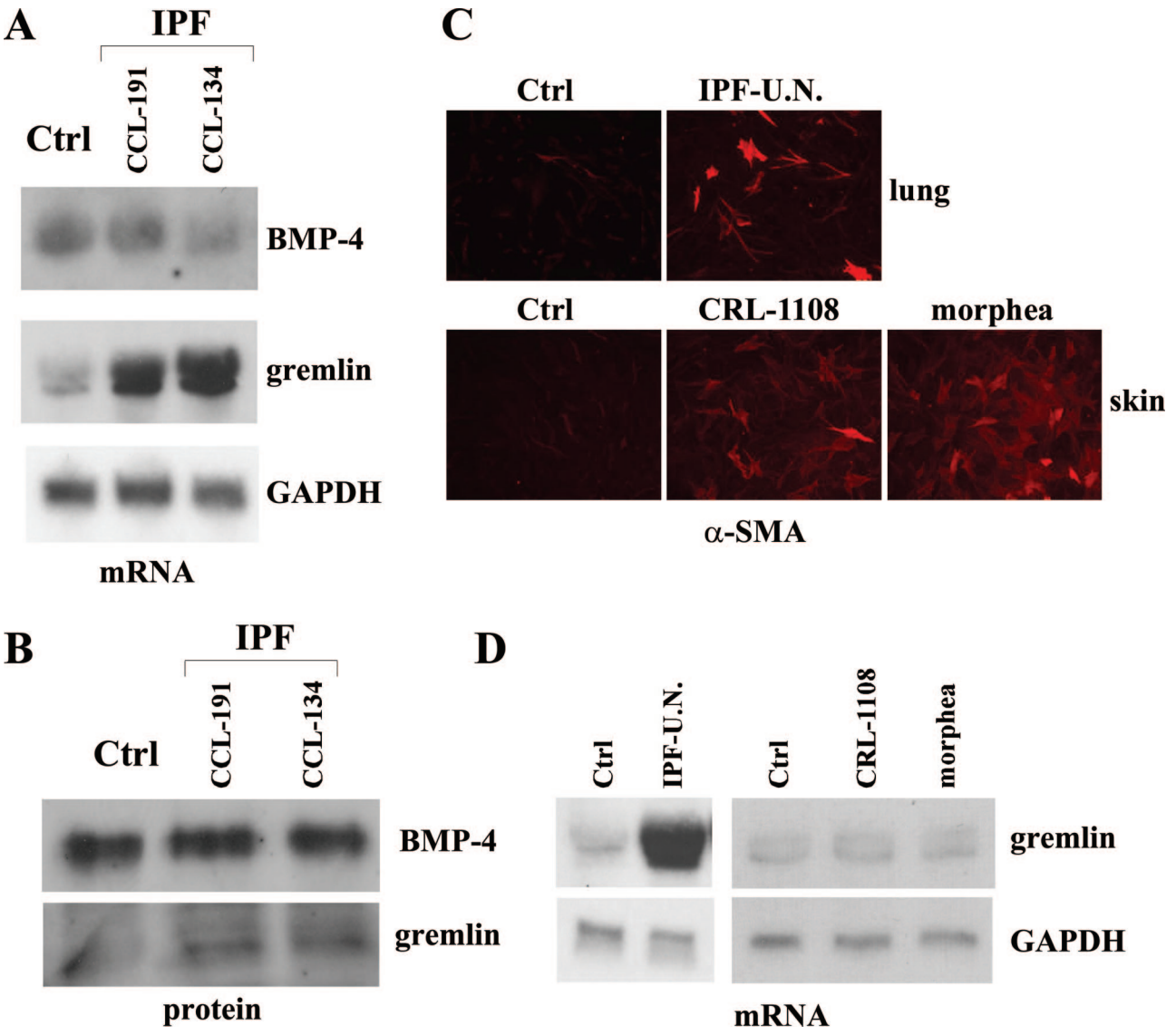


**Figure 2.** IPF fibroblasts overexpress gremlin. **A:** Total cellular RNA was isolated from cultured control (CCL-190) or IPF (CCL-191 and -134) fibroblasts, and mRNA expression levels of gremlin and BMP-4 were analyzed by Northern hybridization. The expression of a constant gene, GAPDH, was used to control loading. **B:** Cell-conditioned medium was collected from the same number of cells and analyzed for gremlin and BMP-4 protein levels by immunoblotting. **C:** Control lung (CCL-190) and skin (4′-strain) fibroblasts as well as primary fibroblasts isolated locally from patients with IPF (IPF-U.N., see Materials and Methods), and systemic (CRL-1108) or localized (morphea) scleroderma were cultured on glass coverslips and stained for the myofibroblast marker α-SMA. Red color indicates positive staining. **D:** Total cellular RNA was isolated from control (CCL-190, 4′-strain) and patient (IPF-U.N., CRL-1108, morphea) fibroblasts, and Northern hybridization analyses were performed as in **A**.

vested at 24 hours, was analyzed directly (active TGF-β) or after activation of the latent TGF-β forms by heat treatment (active plus latent TGF-β).[20] The specificity of the assay was confirmed using neutralizing antibodies against TGF-β (see Materials and Methods). In control fibroblasts the proportion of active TGF-β was ~5% of total TGF-β (data not shown). The IPF fibroblasts were found to secrete approximately threefold higher levels of active TGF-β, whereas the total TGF-β levels increased only ~1.5-fold (Figure 1B). This implies that IPF fibroblasts, in addition to producing more TGF-β, are able to activate a larger proportion of the latent TGF-β forms. Analyses of the mRNA expression levels of TGF-β isoforms by Northern hybridization indicated that lung fibroblasts produce mainly TGF-β1 (Figure 1C). The mRNA expression levels of the different latent TGF-β-binding proteins (LTBPs) were not altered in IPF fibroblasts (data not shown). The expression of CTGF, which is a TGF-β1 target gene, was enhanced twofold to threefold in IPF fibroblasts, further suggestive of increased TGF-β1 signaling (Figure 1C).

### *IPF Fibroblasts Overexpress Gremlin mRNA*

We have recently observed that the disruption of TGF-β1 activation in mouse lung fibroblasts results in alterations in the BMP-4 signaling pathway. In the absence of LTBP-4, the secretion of active TGF-β was reduced, which led to increased BMP-4 expression in parallel with

the loss of expression of the BMP inhibitor gremlin.[19] Previously, TGF-$\beta$1 has been suggested to regulate gremlin expression in kidney mesangial cells.[25] The expression of BMP-4 and gremlin mRNA in IPF fibroblasts was therefore analyzed by Northern hybridization. We found that BMP-4 mRNA levels were not notably altered, whereas gremlin mRNA levels were increased fourfold to fivefold (Figure 2A). Quantitative real-time RT-PCR analyses of gremlin mRNA levels confirmed the increase to be approximately fivefold (data not shown). Accordingly, immunoblotting analyses suggested that increased levels of gremlin were secreted by the IPF fibroblasts, while BMP-4 levels did not differ significantly (Figure 2B). To explore further the altered expression pattern of gremlin we established fresh fibroblast cultures from a patient with IPF (IPF-U.N., see Materials and Methods). Overexpression of the myofibroblast marker $\alpha$-SMA (Figure 2C) was noted in parallel with very high levels of gremlin mRNA expression (Figure 2D). To assess the question whether gremlin overexpression is a general feature of fibrotic diseases involving increased myofibroblast population, we analyzed gremlin mRNA expression in dermal fibroblasts from patients with systemic (CRL-1108) or localized (morphea) scleroderma. As in IPF, the pathology of these diseases includes excessive ECM accumulation and the persistence of fibroblasts.[26] $\alpha$-SMA was found to be overexpressed in scleroderma fibroblasts (Figure 2C), but gremlin mRNA levels were comparable to control dermal fibroblast (Figure 2D), suggesting that gremlin overexpression may not be associated with fibrotic diseases in general.

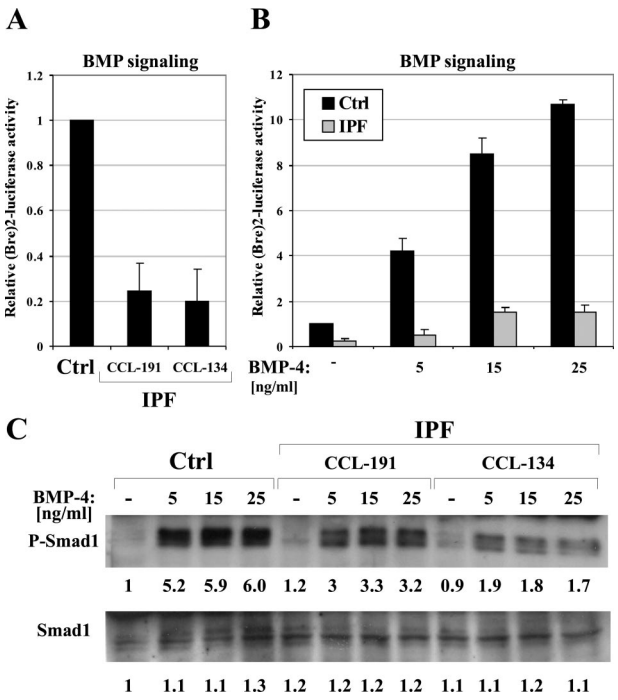


**Figure 3.** BMP-4 signaling is impaired in IPF fibroblasts. **A:** Control (CCL-190) or IPF (CCL-191 and -134) fibroblasts were transiently transfected with a BMP-responsive $(BRE)_2$-luciferase promoter construct (see Materials and Methods). Luciferase activities were measured 48 hours after transfection. The activities were normalized by comparing them with the activities of co-transfected *Renilla* luciferase. The results are expressed as relative luciferase activities (the activity in control fibroblasts is set to 1). The error bars represent the SEM of the samples. **B:** Control (CCL-190) or IPF (CCL-191) fibroblasts were transiently transfected, treated with the indicated concentration of BMP-4 for 16 hours, and analyzed for luciferase activity as in **A**. **C:** Fibroblasts were treated with the indicated concentrations of BMP-4 for 1 hour, and the levels of phosphorylated Smad1 (P-Smad1) and total Smad1 were analyzed from cell lysates by immunoblotting. Densitometric scanning results normalized to the untreated CCL-190 control are shown below the blots.

## *BMP-4 Signaling Is Severely Impaired in IPF Fibroblasts*

Because overexpression of gremlin is likely to suppress BMP signaling pathways, we used a BMP-responsive promoter construct [$(BRE)_2$-luciferase, see Materials and Methods] to measure BMP signaling activity in IPF fibroblasts. Cells were transiently transfected with the promoter construct, and the luciferase indicator gene activity was measured after 2 days. The promoter was considerably less active in IPF fibroblasts, suggesting that gremlin overexpression may lead to decreased levels of endogenous BMP signaling (Figure 3A). To test whether the responsiveness to exogenously added BMP-4 would be altered, the cells were treated with increasing concentrations of BMP-4 for 16 hours followed by indicator gene analyses. The responsiveness of the IPF fibroblasts to BMP-4 was significantly reduced (Figure 3B). Smad1 is a downstream signaling molecule of BMPs that is phosphorylated and transported into the nucleus rapidly after ligand stimulation.[27] Fibroblasts were treated with BMP-4 for 1 hour, after which the phosphorylation level of Smad1 was analyzed by immunoblotting. In normal lung fibroblasts BMP-4 (5 ng/ml) caused a significant induction of Smad1 phosphorylation, while the total Smad1 protein levels remained unaltered (Figure 3C). Higher concentrations of BMP-4 further enhanced Smad1 phosphorylation to some extent. In IPF fibroblasts the BMP-4 responsiveness was noticeably reduced, and the total levels of Smad1 phosphorylation were decreased, even with relatively high BMP-4 concentrations. These experiments suggest that the responsiveness to endogenous and exogenous BMP-4 is severely impaired in IPF fibroblasts.

## *IPF Lungs Show Increased Gremlin Immunoreactivity in Mesenchymal Cells*

Immunohistochemical staining was performed to visualize BMP-4 and gremlin expression and localization in normal and IPF lungs. BMP-4 immunoreactivity was found in the IPF lung in occasional epithelial cells and did not differ from the localization in normal lung (Figure 4). Gremlin immunoreactivity was observed in the normal lung tissue at the alveolar epithelial lining and alveolar macrophages, but not in the lung interstitium (Figure 4). In contrast, in lung biopsies from patients with IPF, the gremlin immunoreactivity was strong and was observed at areas of relatively well preserved lung tissue in early fibrotic lesions, but also in advanced fibrotic lesions.

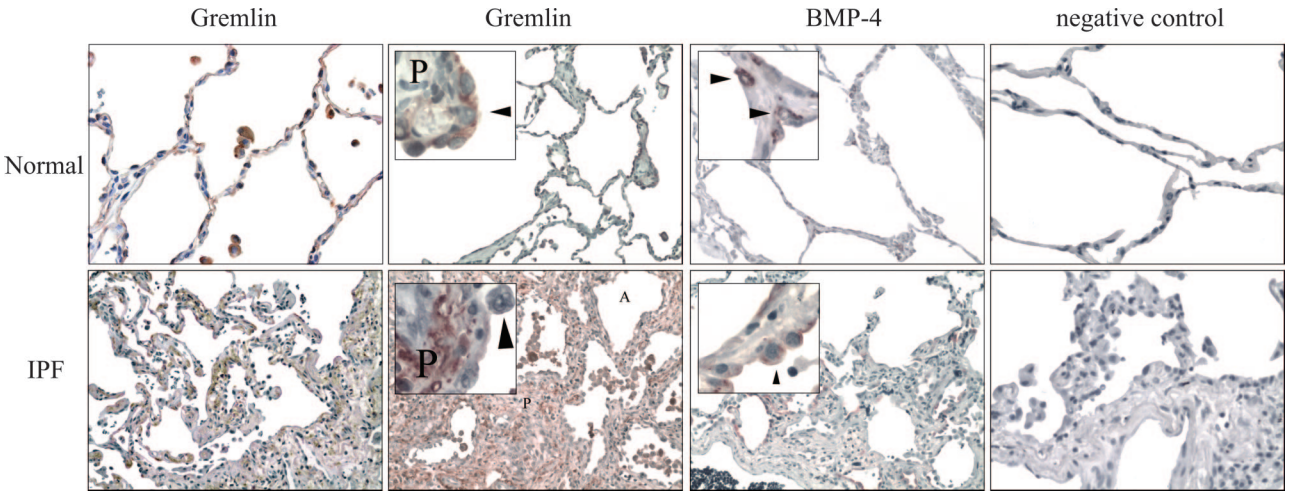


**Figure 4.** Paraffin sections from normal adult and IPF patient lungs were stained for gremlin and BMP-4. Positive staining is reddish-brown. In the normal lungs (two sections) gremlin immunoreactivity was located in alveolar macrophages and cells lining the alveolar wall (**arrow**). No gremlin immunoreactivity was observed in the parenchyme (P). IPF lungs showed strong immunoreactivity in early parenchymal lesions where the alveolar structure was still preserved (**bottom left**), whereas in areas of more advanced pulmonary fibrosis staining was intense throughout the parenchyme (**second bottom panel**, IPF-U.N. cell line was propagated from this patient). In IPF lungs, no gremlin immunoreactivity was observed in the epithelial cells (**arrow**). BMP-4 immunoreactivity was observed in the normal and IPF lungs in cells lining the alveoli (**A**, **small arrows**). The amount of BMP-4-positive cells was similar in both normal and IPF lungs. Control sections were treated with mouse isotype control (not shown) or goat IgG (**right**). Original magnifications: ×10 or ×40; except the **top left panel** at ×20.

### *Increased Expression of Gremlin mRNA in the Lungs of IPF Patients*

To confirm gremlin overexpression in IPF patients, real-time RT-PCR was performed. mRNA was isolated from the lung biopsies of six IPF patients and six control subjects (see Materials and Methods) followed by quantitative gremlin mRNA analyses. Gremlin mRNA levels were low in all control samples, whereas in five of six IPF patient samples the gremlin mRNA levels were clearly elevated (Figure 5A). Data analyses using the unpaired *t*-test confirmed that the inductions were statistically significant ($P < 0.05$). The highest level noted was ~35-fold overexpression. BMP-2, -4, and -7 mRNA expression levels were analyzed next from the same set of samples. More variation was observed between the expression levels of BMPs in the biopsy samples (Figure 5B). BMP-4 and -7 expression levels did not seem to be altered in IPF, while BMP-2 levels were reduced (average level of BMP-2 mRNA in IPF samples was ~40% of control level).

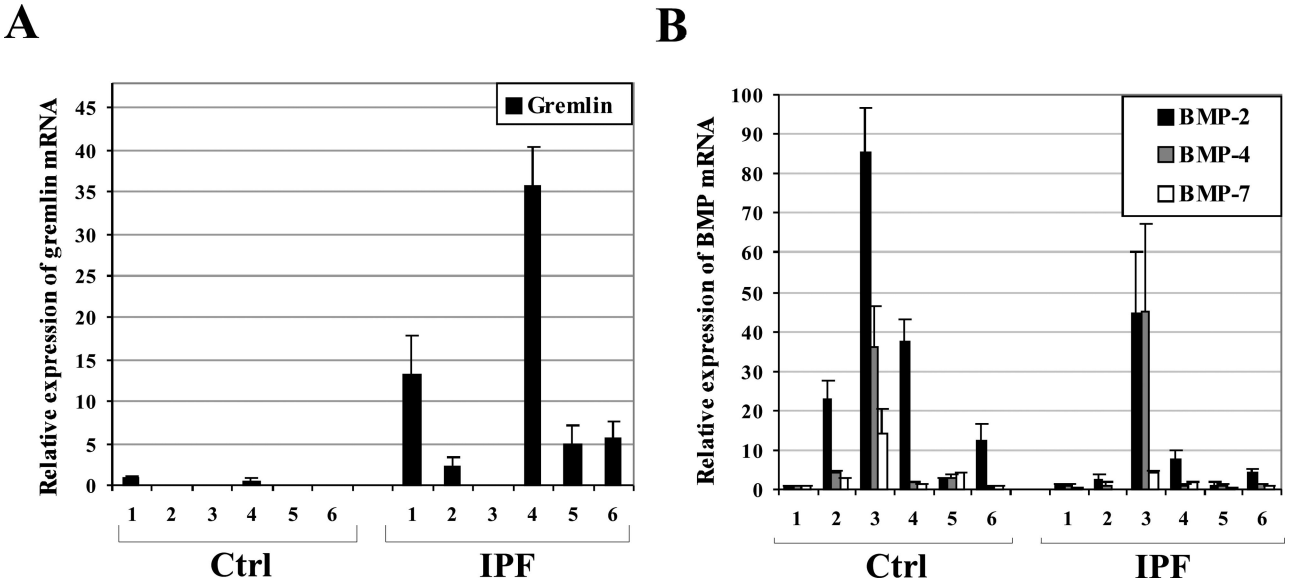


**Figure 5.** Increased expression of gremlin in the lungs of IPF patients. **A:** Total cellular RNA was isolated from control (Ctrl) or IPF patient lung (IPF) tissues from six subjects, and the expression of gremlin (**A**) or BMP-2, -4, and -7 (**B**) was analyzed by quantitative real-time RT-PCR. The mRNA expression levels were normalized to the expression of a control gene (actin), and the results are expressed as relative values (gremlin expression in Ctrl-1 is set to 1). The error bars represent SEM of the samples.

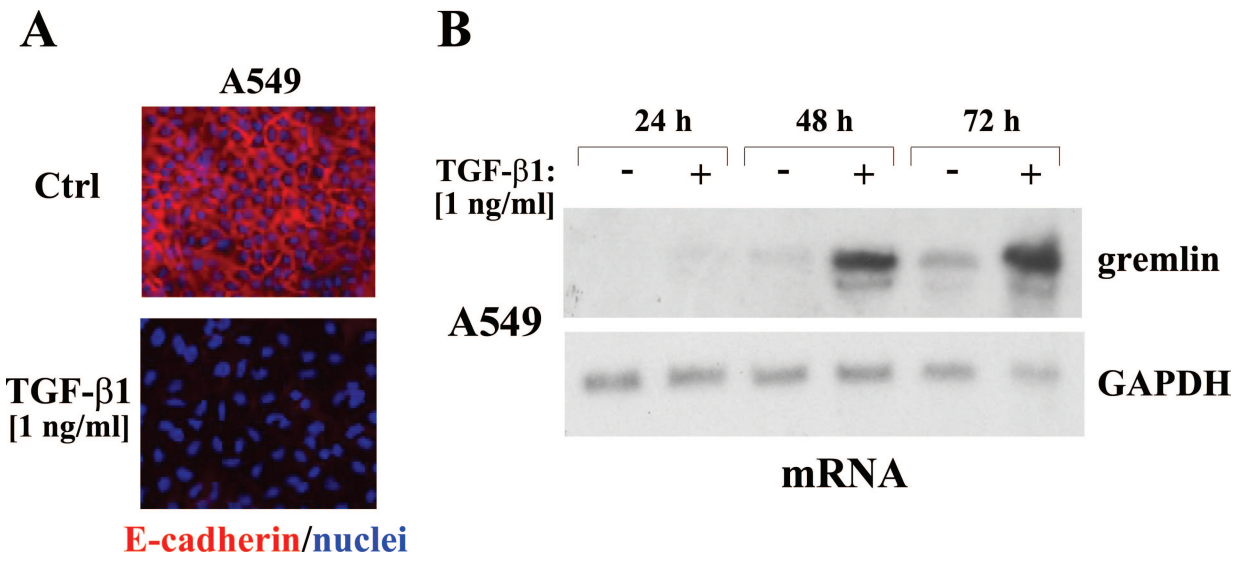


**Figure 6.** TGF-β1 induces gremlin expression in association with EMT in lung epithelial cells. **A:** A549 lung epithelial cells were cultured on glass coverslips, treated with 1 ng/ml TGF-β1 for 48 hours, and stained for E-cadherin. The staining indicates E-cadherin protein (red) and nuclei (blue). **B:** A549 cells were treated with TGF-β1 for the indicated times, after which total cellular RNA was isolated and mRNA expression levels of gremlin were analyzed by Northern hybridization. mRNA expression of a constant gene, GAPDH, was used to control loading.

### *TGF-β Induces Gremlin Expression in Association with EMT in Lung Epithelial Cells*

Lung epithelial cells can undergo EMT in response to TGF-β stimulation.[28] We analyzed here whether the TGF-β-induced differentiation processes would be associated with altered gremlin mRNA expression levels. A549 lung epithelial cells are sensitive to TGF-β-induced EMT, which is characterized by the loss of cell surface expression of E-cadherin.[29] A549 cells were treated with TGF-β1 (1 ng/ml) for 48 hours, and E-cadherin levels were analyzed by immunofluorescence analyses. As reported earlier the morphology of the cells changed to that of spindle-like fibroblasts[29] (data not shown), and E-cadherin disappeared from the cell surfaces (Figure 6A). The basal expression levels of gremlin were low in A549 cells, whereas TGF-β1 induced prominent expression of gremlin mRNA (Figure 6B). The induction was detectable within 48 hours and persisted at the same level during the 72 hours of incubation.

To analyze whether gremlin expression alone could change the cellular phenotype, we produced a stable A549 cell pool (A549/gremlin) that overexpressed gremlin (see Materials and Methods). The growth rate of the A549/gremlin cells was comparable to A549 cells (data not shown), and gremlin mRNA analyses indicated that the transgene was expressed as expected (Figure 7A). BMP signaling activity was measured using the BMP responsive promoter construct and found to be reduced to ~20% of control level (Figure 7B) suggesting that gremlin overexpression reduced BMP signaling. Interestingly, the cell surface expression of E-cadherin was somewhat reduced in A549/gremlin cells (Figure 6D), suggesting that gremlin can interfere with cellular pathways regulating the early signs of EMT. When low concentrations of TGF-β1 (0.05 to 0.5 ng/ml) were used to induce EMT, the A549/gremlin cells were found to be more sensitive to the loss of cell surface E-cadherin (Figure 6D). These results further suggest that enhanced expression of gremlin is associated with EMT of A549 lung epithelial cells.

## *Discussion*

IPF is a devastating disease with treatment options limited to corticosteroids in combination with cytotoxic drugs and in rare cases lung transplantation. Development of new diagnostic and therapeutic strategies requires better knowledge of the molecular mechanisms of the disease. Recent studies have suggested that IPF may result from abnormal reparative processes after injury in the lung.[2] The cross-talk between epithelial and mesenchymal cells of the lung is crucial for the normal regenerative processes. Many growth factor pathways involved in this cross-talk are altered in IPF. The production of profibrotic molecules including TGF-β, PDGF, and CTGF is frequently increased.[6] Inducers of epithelial cell proliferation such as HGF, which have a protective effect against fibrosis, are down-regulated in IPF fibroblasts.[30] Imbalance of proteolytic systems including plasminogen activators and matrix metalloproteinases and their specific inhibitors can favor the accumulation of ECM.[10,31,32] The best known mediator of fibrotic activities in the IPF lung is TGF-β, which, in addition to increasing ECM synthesis, can also regulate the proteolytic activities in the lung.[6,33] We find here that lung fibroblasts isolated from IPF patients secrete increased amounts of TGF-β and also activate a larger proportion of the latent TGF-β forms. mRNA analyses of the TGF-β isoform expression indicated that lung fibroblasts produce mainly TGF-β1. The expression of TGF-β2 and -β3 may be suppressed by TGF-β1 as has been observed in mouse fibroblasts.[34] The profibrotic TGF-β1 target gene, CTGF, was induced in IPF fibro-

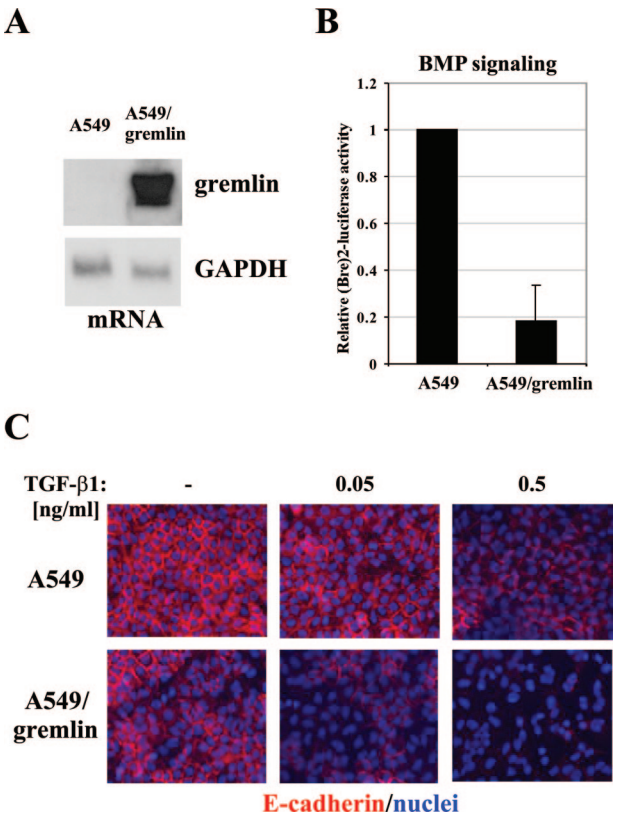


**Figure 7.** Gremlin overexpression sensitizes A549 cells to TGF-$\beta$1-induced EMT. **A:** Total cellular RNA was isolated from A549 or A549/gremlin cells, and mRNA expression levels of gremlin were analyzed by Northern hybridization. mRNA expression of a constant gene, GAPDH, was used to control loading. **B:** Cells were transiently transfected with a BMP-responsive $(BRE)_2$-luciferase promoter construct, and luciferase activities were measured as in Figure 3A. The error bars represent the SEM of the samples. **C:** A549 and A549/gremlin cells were cultured on glass coverslips, treated with the indicated concentrations of TGF-$\beta$1 for 48 hours, and stained for E-cadherin. The staining indicates E-cadherin protein (red) and nuclei (blue).

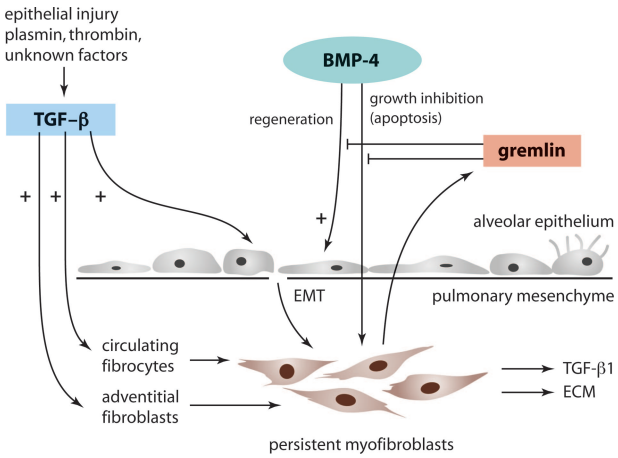


**Figure 8.** Potential roles of the TGF-$\beta$ and BMP-4 signaling pathways in IPF. The expression of TGF-$\beta$ is already induced during early stages of IPF. TGF-$\beta$ recruits fibroblasts to the site of injury and induces their activation. TGF-$\beta$ also stimulates the EMT of alveolar epithelial cells. Pulmonary myofibroblasts persist in IPF and overexpress gremlin, which can inhibit BMP-4-induced regeneration of the epithelium. This may lead to a vicious circle of sustained epithelial injury and TGF-$\beta$1 activation. In addition, gremlin blocks BMP-4-mediated myofibroblast apoptosis, which further enhances parenchymal fibrosis. Excessive fibroproliferation leads to the excessive accumulation of the ECM.

blasts, confirming increased TGF-$\beta$ signaling.[35,36] Possible mediators of TGF-$\beta$ activation in the IPF lung include thrombospondin-7,[37,38] integrin $\alpha_v\beta_6$,[39] as well as the increased matrix metalloproteinase activities associated with IPF.[40,41]

Our previous studies suggested that decreased TGF-$\beta$ activation in mouse lung fibroblasts leads to the loss of expression of gremlin and the increase in BMP-4 signaling.[19] This prompted us to analyze the expression levels of gremlin and BMP-4 in IPF fibroblasts, which were now found to secrete more active TGF-$\beta$. Although there may be heterogeneity among fibroblasts and a relatively few different IPF fibroblast cell lines were analyzed, a consistent up-regulation of gremlin mRNA levels was observed in each of these IPF fibroblast lines. The mechanism of this up-regulation, however, remained unclear. Overexpression of gremlin probably resulted in decreased responsiveness of the IPF fibroblasts to endogenous and exogenous BMP-4. Although we cannot exclude the possible nonspecific activation of the BMP responsive reporter promoter in control fibroblasts, the data on gremlin expression in fibroblasts and epithelial cells suggests the involvement of autocrine BMP signaling. To assess the correlation between the *in vitro* results using cultured IPF fibroblasts with the IPF disease in patients, immunohistochemical stainings of lung sections were performed. Although BMP-4 immunoreactivity was moderate and confined to the epithelial layer in both normal and IPF lung, the gremlin immunoreactivity was enhanced in the IPF lung, and the localization switched from the epithelium to the interstitium. In agreement with the immunohistochemical staining, quantitative mRNA analyses of lung tissue samples indicated a high occurrence of overexpression of gremlin in IPF. We also observed lower levels of BMP-2 in the IPF lung, which correlates well with the microarray data obtained from the Gene Expression Omnibus database.[42] These changes together probably alter the BMP signaling activities in the IPF lung.

Gremlin has an important role in epithelial and mesenchymal signaling cross-talk in branching morphogenesis during lung and kidney development.[15,43] Knockout and overexpression studies indicate that gremlin induces epithelial cell growth arrest and differentiation. In addition, gremlin-mediated BMP antagonism promotes the survival of mesenchymal cells. Gremlin expression was detected in the epithelial cells in normal lung, where it may function to maintain epithelial integrity. In IPF lung gremlin was overexpressed specifically in the mesenchymal cells, where it may promote the differentiation and persistence of myofibroblasts (illustrated in Figure 8). Both BMP and gremlin are secreted proteins, and the mesenchymally produced gremlin may decrease BMP-mediated epithelial regeneration after injury. High expression of gremlin may also be associated with accelerated fibrosis because the patients in this study represented advanced stages of pulmonary fibrosis. Recently, gremlin overexpression has been associated with fibrotic kidney diseases,[44,45] which share many pathological features with IPF.

Dermal fibroblasts from patients with systemic or localized scleroderma, other fibrotic diseases characterized by the accumulation of myofibroblasts, did not show increased expression of gremlin mRNA. Thus fibrotic diseases of the lung (IPF) and kidney, but probably not of skin, seem to be associated with gremlin overexpression, which may suggest the reappearance of a developmental gene in disease.

The increased population of fibroblasts and differentiated myofibroblasts in IPF show heterogeneity and may be derived from various sources. These include peribronchial and perivascular adventitial fibroblasts, circulating fibrocytes,[46,47] as well as lung epithelial cells that can undergo EMT (illustrated in Figure 8). There is emerging *in vivo* evidence to suggest an important role for EMT during embryonic development and in malignant diseases.[29] Overexpression of matrilysin (MMP-7) has been described in IPF,[40] and it may have a role in E-cadherin ectodomain shedding and in mediating the induction of EMT.[48] In addition, TGF-$\beta$ is a potent inducer of EMT in lung epithelial cells.[28] We find here that gremlin overexpression is associated with TGF-$\beta$1-induced EMT of lung epithelial cells. It appears that gremlin expression can sensitize cells to TGF-$\beta$1-induced EMT. The relevance of our results with A549 cells to normal lung epithelial cells *in vitro* and *in vivo* remains to be evaluated. BMP-7, on the other hand, can induce mesenchymal-to-epithelial transition in renal fibroblasts and also counteract TGF-$\beta$1-induced EMT.[5,49] Apparently the balance between TGF-$\beta$ and BMP signaling pathways plays a major role during these phenotypic transitions.

Our results reveal for the first time that enhanced gremlin-mediated BMP antagonism may be associated with IPF. Further studies are needed to evaluate whether gremlin overexpression plays an important role in the pathogenesis of IPF. Gremlin antagonism may prove to be beneficial for IPF patients, as suggested by studies showing that BMP-7 or a BMP signaling enhancer, kielin/chordin-like protein, can inhibit or attenuate renal fibrotic diseases in mouse models.[50,51] Future studies will reveal whether gremlin is a potential therapeutic target for the treatment of IPF.

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## References

1. American Thoracic Society and the European Respiratory Society: Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. Am J Respir Crit Care Med 2000, 161:646–664
2. Geiser T: Idiopathic pulmonary fibrosis—a disorder of alveolar wound repair? Swiss Med Wkly 2003, 133:405–411
3. American Thoracic Society and the European Respiratory Society: International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. Am J Respir Crit Care Med 2002, 165:277–304
4. Daniels CE, Wilkes MC, Edens M, Kottom TJ, Murphy SJ, Limper AH, Leof EB: Imatinib mesylate inhibits the profibrogenic activity of TGF-$\beta$ and prevents bleomycin-mediated lung fibrosis. J Clin Invest 2004, 114:1308–1316
5. Zeisberg M, Shah AA, Kalluri R: Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. J Biol Chem 2005, 280:8094–8100
6. Allen JT, Spiteri MA: Growth factors in idiopathic pulmonary fibrosis: relative roles. Respir Res 2002, 3:13
7. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH: Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor-$\beta$. J Exp Med 1987, 165:251–256
8. Ignotz RA, Massague J: Transforming growth factor-$\beta$ stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 1986, 261:4337–4345
9. Raghow R, Postlethwaite A, Keski-Oja J, Moses H, Kang A: Transforming growth factor-$\beta$ increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. J Clin Invest 1987, 79:1285–1288
10. Keski-Oja J, Koli K, Lohi J, Laiho M: Growth factors in the regulation of plasminogen-plasmin system in tumor cells. Semin Thromb Hemost 1991, 17:231–239
11. Koli K, Saharinen J, Hyytiäinen M, Penttinen C, Keski-Oja J: Latency, activation, and binding proteins of TGF-$\beta$. Microsc Res Tech 2001, 52:354–362
12. Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL: Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development 1996, 122:1693–1702
13. Shannon JM, Hyatt BA: Epithelial-mesenchymal interactions in the developing lung. Annu Rev Physiol 2004, 66:625–645
14. Topol LZ, Marx M, Laugier D, Bogdanova NN, Boubnov NV, Clausen PA, Calothy G, Blair DG: Identification of drm, a novel gene whose expression is suppressed in transformed cells and which can inhibit growth of normal but not transformed cells in culture. Mol Cell Biol 1997, 17:4801–4810
15. Lu MM, Yang H, Zhang L, Shu W, Blair DG, Morrisey EE: The bone morphogenic protein antagonist gremlin regulates proximal-distal patterning of the lung. Dev Dyn 2001, 222:667–680
16. Chen B, Blair DG, Plisov S, Vasiliev G, Perantoni AO, Chen Q, Athanasiou M, Wu JY, Oppenheim JJ, Yang D: Cutting edge: bone morphogenic protein antagonists Drm/gremlin and Dan interact with Slits and Act as negative regulators of monocyte chemotaxis. J Immunol 2004, 173:5914–5917
17. Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engstrom U, Heldin CH, Funa K, ten Dijke P: The L45 loop in type I receptors for TGF-$\beta$ family members is a critical determinant in specifying Smad isoform activation. FEBS Lett 1998, 434:83–87
18. Korchynskyi O, ten Dijke P: Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 2002, 277:4883–4891
19. Koli K, Wempe F, Sterner-Kock A, Kantola A, Komor M, Hofmann WK, von Melchner H, Keski-Oja J: Disruption of LTBP-4 function reduces TGF-$\beta$ activation and enhances BMP-4 signaling in the lung. J Cell Biol 2004, 167:123–133
20. Brown P, Wakefield L, Levinson A, Sporn M: Physicochemical activation of recombinant latent transforming growth factor-$\beta$s 1, 2, and 3. Growth Factors 1990, 3:35–43
21. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-$\beta$ using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 1994, 216:276–284
22. Penttinen C, Saharinen J, Weikkolainen K, Hyytiäinen M, Keski-Oja J: Secretion of human latent TGF-$\beta$ binding protein-3 (LTBP-3) is dependent on co-expression of TGF-$\beta$. J Cell Sci 2002, 115:3457–3468
23. Khalil N, O'Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Bereznay OH, Greenberg AH: Increased production and immunohistochemical localization of transforming growth factor-$\beta$ in idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol 1991, 5:155–162

24. Fireman E, Shahar I, Shoval S, Messer G, Dvash S, Grief J: Morphological and biochemical properties of alveolar fibroblasts in interstitial lung diseases. Lung 2001, 179:105–117
25. McMahon R, Murphy M, Clarkson M, Taal M, Mackenzie HS, Godson C, Martin F, Brady HR: IHG-2, a mesangial cell gene induced by high glucose, is human gremlin. Regulation by extracellular glucose concentration, cyclic mechanical strain, and transforming growth factor-$\beta$1. J Biol Chem 2000, 275:9901–9904
26. Trojanowska M: Molecular aspects of scleroderma. Front Biosci 2002, 7:608–618
27. Itoh S, Itoh F, Goumans MJ, ten Dijke P: Signaling of transforming growth factor-$\beta$ family members through Smad proteins. Eur J Biochem 2000, 267:6954–6967
28. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z: Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-$\beta$1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 2005, 166: 1321–1332
29. Zavadil J, Böttinger EP: TGF-$\beta$ and epithelial-to-mesenchymal transitions. Oncogene 2005, 24:5764–5774
30. Marchand-Adam S, Marchal J, Cohen M, Soler P, Gerard B, Castier Y, Leseche G, Valeyre D, Mal H, Aubier M, Dehoux M, Crestani B: Defect of hepatocyte growth factor secretion by fibroblasts in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2003, 168:1156–1161
31. Kotani I, Sato A, Hayakawa H, Urano T, Takada Y, Takada A: Increased procoagulant and antifibrinolytic activities in the lungs with idiopathic pulmonary fibrosis. Thromb Res 1995, 77:493–504
32. Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R, Pardo A: TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? Am J Physiol 2000, 279:L562–L574
33. Laiho M, Saksela O, Andreasen P, Keski-Oja J: Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-$\beta$. J Cell Biol 1986, 106:2403–2410
34. Bascom CC, Wolfshohl JR, Coffey Jr RJ, Madisen L, Webb NR, Purchio AR, Derynck R, Moses HL: Complex regulation of transforming growth factor-$\beta$1, -$\beta$2, and -$\beta$3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors-$\beta$1 and -$\beta$2. Mol Cell Biol 1989, 9:5508–5515
35. Grotendorst GR, Okochi H, Hayashi N: A novel transforming growth factor-$\beta$ response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ 1996, 7:469–480
36. Watts KL, Spiteri MA: Connective tissue growth factor expression and induction by transforming growth factor-$\beta$ is abrogated by simvastatin via a Rho signaling mechanism. Am J Physiol 2004, 287:L1323–L1332
37. Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N: Thrombospondin-1 is a major activator of TGF-$\beta$1 in vivo. Cell 1998, 93:1159–1170
38. Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y, Goto Y, Masuyama K, Zhang MJ, Hirano K, Mochizuki M, Ohtsuka M, Sekizawa K: Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. Am J Respir Cell Mol Biol 2001, 24:1–11
39. Munger J, Huang X, Kawakatsu H, Griffiths M, Dalton S, Wu J, Pittet J-F, Kaminski N, Garat C, Matthay M, Rifkin DB, Sheppard D: The integrin $\alpha v\beta 6$ binds and activates latent TGF-$\beta$1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999, 96:319–328
40. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, Sheppard D, Pardo A, Selman M, Heller RA: Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci USA 2002, 99:6292–6297
41. Atkinson JJ, Senior RM: Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 2003, 28:12–24
42. Pardo A, Gibson K, Cisneros J, Richards TJ, Yang Y, Becerril C, Yousem S, Herrera I, Ruiz V, Selman M, Kaminski N: Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. PLOS Medicine 2005, 2:891–903
43. Michos O, Panman L, Vintersten K, Beier K, Zeller R, Zuniga A: Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. Development 2004, 131:3401–3410
44. Dolan V, Murphy M, Sadlier D, Lappin D, Doran P, Godson C, Martin F, O'Meara Y, Schmid H, Henger A, Kretzler M, Droguett A, Mezzano S, Brady HR: Expression of gremlin, a bone morphogenetic protein antagonist, in human diabetic nephropathy. Am J Kidney Dis 2005, 45:1034–1039
45. Dolan V, Murphy M, Alarcon P, Brady HR, Hensey C: Gremlin—a putative pathogenic player in progressive renal disease. Expert Opin Ther Targets 2003, 7:523–526
46. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH: Bone marrow-derived progenitor cells in pulmonary fibrosis. J Clin Invest 2004, 113:243–252
47. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM: Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 2004, 114:438–446
48. McGuire JK, Li Q, Parks WC: Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. Am J Pathol 2003, 162:1831–1843
49. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF-$\beta$-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003, 9:964–968
50. Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Muller GA, Kalluri R: Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol 2003, 285:F1060–F1067
51. Lin J, Patel SR, Cheng X, Cho EA, Levitan I, Ullenbruch M, Phan SH, Park JM, Dressler GR: Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. Nat Med 2005, 11:387–393