GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Altered Expression of Bone Morphogenetic Protein Accessory Proteins in Murine and Human Pulmonary Fibrosis

Noelle Murphy,^{*†} Katherine U. Gaynor,^{*} Simon C. Rowan,^{*} Sinead M. Walsh,^{*‡} Aurelie Fabre,[§] John Boylan,[†] Michael P. Keane,^{*‡} and Paul McLoughlin^{*‡}

From the School of Medicine,^{*} Conway Institute, University College Dublin, Dublin; and the Departments of Anaesthesia and Intensive Care Medicine[†] and Histopathology[§] and the School of Medicine,[‡] St Vincent's University Hospital, Dublin, Ireland

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Address correspondence to
Paul McLoughlin, M.B.,
B.Ch., Ph.D., UCD School of
Medicine, Health Sciences
Centre, University College
Dublin, Belfield, Dublin 4,
Ireland. E-mail: paul.mcloughlin@ucd.ie.

Idiopathic pulmonary fibrosis is a chronic, progressive fibrotic disease with a poor prognosis. The balance between transforming growth factor β 1 and bone morphogenetic protein (BMP) signaling plays an important role in tissue homeostasis, and alterations can result in pulmonary fibrosis. We hypothesized that multiple BMP accessory proteins may be responsible for maintaining this balance in the lung. Using the bleomycin mouse model for fibrosis, we examined an array of BMP accessory proteins for changes in mRNA expression. We report significant increases in mRNA expression of gremlin 1, noggin, follistatin, and follistatin-like 1 (*Fstl1*), and significant decreases in mRNA expression of chordin, kielin/chordin-like protein, nephroblastoma overexpressed gene, and BMP and activin membrane-bound inhibitor (*BAMBI*). Protein expression studies demonstrated increased levels of noggin, *BAMBI*, and *FSTL1* in the lungs of bleomycin-treated mice and in the lungs of idiopathic pulmonary fibrosis patients. Furthermore, we demonstrated that transforming growth factor β stimulation resulted in increased expression of noggin, *BAMBI*, and *FSTL1* in human small airway epithelial cells. These results provide the first evidence that multiple BMP accessory proteins are altered in fibrosis and may play a role in promoting fibrotic injury. (*Am J Pathol* 2016, 186: 600–615; <http://dx.doi.org/10.1016/j.ajpath.2015.10.032>)

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrotic disease of unknown cause that is limited to the lungs. Prognosis is extremely poor, with a median survival of 3 to 5 years after diagnosis. Transforming growth factor (TGF)- β 1 has been shown to be a key mediator of fibrosis in the lung. Bone morphogenetic proteins (BMPs) are multifunctional growth factors belonging to the TGF- β superfamily, and it has been postulated that increased TGF- β 1 signaling with a concomitant reduction in BMPs is an important driver of inflammation and fibrosis.¹ Moreover, there is growing evidence that the interactions between members of the TGF- β superfamily and the accessory proteins that modulate TGF- β superfamily signaling are important in the development of an array of inflammatory and fibrotic diseases in the lung.² It is clear that members of the TGF- β superfamily are important regulators of pulmonary homeostasis, and alterations in TGF- β superfamily signaling contribute to the development of

pulmonary pathology. In particular, reductions in BMP signaling are pivotal in the development of pulmonary hypertension, pulmonary fibrosis, and pulmonary inflammation.^{1,3,4} Furthermore, attenuated BMP signaling is a recognized feature of inflammatory and fibrotic conditions in other organ systems, including the joints, kidneys, and liver.^{5–7}

In many disorders, reduced BMP signaling is associated with a concomitant rise in TGF- β 1/activin signaling. Thus, balanced BMP and TGF- β 1/activin signaling may be a

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crucial determinant of regeneration or repair after injury versus maladaptive healing by fibrosis. It has already been shown that one BMP accessory protein, gremlin1 (a BMP antagonist), is up-regulated in IPF.¹ Gremlin1 is one of a larger group of BMP accessory proteins, some of which also bind to TGF- β 1 or activin.^{8,9} We hypothesized that during the disease process, the altered expression of a number of different BMP accessory proteins may lead to an imbalance in TGF- β 1/BMP signaling, resulting in fibrosis. To test this hypothesis, we utilized a well-established mouse model of fibrosis in which intratracheal administration of bleomycin provides an initial inflammatory response that heals by fibrosis, resembling the changes seen in human lung injury.¹⁰ We examined the expression levels of a number of BMP accessory proteins that are known to play a role in lung diseases, are implicated in lung organogenesis, or are known to be expressed in the lung. We selected key targets to investigate further, looking at levels of protein expression in fibrotic mouse and human tissue and at the effects of TGF- β 1 induction on expression of these inhibitors in human small airway epithelial cells (SAECs).

Materials and Methods

Animals

All procedures involving animals were approved by the University College Dublin Animal Research Ethics Committee and licensed by the Department of Health and Children in accordance with European Communities Regulations 2002 (Amendment of Cruelty to Animals Act 1876) and EU Directive 2010/63/EU. Specific pathogen-free adult male C57Bl/6J wild-type mice (25 to 32 g at 10 to 15 weeks old) were used in all experiments. Specific pathogen-free status was as defined in the Federation of Laboratory Animal Science Association guidelines.¹¹ All animals were exposed to the same 12:12-hour light-dark cycle, and food and water were provided *ad libitum*. Animals were housed singly in individually ventilated cages for the duration of the study, maintaining specific pathogen-free status. All mice used in the study were reared in the animal facility from birth, and specific pathogen-free status was according to the Federation of Laboratory Animal Science Association guidelines.¹¹

Bleomycin Mouse Model

Mice were randomly allocated by grab sampling to either a saline (control) or bleomycin-inoculated group ($n = 12$ per group). Mice were anesthetized by i.p. injection of 100 mg/kg ketamine (Vetalar; Pfizer, Sandwich, Kent, UK). Intratracheal instillation was used to deliver either 1 U/kg of bleomycin (Bleo-Kyowa; Kyowa Hakko Ltd, Slough, UK) in 50 μ L of normal saline or 50 μ L of normal saline alone, as described.¹² In brief, once anesthetized, the mice were suspended on a custom-made Perspex intubation stand (made in-house) at an angle of 70 degrees by their front

incisors. The tongue was carefully pulled out and an otoscope (Welch Allyn 3.5 V Diagnostic Otoscope; Welch Allyn Ltd, Meath, Ireland) with a pediatric tip was inserted into the mouth until the epiglottis came into view. A metal applicator was used to apply lidocaine jelly (Instillagel; Farco-Pharma, Berlin, Germany) posterior to the epiglottis. The otoscope was removed for 2 minutes, giving the lidocaine time to anesthetize the vocal cords, and then reinserted into the mouth, after which a 22-gauge catheter (Jelco, Dublin, Ireland) mounted with a guide wire was passed through the vocal cords. On confirmation of correct placement of the catheter, either bleomycin (1 U/kg in 50 μ L of normal saline) or 50 μ L normal saline alone was instilled down the intratracheal catheter. A Stepper Repetitive Dispensing Pipette (Dymax, Kildington, Oxfordshire, UK) was used to ensure that the exact volume was administered to each mouse. The mice were extubated and allowed to recover from anesthesia in a warming chamber. One mouse from each group died during or immediately after the inoculation procedure; thus, lungs from 11 animals were available for analysis. Mice were housed individually after inoculation in individually ventilated cages and observed daily for adverse clinical signs, including the following: appearance (eg, signs of dehydration, piloerection, lack of grooming), body weight, behavior (eg, alterations in mobility, vocalization), respiratory rate, and signs of pain. If animals were deemed to be suffering more than moderate adverse effects or had lost more than 20% of their initial body weight, they were sacrificed by overdose of anesthesia before the predefined study time had elapsed.

Fourteen days after instillation, mice were sedated by brief inhalation of 2% isoflurane (Vetflurance, Virbac, Suffolk, UK) in oxygen vapor and then given an i.p. injection of 60 mg/kg sodium pentobarbital (Rhône Merieux Ltd, Harlow, UK) and 1000 IU/kg anticoagulated with heparin (Leo Laboratories Ltd, Maidenhead, Berkshire, UK). They were then returned to their cage and breathed room air until confirmation of an adequate depth of anesthesia by absence of response to tail compression; the femoral artery was then incised and the mice sacrificed by exsanguination. Post mortem a ligature was passed around the right main bronchus to tie off the right lung. The right lung was removed, snap-frozen in liquid nitrogen, weighed, and then used for mRNA analysis. The remaining lung was fully inflated via a tracheal cannula with fixative [4% (w/v) paraformaldehyde] at a pressure of 25 cm H₂O for 30 minutes. After lung inflation, the left main bronchus was ligated at the level of the hilum. The left lung was left in fixative for 24 hours. The volumes of the left lungs were then measured by water displacement.¹³ Two lungs from the bleomycin-inoculated group were punctured at the time of isolation and thus could not be inflated; consequently, they were not available for further histological study. One additional lung was excluded from each group for technical reasons, leaving 10 and 8 lungs available for stereological analysis in the control and bleomycin groups, respectively.

Table 1 Antibodies Used in the Study

Primary Ab	Company	Code	Technique	Dilution
PO ₄ Smad 2	Cell Signaling Technologies	3107	WB (mouse)	1:500*
Total Smad2	Cell Signaling Technologies	3108	WB (mouse)	1:1000 [†]
PO ₄ Smad 3	Cell Signaling Technologies	9520	WB (mouse)	1:500*
Total Smad 3	Cell Signaling Technologies	9513	WB (mouse)	1:1000*
PO ₄ Smad 1/5/8	Cell Signaling Technologies	95115	WB (mouse)	1:2000 [‡]
Total Smad 1/5/8	Cell Signaling Technologies	sc6031R	WB (mouse)	1:200 [‡]
Noggin	R&D Systems	AF719	WB (mouse)	1:400*
			IHC (mouse)	1:40 [§]
	Abcam	Ab16054	IHC (human)	1:200
BAMBI	R&D Systems	AF2387	WB (mouse)	1:200*
			IHC (mouse)	1:120 [§]
	R&D Systems	AF921	IHC (human)	1:25
Follistatin-like 1	R&D Systems	AF1738	WB (mouse)	1:500 [¶]
			IHC (mouse)	1:90
	R&D Systems	AF1694	IHC (human)	1:25
GAPDH	Cell Signaling Technologies	2118S	WB (mouse)	1:5000**

*Secondary antibody raised against rabbit, dilution 1:2000.

[†]Secondary antibody raised against mouse, dilution 1:2000.

[‡]Secondary antibody raised against rabbit, dilution 1:5000.

[§]Secondary antibody raised against rabbit, dilution 1:150.

[¶]Secondary antibody raised against goat, dilution 1:2000.

^{||}Secondary antibody raised against goat, dilution 1:150.

**Secondary antibody raised against rabbit, dilution 1:10,000.

Lung injury was quantified with stereological techniques to determine the volume of the alveolar wall, including all of the inflammatory cell infiltrate and fibrotic extracellular matrix within the gas exchange regions of the lungs, as recommended by the joint American Thoracic Society/European Respiratory Society policy statement on tissue quantification.¹⁴ After fixation, the vertical axis of the lung was identified and the lung was cut into 3-mm-thick slabs perpendicular to the vertical axis of the lung, with a random starting point chosen from within the first 3 mm of lung. This resulted in five to six slabs per left lung. These lung slabs were then embedded in paraffin wax, using the characteristic shape of each slab to maintain the vertical orientation. Sections (5 μ m thick) were cut from each slab until a series of complete transverse sections (ie, completely surrounded by pleural surface) had been obtained, and then one was stained with hematoxylin and eosin for histological assessment.

Multiple microscopic images of lung tissue were selected from within each section by using a systematic random selection strategy (Olympus BX61 motorized microscope with an $\times 20$ objective; Olympus, Albertslund, Denmark). These images were selected using the Computer-Assisted Stereological Tool system software (Visopharm Integrator System version 2.9.11.0, Olympus), which controls movement of the microscope stage so that the selection of the fields of view is independent of the observer. The number of fields of view taken from each section was proportional to the area of that section. The computer program superimposed a 6 $\times$ 6-point counting grid on each histological image. Because the position of the sections within the lung, the microscopic fields

of view, and the position of the counting grid were randomly selected, the probability of a point being superimposed on lung tissue or airspace was directly proportional to the fraction of the total volume of the lung occupied by that feature. The observer who counted the number of points falling on tissue or airspace was not informed of the treatment received by that lung. Determination of the fraction of the lung occupied by lung tissue (including all cells, extracellular matrix, and inflammatory cells) and the volume of each lung (measurement by water displacement) permitted calculation of the total tissue volume in that lung.

Additional sections were stained with picrosirius red to identify collagen. Sections (4 μ m) were deparaffinized in xylene, rehydrated, and treated with in 0.2% (v/v) aqueous phosphomolybdic acid. Sections were stained using a 0.1% (w/v) solution of Sirius Red (Sigma Aldrich, Dublin, Ireland) in saturated aqueous picric acid (Sigma Aldrich) for 90 minutes. Subsequently, sections were rinsed in 0.4% (v/v) HCl solution and were then dehydrated in ascending concentrations of ethanol, cleared with xylene, and coverslipped. Sections were viewed under polarized light, and 20 random images were acquired ($\times 10$ objective) from each lung, digitized, and analyzed using ImageJ software version 1.48v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). The signal in the green channel was segmented to allow measurement of the percentage of the area of each image that contained collagen, as identified by birefringence.

A second independent set of intratracheal inoculations of saline ($n = 8$) control or bleomycin ($n = 8$) was then completed, as previously described. Fourteen days after saline or bleomycin instillation, the left and right lungs were

Table 2 Applied Biosystems Gene Expression Assays

Gene	Symbol	Aliases	Assay ID
Transforming growth factor, beta 1	<i>Tgfb1</i>		Mm01178820_m1
Collagen, type I, alpha 1	<i>Col1a1</i>	<i>Cola-1, Mov-13</i>	Mm00801666_g1
Connective tissue growth factor	<i>Ctgf</i>	<i>Ccn2, Fisp12, Hcs24</i>	Mm01192932_g1
BMP-binding endothelial regulator	<i>Bmper</i>	<i>Cv-2, Crim3</i>	Mm01175806_m1
Chordin	<i>Chrd</i>	<i>Chd</i>	Mm00438203_m1
Gremlin 1	<i>Grem1</i>	<i>Cktsf1b1, Dm, Ld</i>	Mm00488615_s1
Matrix gla protein	<i>Mgp</i>	<i>Mglap</i>	Mm00485009_m1
Noggin	<i>Nog</i>		Mm01297833_s1
BMP and activin membrane-bound inhibitor	<i>Bambi</i>		Mm03053974_s1
Follistatin	<i>Fst</i>	<i>Fs</i>	Mm00514982_m1
Follistatin-like 1	<i>Fstl1</i>	<i>Fstl, TSC-36</i>	Mm00433371_m1
Follistatin-like 3	<i>Fstl3</i>	<i>Flrg</i>	Mm00473194_m1
Kielin/Chordin-like protein	<i>Kcp</i>	<i>Crim2</i>	Mm01159615_m1
Nephroblastoma overexpressed gene	<i>Nov</i>	<i>Ccn3</i>	Mm00456855_m1
Collagen, type I, alpha 1	<i>COL1A1</i>	<i>OI4</i>	Hs00164004_m1
Connective Tissue Growth Factor	<i>CTGF</i>	<i>CCN2, HCS24, IGFBP8, NOV2</i>	Hs01026927_g1
Noggin	<i>NOG</i>	<i>SYM1, SYNS1</i>	Hs00271352_s1
BMP and activin membrane bound inhibitor	<i>BAMBI</i>	<i>NMA</i>	Hs01026927_g1
Follistatin-like 1	<i>FSTL1</i>	<i>FRP, FSL1</i>	Hs00907496_m1

isolated post mortem and immediately snap-frozen. Protein was extracted from the left lungs for use in enzyme-linked immunosorbent assay and from corresponding right lungs for use in Western blot analysis.

Enzyme-Linked Immunosorbent Assay

Protein suitable for enzyme-linked immunosorbent assay was extracted from lung homogenized in phosphate-buffered saline with added protease inhibitors and used in commercially produced kits (R&D Systems, Abingdon, Oxfordshire, UK) to quantify Bmp2, activin, and TGF- β 1 concentrations.

Western Blot Analysis

Flash-frozen whole lung lysates were prepared in radio-immunoprecipitation assay buffer (Merck Millipore, Billerica, MA), homogenized by mechanical disruption (Tissue Ruptor; Qiagen, Hilden, Germany), and sonicated to ensure adequate lysis. Equal amounts of protein extract were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and detected with appropriate antibodies and secondary antibodies labeled with horseradish peroxidase (Table 1). After incubation with secondary antibody, proteins were visualized with an enhanced chemiluminescent signal kit (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Waltham, MA). An identical amount of protein from a single standard sample formed by pooling homogenate from lungs isolated from mice in the saline control group was loaded onto each gel to act as a loading and transfer control. Densitometric analysis was performed using ImageJ software in two stages: the density of the target band was normalized to the endogenous loading control (GAPDH) for that sample, and the resultant value was

normalized to the corresponding value for the pooled standard sample on that gel.

Analysis of Gene Expression

Total mRNA was extracted from tissue and cell pellets were snap-frozen in liquid nitrogen using the Qiagen RNeasy Mini-kit (Qiagen) according to the manufacturer's protocol. Total RNA (1 μ g) was reverse transcribed to cDNA using a SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed on 384-well plates, and each sample was measured in duplicate. An initial minus reverse transcriptase reaction confirmed that all RNA samples were free of contaminating genomic DNA. Probes were ordered as Assay-On-Demand Gene Expression Assays (Applied Biosystems, Foster City, CA) (Table 2). Reactions were performed on the Applied Biosystems PRISM 7900 Sequence Detection System (Applied Biosystems), and mRNA levels were determined using the comparative threshold method.¹⁵ We selected BMP accessory proteins previously reported to play a role in lung diseases, including gremlin 1, follistatin, noggin, BMP-binding endothelial regulator (BMPER), and BMP and activin membrane-bound inhibitor (BAMBI)^{1,16–18}; BMP accessory proteins implicated in lung organogenesis, including follistatin-like 1 (FSTL1), matrix GLA protein (MGP), and chordin in the bleomycin model of lung injury^{19–21}; and BMP accessory proteins known to be expressed in the normal lung: follistatin-like 3 (FSTL3), nephroblastoma overexpressed gene (NOV), and kielin/chordin-like protein (KCP).^{22,23}

Immunostaining

Immunohistochemical localization of BAMBI, FSTL1, and noggin was performed on paraffin-embedded lung sections

from wild-type mice sacrificed 14 days after instillation of bleomycin or saline, as previously described.²⁴ Briefly, 5-μm-thick sections were cut from the surface of each slice and mounted onto poly-L-lysine-coated glass slides (Sigma, St. Louis, MO). Slides were baked for 18 hours at 37°C, dewaxed, and hydrated. Endogenous peroxidase activity was blocked by exposure to hydrogen peroxide (0.3% v/v) in ethanol (70%). Antigen retrieval was undertaken for 20 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) at a controlled temperature (95°C) in a water bath. Slides were washed in phosphate-buffered saline and blocked in appropriate serum (10% diluted in phosphate-buffered saline) for 1 hour. Tissue sections were incubated in primary antibody (Table 1), diluted in phosphate-buffered saline, and left for 18 hours at 4°C in humidified Petri dishes. Appropriate biotin-labeled secondary antibodies were used to detect specific binding using streptavidin-linked horseradish peroxidase and diaminobenzidine (Sigma Aldrich). The slides were then counterstained with hematoxylin (Lennox Laboratory Supplies, Dublin, Ireland) before being examined microscopically on an Olympus BX61 microscope (×40 objective).

Human Lung Tissue

Human IPF tissue was collected from explanted lungs at the time of lung transplantation [$n = 6$, all male, mean age 59.6 years (range 41 to 68 years)]. Control lung tissue was obtained by taking tissue blocks from lobectomy samples removed for surgical excision of cancer at a site remote from the tumor ($n = 3$, anonymized data). The protocols were approved by the hospital ethics committee (Mater Misericordiae University Hospital, Dublin, Ireland), and all patients gave written informed consent. IPF was diagnosed according to the 2011 official American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association statement “Idiopathic Pulmonary Fibrosis:

Figure 1 Treatment with intratracheal bleomycin results in lung damage and fibrosis. **A:** Absolute right lung weights 14 days after intratracheal administration of bleomycin. **B:** Right lung weights relative to final body weight 14 days after intratracheal administration of bleomycin. **C:** Representative images of hematoxylin and eosin-stained lungs 14 days after intratracheal administration of bleomycin showing bronchocentric injury (black arrow), small patches adjacent to the plural surface (gray arrow), perivascular and peribronchial inflammatory cells, and significant alveolar injury with loss of airspace, thickening of alveolar walls, and intra-alveolar inflammatory cells in bleomycin-treated mice compared with in normal saline controls. Bottom two panels show polarized light images of picrosirius red staining. **D:** Stereological analysis of volume of lung tissue plus inflammatory cells 14 days after intratracheal administration of bleomycin. **E** and **F:** mRNA expression of the TGF-β-inducible genes *Col1a1* and *Ctgf* after bleomycin instillation. Data are expressed as medians (**A** and **B**) and medians ± interquartile range (**D**–**F**). mRNA values (**E** and **F**) are expressed as fold change relative to mean saline-inoculated control value. * $P < 0.05$ and ** $P < 0.01$, U -test. $n = 11$ per group (**A**, **B**, **E**, and **F**); $n = 10$ and 8 for control and bleomycin groups, respectively (**D**). TGF-β, transforming growth factor β.

Table 3 mRNA Expression of a Panel of BMP-Related Proteins in Wild-Type Mouse Lung Lysates, 14 Days after Intratracheal Instillation of Bleomycin

TGF- β superfamily ligand group	Accessory protein	Relative mRNA expression	
		Control	Bleomycin treated
BMP only	BMPER ^{29,30}	1.1 (0.6–1.4)	1.1 (0.7–1.3)
	Chordin ³¹	1.0 (0.8–1.2)	0.7 (0.5–1.1)*
	Gremlin 1 ³²	1.1 (0.9–1.2)	12.6 (4.0–15.0)**
	MGP ^{33,34}	1.0 (0.9–1.2)	1.3 (1.1–1.5)
	Noggin ³⁵	0.9 (0.8–1.4)	1.5 (1.3–2.5)*
BMP and TGF- β /activin	BAMBI ⁹	1.0 (0.5–1.8)	0.1 (0.1–0.3)*
	Follistatin ^{8,36,37}	1.1 (0.9–1.2)	6.5 (2.2–10.0)**
	Follistatin-like 1 ^{22,38,39}	1.0 (0.9–1.3)	1.7 (1.3–2.0)**
	Follistatin-like 3 ^{22,40}	1.0 (0.9–1.1)	0.9 (0.7–1.4)
	KCP ^{41–43}	1.0 (0.8–1.5)	0.5 (0.3–0.6)**
	NOV ⁴⁴	0.9 (0.8–1.2)	0.3 (0.2–0.4)**

Data are expressed as medians $\pm$ interquartile range. Values are displayed as fold change relative to mean saline-inoculated control value. $n = 11$ per group, except for NOV, where $n = 10$ for technical reasons.

* $P < 0.05$, ** $P < 0.01$, U -test.

Evidence-Based Guidelines for Diagnosis and Management.”²⁵ Human tissue slides were stained on the Automated Dako Autostainer Link 48 (DakoCytomation Ireland Ltd, Galway, Ireland) by using Dako antigen retrieval buffer at pH 6.0 and EnVision Flex and LSAB+ kits (DakoCytomation Ireland Ltd).

Cell Culture Experiments

Cell culture experiments were performed with primary human SAECs (Lonza, Basel, Switzerland) obtained from a 19-year-old male nonsmoker. The SAECs were maintained in SAEC growth medium (Lonza) at 37°C in 5% CO₂. All cells used in these experiments were between passages 5 and 7 and were routinely checked to confirm absence of *Mycoplasma* contamination by the MycoAlert assay (Cambrex Bioscience, Nottingham, UK). SAECs were seeded onto 12-well plates and grown until 80% confluency. The medium was changed, and the cells were either treated with 10 ng/mL TGF- β 1 (R&D Systems) in 4 mmol/L HCl containing 1% bovine serum albumin or treated with vehicle alone (sterile 4 mmol/L HCl containing 1% bovine serum albumin), as a control. After 72 hours of treatment, the cells were lysed and RNA extracted for further analysis, as described above in *Analysis of Gene Expression*. These conditions were chosen because they had previously been shown to lead to the expression of markers of epithelial-mesenchymal transition in these cells by RT-qPCR (cadherin 1, vimentin, and α -smooth muscle actin) and Western blot analysis with densitometry (cadherin 1, zona occludens 1, vimentin, and α -smooth muscle actin).²⁶

Statistical Analysis

Normally distributed data are reported as means $\pm$ SEM, whereas non-normally distributed data are presented as medians $\pm$ interquartile range. For normally distributed

data, statistical significance of differences between two group means was determined with t -tests. For non-normally distributed data, statistical significance was determined by using the U -Test (unpaired test); P values were computed using the exact permutation method. $P < 0.05$ was accepted as statistically significant.

Results

Treatment with Intratracheal Bleomycin Results in Lung Fibrosis

At the start of the study there was no significant difference in body weight between groups: median weight was 29.0 g (27.5 to 30.5 g) in the control group ($n = 11$) and 29.0 g (27.8 to 30.3 g) in the bleomycin-treated group ($n = 11$). Intratracheal bleomycin treatment led to significant weight loss ($-3.75 \text{ g} \pm 0.63 \text{ g}$) compared with saline-inoculated mice ($-0.45 \text{ g} \pm 0.36 \text{ g}$; $P < 0.01$). Two of the bleomycin-treated animals had to be sacrificed humanely before the 14-day period after instillation had elapsed because of excessive ($>20\%$) weight loss (on day 7 and day 11).

In the second group of mice used to investigate protein levels of BMP accessory proteins, all mice survived the course of the study, although the bleomycin-treated mice still had significant weight loss ($-3.80 \text{ g} \pm 0.73 \text{ g}$) compared with the saline-inoculated mice ($-0.25 \text{ g} \pm 0.18 \text{ g}$; $P < 0.001$). Median lung weight was significantly increased in animals treated with bleomycin compared with in those treated with saline, both when expressed as absolute lung weight and when lung weight was corrected for the final body weight (Figure 1, A and B). This was replicated in the later 14-day study.

Histological examination of the lungs (Figure 1C) demonstrated bronchocentric injury, small patches adjacent to the plural surface, perivascular and peribronchial

Figure 2 Treatment with intratracheal bleomycin results in changes in TGF- β 1 and BMP expression and signaling. **A:** Concentrations of BMP2, TGF- β , and activin proteins measured by enzyme-linked immunosorbent assay in lung lysates 14 days after intratracheal administration of bleomycin compared with in saline-inoculated controls. **B:** Representative Western blot showing PO₄ Smad2, total Smad2, and GAPDH expression and densitometry analysis of the ratio of PO₄Smad2 to total Smad2. **C:** Representative Western blot showing PO₄ Smad1/5/8, total Smad1/5/8, and GAPDH expression and densitometry analysis of the ratio of PO₄Smad1/5/8 to total Smad1/5/8. Data expressed as medians $\pm$ interquartile range (**B** and **C**). $n = 8$ per group (**A–C**). * $P < 0.05$, ** $P < 0.01$, U -test. B, bleomycin-treated; BMP, bone morphogenetic protein; C, saline control; PS, pooled sample; TGF- β , transforming growth factor β .

inflammatory cells, and significant alveolar injury with loss of airspace, thickening of alveolar walls, and intra-alveolar inflammatory cells in the bleomycin-treated mice compared with in the normal saline controls, which was consistent with fibrotic injury. Stereological analysis confirmed that the bleomycin-treated mice had an increase in the total volume of lung tissue, reflecting inflammatory cell infiltration and fibrotic matrix deposition (Figure 1D). Picrosirius red staining showed increased collagen in sections from bleomycin-inoculated lungs when compared with saline controls (Figure 1C). The mean area of collagen identified by birefringence in picrosirius red–stained sections was significantly ($P < 0.01$) elevated in the bleomycin-injured lungs ($3.84 \pm 0.51\%$, $n = 8$) when compared with saline-inoculated lungs ($3.00 \pm 0.45\%$, $n = 7$).

Collagen accumulation is a major feature of fibrosis, and one of the most important mediators of collagen synthesis is TGF- β 1.^{27,28} We therefore investigated the levels of expression of two TGF- β 1–regulated genes, *Colla1* and *Ctgf*, which are characteristically elevated in the bleomycin-induced pulmonary fibrosis model. *Colla1* and *Ctgf* were significantly increased in the bleomycin-treated mice (Figure 1, E and F), which was in keeping with an established profibrotic state. Taken together with

the histological findings, these results show a pattern typical of bleomycin-induced fibrosis.

The mRNA Expression of BMP-Related Signaling Molecules Is Altered in Fibrotic Lungs

To investigate the expression of BMP accessory proteins in fibrosis and TGF- β 1/BMP signaling, we examined the mRNA expression of a panel of extracellular BMP–related signaling molecules (Table 3). With regard to RNA of the BMP accessory proteins known to interact with BMP alone, gremlin 1 and noggin mRNA was significantly up-regulated after bleomycin treatment compared with saline-inoculated controls, chordin mRNA expression was significantly decreased in bleomycin-treated mice, and there was no significant difference in expression of *Bmper* and *Mgp* mRNA (Table 3). With regard to the RNA of the BMP accessory proteins that predominantly interact with BMP and TGF- β 1/activin, follistatin and *Fstl1* mRNA were significantly up-regulated in bleomycin-treated mice, whereas *Bambi*, *KCP*, and *Nov* mRNA were significantly down-regulated in bleomycin-treated mice. There was no change in *FSTL3* mRNA in the bleomycin-treated mice compared with in the saline controls.

Figure 3 Investigation of noggin protein levels in intratracheal bleomycin-treated lungs. **A:** Localization of noggin by immunohistochemical staining. Upper six microscopic images ($\times 40$ and $\times 60$ objectives) show noggin present in the epithelial cells of airways and alveolar macrophages in control and bleomycin-treated lungs and in the alveolar cells of bleomycin-treated animals. Bottom panels present concentration-matched nonimmune goat IgG showing absence of staining. **Blue arrow** indicates macrophage; **green arrow**, bronchial epithelium; **yellow arrow**, pneumocytes; and **red arrow**, fibroblasts. **B:** Representative Western blot showing expression of noggin protein in lungs 14 days after intratracheal administration of bleomycin compared with saline-inoculated controls. **C:** Densitometry analysis of noggin protein expression normalized to GAPDH. Data are expressed as medians $\pm$ interquartile range (**C**). $n = 8$ per group (**C**). $*P < 0.05$, U -test. B, bleomycin-treated; C, saline control; Obj, objective; PS, pooled sample.

The Protein Expression of BMP-Related Signaling Molecules Is Altered in Murine Fibrotic Lungs

To further investigate the BMP-related signaling molecules in fibrosis, we selected three targets for examination of protein changes: noggin, BAMBI, and FSTL1. In a separate series of experiments, we collected lungs from bleomycin-inoculated mice ($n = 8$) and saline-inoculated mice ($n = 8$) and extracted protein suitable for Western blot analysis and enzyme-linked immunosorbent assay. Protein levels of TGF- β 1 were significantly increased in the bleomycin-treated mice

compared with in the controls (Figure 2A). Concomitant with this, we found increased levels of activin, which is also implicated in fibrotic disease,⁴⁵ and reduced levels of BMP2 (Figure 2A). TGF- β 1 and BMP signal through a heteromeric cell surface serine/threonine kinase complex,⁴⁶ resulting in the receptor-mediated phosphorylation and activation of either Smad2/3 (TGF- β 1 and activin) or Smad1/5/8 (BMPs) transcription factors and alterations in gene transcription. Corresponding to increased levels of TGF β 1 and activin, we found that the ratio of phospho-Smad2 to total Smad2 was significantly increased in the bleomycin-treated lungs (Figure 2B).

Table 4 Comparison of Cellular Location of Noggin, BAMBI, and FSTL1 in Human Lung Specimens and Saline-Inoculated (Control) and Bleomycin-Inoculated Mouse Lungs

Cellular location	Noggin				BAMBI				FSTL1			
	Cont	IPF	C	Bleo	Cont	IPF	C	Bleo	Cont	IPF	C	Bleo
Macrophages	+	+	+	+	+	+	+	+	++	+	+	+
Bronchial epithelium	+	+	+	+	+	+	+	+	++	+	+	+
Endothelial cells	+	+	—	+	—	+	—	—	++	+	—	—
Smooth muscle	+	+	—	—	—	+	—	—	++	+	+	+
Pneumocytes*	—	+	+	++	—	+	+	—	—	+	+	+
Inflammatory cells	NA	+	NA	+	NA	+	NA	—	NA	+	NA	—
Fibroblasts	NA	+	NA	+	NA	+	NA	+/-	NA	+	NA	+/-
Honeycomb metaplastic epithelium	NA	+	NA	NA	NA	+	NA	NA	NA	+	NA	NA

*Type 2 pneumocyte hyperplasia in IPF and bleomycin-injured lungs.
+, present; —, absent; Bleo, bleomycin-inoculated mice; C, saline-inoculated control mice; Cont, control human lung tissue; IPF, lung tissue from IPF lungs; NA, not applicable.

However, there was no significant difference in the ratio of phospho-Smad3 to total Smad3 (data not shown). The ratio of phospho-Smad 1/5/8 to total Smad 1/5/8 was significantly reduced after bleomycin treatment (Figure 2C), which is consistent with reduced BMP signaling.

Noggin was detected in macrophages and bronchial epithelial cells of control and bleomycin-treated lungs, type II pneumocytes, inflammatory cells, and fibroblasts of bleomycin-treated lungs (Figure 3A, Table 4). In addition, noggin was detected in the vascular endothelium of the bleomycin-treated lungs (Supplemental Figure S1). There was no staining in specimens stained with nonimmune goat IgG instead of primary antibody (Figure 3A). Immunohistochemical analysis showed more intense staining of protein in bleomycin-treated lungs (Table 4). Western blot analysis detected a single band at the expected molecular weight of noggin (Figure 3B), which showed decreased levels of noggin in the bleomycin-treated lungs (Figure 3C).

BAMBI was localized in the macrophages and bronchial epithelium of control and bleomycin-treated lungs. Occasional fibroblasts positive for BAMBI were also seen in bleomycin-treated lungs (Figure 4A, Table 4). There was no staining in specimens when nonimmune goat IgG was substituted for primary antibody at the same concentration (Figure 4A). Western blot analysis visualized BAMBI as two bands, which is consistent with N-glycosylation (Figure 4B). The higher-molecular-weight band, which represents the glycosylated form of protein, was seen to be more highly expressed in bleomycin-treated mice (Figure 4C), whereas the lower molecular weight band was only detected in saline-inoculated mice.

FSTL1 was localized in the macrophages, bronchial epithelium, and smooth muscle cells of control and bleomycin-treated lungs, but in type II pneumocytes and occasional fibroblasts of just the bleomycin-treated lungs (Figure 5A, Table 4). There was no staining in sections in which the same concentration of nonimmune goat IgG was used instead of primary antibody (Figure 5A). Immunohistochemical analysis showed more intense

staining in bleomycin-treated lungs compared with in saline controls (Figure 5). FSTL1 is a 46-kDa protein that appeared as a doublet on immunoblot because of glycosylation (Figure 5B), which is consistent with previous reports.⁴⁷ Densitometry demonstrated a significant increase of expression of FSTL1 protein (both bands) in the bleomycin-treated lungs (Figure 5C).

Noggin, FSTL1, and BAMBI Are Expressed in Human Fibrotic Disease

To see whether alterations of BMP accessory proteins occur in humans with fibrotic lung disease, we used sections from patients with IPF (*n* = 6) and control lung (*n* = 3). Noggin, BAMBI, and FSTL1 were all present in macrophages, type II pneumocytes, endothelial cells, and smooth muscle cells of both healthy lungs and those for individuals with IPF (Table 4 and Figure 6). In addition, these proteins were also present in areas of fibroblastic focus and honeycomb metaplastic epithelium, which is characteristic of fibrotic lung disease.

TGFB1 Stimulation Increases the Expression of Noggin, FSTL1, and BAMBI mRNA Expression in Human SAECs

TGFB1 is known to regulate many BMP accessory proteins. This means that there may be a feedforward loop whereby increased BMP antagonism results in increased TGFB1 signaling, which in turn increases levels of BMP accessory proteins further. We therefore tested whether TGFB1 induced changes in the expression of noggin, FSTL1, and BAMBI by using human SAECs. To confirm TGF-β1–induced responses in the SAECs, we first measured mRNA expression levels of the TGF-β1–inducible genes *COL1A1* and *CTGF*, and demonstrated significant increases of both genes (twofold and threefold, respectively). Further measurement of the BMP accessory proteins noggin, FSTL1, and BAMBI (Figure 7), demonstrated that mRNA expression was

Figure 4 Investigation of BAMBI protein levels in intratracheal bleomycin-treated lungs. **A:** Localization of BAMBI by immunohistochemical staining. Upper six microscopic images (×40 and ×60 objectives) show BAMBI present in the epithelial cells of airways and alveolar macrophages in control and bleomycin-treated lungs and in the alveolar cells of bleomycin-treated animals. Bottom panels present concentration-matched nonimmune goat IgG showing absence of staining. **Blue arrow** indicates macrophage; **green arrow**, bronchial epithelium; **red arrow**, fibroblasts. **B:** Representative Western blot showing expression of BAMBI protein in lungs 14 days after intratracheal administration of bleomycin compared with saline-inoculated controls. **C:** Densitometry analysis of BAMBI protein expression normalized to GAPDH. Data are expressed as medians ± interquartile range (C). $n = 8$ per group (C). * $P < 0.05$, U -test. B, bleomycin-treated; C, saline control; Obj, objective; PS, pooled sample.

significantly increased for all three compared with in nontreated controls. These findings show that for human SAECS, increases in TGF- β 1 stimulate concomitant increases in expression of BMP accessory proteins.

Discussion

The TGF- β 1/BMP signaling cascade plays an important role in maintaining tissue homeostasis in the lung.

Dysregulation of this pathway, increased TGF- β 1 and activin signaling, leads to fibrosis through increased collagen production, epithelial-mesenchymal transition, and fibrotic laydown, whereas increased BMP signaling can reverse fibrotic change.^{1,48} There is significant crosstalk between the members of the TGF- β superfamily, and alterations in expression of BMP accessory proteins are emerging as an important component of pulmonary pathology.^{2,49} Although work has been performed to look at alterations in certain specific BMP

Figure 5 Investigation of levels of FSTL1 protein in intratracheal bleomycin-treated lungs. **A:** Localization of FSTL1 by immunohistochemical staining. Upper six microscopic images (40 $\times$ and 60 $\times$ objectives) show FSTL1 present in the epithelial cells of airways and alveolar macrophages in control and bleomycin-treated lungs, and in the alveolar cells of bleomycin-treated animals. Bottom panels: Concentration-matched nonimmune goat IgG showing absence of staining. **Blue arrow** indicates macrophage; **green arrow**, bronchial epithelium; **yellow arrow**, pneumocytes; **red arrow**, fibroblasts; **black arrow**, smooth muscle cells. **B:** Representative Western blot showing expression of FSTL1 protein in lungs 14 days after intratracheal bleomycin compared to saline inoculate controls. **C:** Densitometry analysis of FSTL1 protein expression normalized to GAPDH. Data are expressed as medians $\pm$ interquartile range (**C**). $n = 8$ per group (**C**). $*P < 0.05$, U -test. B, bleomycin treated; C, saline control; Obj, objective; PS, pooled sample.

accessory proteins in lung fibrosis,¹ this is the first study to examine the expression of multiple BMP accessory proteins during the fibrotic process.

Bleomycin-Induced Pulmonary Fibrosis

Bleomycin-treated mice were used as a model for fibrosis as they have previously been shown to model changes seen in human lung injury.¹⁰ The mice used in these experiments were confirmed to have an established profibrotic state as they had characteristics consistent with fibrosis: increased

weight and volume of lungs (Figure 1, A and B),^{50–52} bronchiocentric fibrotic change, thickening of alveolar walls, obliteration of airspace and inflammatory cell infiltrates (Figure 1C),⁵³ increased volume of lung tissue because of inflammatory cell infiltrates, fibrotic matrix deposition (Figure 1D), and increased collagen as identified by picrosirius red–induced birefringence. In addition, the bleomycin-inoculated lungs showed increased levels of TGF- β , increased phosphorylation of SMAD2, and increased mRNA expression of the TGF- β 1–inducible profibrotic genes *Colla1* and *Ctgf* (Figure 2),^{27,28} all of which are compatible with the

Figure 6 Immunohistochemical localization of bone morphogenetic protein accessory proteins in human lungs. Noggin, BAMBI, and FSTL1 are all present in macrophages and pneumocytes in the normal lung. In IPF lungs, all three proteins are present in macrophages, type II pneumocytes, inflammatory aggregates, and metaplastic epithelia. **Black arrow** indicates macrophage; **long arrow**, alveolar macrophages; **star**, bronchial epithelium; **arrowhead**, endothelial cells; **red arrow**, fibroblasts of the fibroblastic focus; **short, thick black arrow**, overlying type II pneumocyte hyperplasia; **blue arrow**, indicates inflammatory aggregate. All images were acquired on a $\times 20$ objective. Scale bar = 200 μm . E, metaplastic epithelium in honeycomb areas; IPF, idiopathic pulmonary fibrosis; V, vessel.

increased TGF β signaling typically observed during the development of lung fibrosis.

Altered BMP Accessory Protein Expression in Fibrotic Lungs

Initial investigation of BMP accessory proteins found alterations in the mRNA expression of multiple genes in the fibrotic lungs: Gremlin 1, *Noggin*, Follistatin, and *Fstl1* were all significantly up-regulated; whereas Chordin, *Kcp*, *Nov*, and *Bambi* were significantly down-regulated (Table 3). Although levels of mRNA expression can give some indication as to changes occurring during fibrosis, post-translational modifications are an important mechanism regulating expression of many proteins. We therefore looked in greater detail at the protein expression of three targets: noggin, BAMBI, and FSTL1. These BMP accessory proteins were chosen because this was the first time they had been examined in a model of fibrotic lung disease, although the expression of each had been implicated previously in fibrosis in other organs.^{54,55} Noggin is an example of a protein acting solely as a BMP-binding protein; it inhibits the actions of BMP2, 4, and 7.^{35,56} In addition to binding BMP2 and 4, BAMBI and FSTL1 also affected TGF- β 1/activin binding^{9,39} and had opposing

changes in mRNA expression, decreased and increased, respectively.

Alterations in Expression of Noggin Protein

In the bleomycin mouse model, noggin staining was more extensive in immunohistochemical analysis, which is consistent with increased mRNA expression (Figure 3A); however, Western blot analysis suggested that the expression was decreased in bleomycin-treated mice compared with in controls (Figure 3C). This difference between the immunohistochemical and Western blot analyses may be explained by a number of potential mechanisms. First, apparent changes in intensity on immunohistochemical analysis are often difficult to judge unless they are very large (presence or absence), so this technique primarily gives information about cellular location rather than concentration of a protein. Second, increased expression in specific subpopulations of cells within the lung might lead to increased intensity of staining in those cells. However, Western blot analysis provides an index of the concentration averaged throughout all cells within the lung. Thus, noggin is diluted by protein extracted from cells that do not express it and by extracellular soluble proteins. If other such soluble proteins were increased in the fibrotic lung, because of either proliferation or infiltration, the

Figure 7 mRNA expression of noggin, BAMBI, and FSTL1 in SAEs stimulated with transforming growth factor β (TGF- β) or vehicle. Data are expressed as medians $\pm$ interquartile range. Values are normalized to 18s rRNA and expressed as fold change relative to vehicle-treated controls. $n = 12$ per group. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated SAEC control (U -test). SAEC, small airway epithelial cell; TGF- β , transforming growth factor β .

effect would be to reduce noggin levels detected by Western blot analysis. A second possibility is selective loss of noggin during protein extraction for Western blot analysis. For example, this might occur because of very avid binding of noggin to the cellular glycocalyx on cell surfaces.

Noggin is glycosylated, and matrix binding is a property of such proteins. It has been shown previously that noggin binds to the glycocalyx and that this bound protein is not effectively extracted by standard tissue lysis techniques. Moreover, noggin bound to the glycocalyx remains an effective blocker of BMP signaling.⁵⁷ Noggin bound to the cellular glycocalyx in such a manner would be stained in fixed sections and would appear in a cellular distribution.

Finally, nonspecific sticking of the noggin antibody to the tissue section must always be considered in these circumstances. However, nonimmune immunoglobulin of the same type that was prepared from the same species did not produce such nonspecific staining. Furthermore, we used the same anti-noggin antibody in both Western blot and immunohistochemical analysis, and it identified a single band at the correct molecular weight. Further work will be required to determine the exact modulating role of noggin in BMP signaling in pulmonary fibrosis and thus in influencing the abnormal TGF- β 1–BMP balance observed in the fibrotic process.

Of note, our findings in human SAEs demonstrate that TGF- β 1 drives an increase in noggin that could inhibit BMP and potentiate the profibrotic action of TGF- β 1. This represents a feedforward mechanism that could drive fibrosis. Noggin acts as a BMP antagonist by binding directly to BMP molecules, preventing BMP from binding to the BMP receptor.⁵⁶ This leads to a reduction in signaling through the Smad1/5/8 pathway and to a concomitant increase in Smad2/3 pathway. Any local increase in noggin protein in the bleomycin-treated lungs may therefore repress the BMP signaling pathway and boost the TGF- β 1 signaling pathway, thus increasing fibrosis. Moreover, the finding in SAEs that noggin mRNA expression was increased by stimulation with TGF- β 1 (Figure 7) suggests that fibrosis will be further enhanced, favoring maladaptive fibrotic healing rather than repair.

Alterations in BAMBI Protein Expression

In the bleomycin mouse model, BAMBI protein was more highly expressed, as demonstrated by both

immunohistochemical and Western blot analysis (Figure 4), in contrast to the lower levels of mRNA expression. A similar opposing finding has been seen in liver cells, in which low levels of mRNA expression correlated with high protein levels in primary human hepatocytes.⁵⁵ On the Western blot there were two bands: the heavier band was present in both the control and bleomycin-treated mice, although it was more highly expressed in the bleomycin-treated mice. The smaller band was present only in the control mice. It is possible that BAMBI protein becomes glycosylated and membrane bound during fibrotic lung injury, causing the protein to become more stable, with less degradation. So although mRNA expression decreases, and there is less protein production and the overall level of stable glycosylated protein is increased by post-translational modifications. This may be due to the effects of post-translational glycosylation, as the heavier, more glycosylated form of BAMBI had higher expression in the bleomycin-treated mice (Figure 4C), and likewise in the liver, primary human hepatocytes produced a glycosylated form of BAMBI.⁵⁵ BAMBI is a pseudoreceptor, with an extracellular domain similar to that of type I TGF- β 1 receptors but no intracellular kinase domain. It acts as a decoy receptor and has been reported to antagonize both the BMP and TGF- β 1/activin signaling pathways by preventing the formation of active receptor complexes on binding.⁹ In cardiac and hepatic cells, BAMBI protects against fibrosis through blocking of TGF- β signaling.^{55,58} This suggests that the increased levels of BAMBI protein found in both the bleomycin mouse model and IPF patients may be a protective measure to block excessive TGF- β 1 signaling and lessen pathological fibrosis.

Alterations in FSTL1 Protein Expression

In the bleomycin mouse model, FSTL1 protein was up-regulated, as seen by immunohistochemical analysis and Western blot analysis consistent with the mRNA expression (Figure 4). During the course of preparation of this manuscript, another study that also reports increased *Fstl1* expression in bleomycin-treated lungs was published.⁵⁹ FSTL1 has been shown to act by directly binding to BMP, preventing binding to its receptor complex, and thereby blocking downstream SMAD signaling.³⁸ In addition, FSTL1 has been shown to bind to TGF- β 1/activin complex.³⁹ Our study reinforces the finding that FSTL1 likely plays an important role in lung

fibrosis, with increases in the protein helping to promote the fibrotic phenotype.

In the human lung tissue, noggin, BAMBI, and FSTL1 were all detected in fibrotic areas (Figure 6). There is considerable concordance in the cellular distribution of these BMP antagonists in human and mouse lungs. Macrophages and bronchial epithelium express all three glycoproteins in both species, as do the alveolar pneumocytes (Table 4). Furthermore, in both humans and mice, fibroblasts express these three BMP antagonists in fibrotic conditions. In contrast, within the pulmonary vascular cells, only noggin is expressed in both human and mouse endothelium, and in smooth muscle cells only FSTL1 is expressed in both (Table 4). As is well known, the bleomycin model does not reproduce the human disease, and thus no honeycombing or fully developed fibroblastic foci are seen in the mouse lungs. Nonetheless, the extensive concordance observed suggests that the bleomycin model will provide a useful tool to further explore the role of these glycoproteins in fibrotic lung diseases.

TGF- β 1—Induced Changes in Expression of BMP Accessory Proteins

All three of these proteins are glycoproteins, so it is likely that upon glycosylation they will stick to the cellular glyocalyx close to the site of production and act in a local context with specific functions. Thus, the exact role of these secreted proteins needs to be explored separately in cell-specific contexts. Because all three proteins were highly expressed in epithelial cells, we used SAECs to look for regulatory feedback loops that may occur in this cell type and gain greater insight into whether it is only the initial injury that causes increased expression of these proteins or whether TGF- β 1 also plays a role. In this study, we found that TGF- β 1 stimulation increased the expression of noggin, BAMBI, and FSTL1, suggesting that after injury, TGF- β 1 release can further drive the fibrotic phenotype in this cell-specific context.

Potential Influence of Sex in Pulmonary Fibrosis

It is important to note that we used male mice in all experiments and that the explanted lung tissue came from male patients. There are sex-based differences in the severity of pulmonary fibrosis in the bleomycin mouse model, with more severe lung disease developing in male mice.⁶⁰ Therefore, when designing our experiments, we used only male mice so that any differences seen would be due to the effects of bleomycin and not confounded by a sex-based effect. Furthermore, IPF is much more common in males than in females, which is why all our human lung samples are from male lungs. In view of this, further work will be required to determine whether the same changes in BMP antagonists are observed in lung fibrosis in females; caution must be exercised before extrapolating these results directly.

Conclusions

The work presented in this study reveals for the first time that there is altered expression of a number of BMP accessory proteins in the murine model of lung injury. We also provide evidence that, for some of these proteins, post-translational mechanisms also play an important role in regulating expression. Clearly this may also be the case for other proteins in the family that we have not examined. Our results demonstrate that to fully understand the mechanisms underlying the altered TGF- β 1—BMP balance, which plays such an important role in pulmonary fibrosis, the changes in the BMP/TGF- β accessory proteins will need to be fully elucidated. Disturbances in these accessory proteins are likely to play an important role in the pathogenesis of IPF.

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Supplemental Data

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