Reemergence of Hedgehog Mediates Epithelial–Mesenchymal Crosstalk in Pulmonary Fibrosis

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Abstract

Hedgehog signaling plays important roles in cell development and differentiation. In this study, the ability of Sonic Hedgehog (SHH) to induce myofibroblast differentiation was analyzed in isolated human lung fibroblasts, and its *in vivo* significance was evaluated in rodent bleomycin-induced pulmonary fibrosis. The results showed that SHH could induce myofibroblast differentiation in human lung fibroblasts in a Smo- and Gli1-dependent manner. Gel shift analysis, chromatin immunoprecipitation assay, and site-directed mutagenesis revealed that a Gli1 binding consensus in the α -SMA gene promoter was important for mediating SHH-induced myofibroblast differentiation. Analysis of Hedgehog reemergence *in vivo* revealed that of all three Hedgehog isoforms, only SHH was significantly induced in bleomycin-injured lung along with Gli1. The induction of SHH was only noted in epithelial cells, and its expression was undetectable in lung fibroblasts or macrophages. Transforming growth factor (TGF)- β induced SHH significantly in cultured alveolar epithelial cells, whereas SHH induced TGF- β in lung fibroblasts. Pulmonary fibrosis and α -smooth muscle actin (α -SMA) expression were significantly reduced in mice that were

Smo deficient only in type I collagen-expressing cells. Thus, the reemergence of SHH in epithelial cells could result in induction of myofibroblast differentiation in a Smo-dependent manner and subsequent Gli1 activation of the α -SMA promoter.

Keywords: Hedgehog; myofibroblast; fibrosis

Clinical Relevance

The study reports on the importance of the reemergence or reactivation of Hedgehog signaling in pulmonary fibrosis in the context of epithelial–mesenchymal crosstalk. The study also reveals direct action of Sonic Hedgehog (SHH) in regulation of the human α -smooth muscle actin gene, an essential marker of myofibroblast differentiation. The findings using human lung fibroblasts from patients with idiopathic pulmonary fibrosis suggest the relevance of SHH to the pathogenesis of idiopathic pulmonary fibrosis and/or its exacerbation.

Pulmonary fibrosis is often the end result of multiple lung diseases with diverse etiologies but can also be idiopathic (1, 2). It is typically characterized by increased

interstitial wall thickening, inflammatory cell infiltration, increased numbers of fibroblasts, and abnormal deposition of extracellular matrix terminating with severe

distortion of lung architecture (2). The regulatory mechanism of this tissue remodeling process remains unclear. Accumulating evidence suggests that

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fibroblasts are recruited to sites of injury and differentiate to a myofibroblast phenotype, which is important for the initiation and propagation of fibrosis (3, 4). *De novo* expression of α -smooth muscle actin (α -SMA) characterizes this myofibroblast differentiation (4).

Hedgehog signaling plays critical roles in cell development and differentiation and in cancer (5). Three ligands of the Hedgehog signaling pathway—Sonic Hedgehog (SHH) (6), Desert Hedgehog (DHH) (7), and Indian Hedgehog (IHH) (8)—have been identified; these ligands share $\sim 60\%$ amino acid identity (9). In the absence of cognate ligands, the Hedgehog receptor Patched homolog 1 (Ptch-1) prevents activation of the Hedgehog pathway by inhibition of the coreceptor Smoothened (Smo) (10). This inhibition of Smo is released upon binding of the Hedgehog ligand to Ptch-1 (10), resulting in stabilization of Gli transcription factors such as Gli1 (11), which activates target genes through binding to their Gli-binding consensus sequence (GACCACCCA) (12). SHH is synthesized in precursor form and autocatalytically cleaved into a nonglycosylated N-terminal fragment (SHH-N) by a protease in the glycosylated C-terminal fragment (SHH-C) (13, 14). In addition, SHH-C catalyzes the cholesterylation of SHH-N at its C terminal (15). The SHH-N retains all known signaling capabilities, whereas SHH-C has no known signaling activity. Further modification of SHH-N includes addition of palmitate to the α -amino group of the N-terminal cysteine residue during transit through the endoplasmic reticulum (16, 17). The cholesterylation contributes to membrane tethering and intercellular transport, whereas the palmitoylation significantly enhances its cell signaling activity (18).

In most adult tissues, Hedgehog signaling is not activated. However, recent studies indicate that it may be reactivated in tissue remodeling or fibrosis (19–21). Reactivation of Hedgehog has been reported in lungs of patients with idiopathic pulmonary fibrosis (IPF) (21–25), in animals with hyperoxic lung injury (26), in fibrotic skin of patients with scleroderma (27), in human nonalcoholic fatty liver (28), and in animal models of liver fibrosis (29, 30). Recent animal model studies suggest that Hedgehog signaling may play a role

in fibrosis by promoting myofibroblast differentiation (27, 31), but studies in human lung cells have not demonstrated such activity (23, 24). Moreover, although Smo inhibitors have been shown to suppress fibrosis in the liver and skin (27, 30), another study failed to show an effect on pulmonary fibrosis using antibodies to neutralize all Hedgehog ligands, although overexpression of SHH enhances collagen expression (32). A more recent study suggests the importance of Smo in α -SMA-expressing cells in an animal model of liver fibrosis (33), but it remains unclear if Smo is important in the differentiation process. Thus, there is conflicting evidence as to the precise role of Hedgehog signaling in fibrosis and myofibroblast differentiation.

To address this issue, the ability of SHH to induce myofibroblast differentiation in human lung fibroblasts was examined in this study. The results showed that SHH could induce Smo-dependent myofibroblast differentiation by direct regulation of α -SMA gene expression via Gli1 binding to a cognate binding consensus sequence present in the α -SMA promoter. To confirm *in vivo* significance, expression of Hedgehog ligands and the role of Hedgehog signaling were evaluated in a bleomycin-induced model of pulmonary fibrosis. The results showed significant induction of lung SHH expression in bleomycin-treated animals, primarily in epithelial cells. SHH expression was undetectable in fibroblasts. Transforming growth factor (TGF)- β was shown to induce SHH in human lung epithelial cells, whereas SHH induced TGF- β in fibroblasts. Furthermore, conditional deficiency of Smo in type I collagen-expressing cells significantly suppressed fibrosis and myofibroblast differentiation. These results confirm the reemergence of Hedgehog signaling in pulmonary fibrosis, and its importance in fibrosis appears to derive in part from its ability to induce myofibroblast differentiation. Moreover, these results suggest the potential importance of epithelial–mesenchymal crosstalk with a positive feedback loop mediated by epithelial-derived SHH and fibroblast/myofibroblast-derived TGF- β .

Materials and Methods

Ethics

The use of animals was conducted in accordance with the National Institutes of

Health guidelines for Survival Rodent Surgery, the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals, 7th edition. The study was reviewed and approved by the University of Michigan Institutional Biosafety Committee and the University Committee on Use and Care of Animals. Use of human tissues was approved by the Institutional Review Board at the University of Minnesota.

Animals

Mesenchymal cell-specific Smo-deficient mice (*smo* CKO mice) with genotype [*smo*^{fl/fl}, *Col1a2-CreER(T)*^{+/0}] were generated by crossing floxed *smo* mice [*smo*^{fl/fl}] (34) with mice carrying a tamoxifen-inducible Cre recombinase [*Cre-ER(T)*] under the control of a regulatory sequence from the α 2(I) collagen gene [*Col1a2-Cre-ER(T)*^{+/0}] (35). Both parental strains had a C57BL/6 background.

Cell Culture and Treatments

Primary culture-derived lung fibroblasts from patients with IPF or control subjects were isolated and cultured as described previously (36). Mouse lung fibroblasts were isolated from lung tissue samples as described previously (37, 38). Mouse alveolar epithelial type II cells (AECII) were isolated by sorting for GFP-positive cells as previously described (39, 40). Human small airway epithelial cells (PCS-301-010) were purchased from American Type Culture Collection (Manassas, VA).

Plasmids and Constructs

The human α -SMA promoter from $-1,698$ to $+87$ relative to transcription start site was amplified by PCR and inserted into vector pGL420 to form pGL420- α SMAp-luc. Site-directed mutagenesis was conducted to generate the Gli binding site mutated human α -SMA gene promoter mutant pGL420- α SMAp-GBEm (GBEm) or the nonspecific control mutant pGL420- α SMAp-Controlm (Controlm). The human *gli1* cDNA, the lentivirus-based shRNA, and the negative control shRNA constructs were purchased from Thermo Fisher Scientific Inc. (Huntsville, AL).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed as described previously (38). Double-stranded wild-type (WT)

Figure 1. Effects of Sonic Hedgehog (SHH) on Gli1 and α -smooth muscle actin (SMA) expression. (A) Human lung fibroblasts isolated from control subjects ($n = 5$) or patients with idiopathic pulmonary fibrosis (IPF) ($n = 5$) were treated with the indicated doses of SHH and then analyzed for Gli1 mRNA levels by quantitative PCR (qPCR). *Significant difference from corresponding untreated control subjects (SHH dose = 0). †Significant difference between the two indicated groups. (B) IPF lung fibroblasts were transfected with Gli1 expression construct (Gli1 cDNA), control empty vector (Vector), Gli1 shRNA expression construct (Gli1 shRNA), or control shRNA (Control shRNA) and then treated with buffer only (None) or with SHH (500 ng/ml). Analysis for α -SMA mRNA by qPCR is shown. *Significant difference from corresponding vector control. (C) The same cells were treated as in (B), and α -SMA and Gli1 proteins were analyzed by Western blotting. (D) The blots in (C) were scanned, and the quantitative results are shown here. *Significant difference from the corresponding control group without Gli cDNA (left panel) or shRNA (right panel) treatment. (E) The human α -SMA gene promoter construct pGL420-h α SMAp-luc was cotransfected with the Gli1 expression construct (Gli1 cDNA), control empty vector (Vector), Gli1 shRNA expression construct (Gli1 shRNA), or control shRNA (Control shRNA) into IPF lung fibroblasts, and the effect on luciferase activity was analyzed as a measure of promoter activity. The luciferase activity was normalized to its respective Renilla luciferase control activity, and the results were expressed as relative light units. *Statistically significant difference when compared with the corresponding vector control group. (F) The IPF cells were transfected with the wild type α -SMA promoter construct as in (E) and then treated with the indicated agents (IPI is the Smo inhibitor IPI-926) to analyze their effects on α -SMA promoter activity measured as in (E). *Significant difference from the untreated (Buffer) group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

oligonucleotide probes corresponding to the region between -88 and -113 in the human α -SMA gene promoter containing the Gli binding element or the Gli binding element mutated control (GBEm) were synthesized by Integrated DNA Technology, Inc. (Coralville, IA).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation was performed using a kit from Millipore Co. (Billerica, MA) following the manufacturer's protocol as previously described (38). Primers designed to amplify the sequence from -243 to +10 flanking the Gli binding consensus in the human α -SMA gene promoter were used in PCR.

Histopathology

Formalin-fixed, paraffin-embedded lung tissue sections were stained with Masson's trichrome 21 days after bleomycin treatment as described previously (41).

Hydroxyproline Assay

As a measure of fibrosis, total lung hydroxyproline content was determined by colorimetric assay after acid hydrolysis as described previously (41). Results were expressed as total hydroxyproline content per lung.

Flow Cytometry Analysis

Flow cytometry analysis was performed on whole lung cell suspensions as described previously (37). Data collected were analyzed using FlowJo software (Tree Star Inc., Ashland, OR), and results are expressed as mean $\pm$ SE from triplicates.

Statistical Analysis

Statistical analysis was undertaken with ANOVA followed by *post hoc* testing using Scheffé's test as described previously (37, 41). qPCR, Western blot analysis, and detailed methods can be found in the online supplement.

Results

SHH Stimulated Myofibroblast Differentiation

The functional role of the reemergence of SHH and Hedgehog signaling in pulmonary fibrosis is unclear, especially *vis à vis* its potential role in myofibroblast differentiation (23, 24, 27). To address this

Figure 2. Binding of Gli1 to the α -SMA gene and its effect on promoter activity. (A) A ^{32} P-labeled, double-stranded wild-type (WT) oligo DNA probe spanning the Gli1 binding consensus in the human α -SMA gene promoter or a Gli binding consensus mutated oligo DNA probe (GBEm) were incubated with nuclear extracts of control (CON) or IPF lung fibroblasts in an electrophoretic mobility shift assay. Where indicated, 100-fold excess cold probe, anti-Gli1 antibodies, or IgG control (IgG) were incubated with the nuclear extracts, respectively, before adding the labeled probe. A single specific DNA-protein complex is noted. (B) The same cells as in (A) were fixed with formaldehyde and

issue, the effects of SHH on the expression of Gli1 were evaluated in cultured human lung fibroblasts isolated from control subjects and patients with IPF, given its role as the downstream trans-acting factor for activation of SHH target gene transcription (42). The results confirmed the expected SHH induction of Gli1 expression in control and IPF lung fibroblasts, although the response was significantly greater in the latter (Figure 1A). Further analysis revealed that SHH caused significant induction of α -SMA expression at mRNA and protein levels (Figures 1B–1D) and myofibroblast differentiation with α -SMA-positive stress fibers (see Figure E1 in the online supplement). This effect was enhanced by Gli1 overexpression by transfection with an expression plasmid but was almost dramatically suppressed by Gli1 shRNA. To confirm that Gli1 regulated α -SMA gene expression at the transcriptional level, the Gli1 expression plasmid or shRNA were cotransfected with the α -SMA promoter–luciferase fusion plasmid into the fibroblasts. Consistent with the mRNA and protein data (Figures 1B and 1C), the α -SMA promoter activity was stimulated significantly by SHH, which was enhanced by overexpression of Gli1 but suppressed by the Gli1 shRNA (Figure 1E). Finally, the stimulatory effect of SHH on α -SMA promoter activity was significantly inhibited by the Smo inhibitor IPI-926 (Figure 1F). Thus, SHH could induce myofibroblast differentiation in human lung fibroblasts by inducing α -SMA gene transcription in a Smo- and Gli1-dependent manner.

Gli1 Directly Regulated the α -SMA Promoter via a Binding Consensus

To further evaluate if SHH-induced myofibroblast differentiation is dependent on direct Gli1 interaction with the α -SMA promoter, its sequence was scanned for the presence of GACCACCCA, the Gli1 binding consensus sequence (12). This resulted in identification of a homologous Gli binding consensus in the upstream

region of the human α -SMA gene promoter between –104 and –96 from the transcriptional start site, which is conserved in the rat and mouse (Figure E2). To confirm Gli1 binding to this Gli binding consensus found in the α -SMA gene promoter, electrophoresis mobility shift assay was performed with a double-stranded oligo DNA probe spanning the Gli binding consensus region in the human α -SMA promoter and human lung fibroblast nuclear extracts isolated from patients with IPF or control subjects. The results showed a major shifted band of the same mobility when either control or IPF fibroblast samples were incubated with the probe, indicative of a protein–DNA complex (Figure 2A). Incubation with 100 $\times$ excess of unlabeled DNA probe reduced band intensity markedly, indicating specificity of binding. Moreover, preincubation of the nuclear extracts with anti-Gli1 antibodies also caused significant reduction of band intensity but not with nonspecific control IgG. No shifted band was observed when the labeled probe was mutated, as indicated in the Gli binding consensus (Figure 2C, upper panel). To further confirm Gli1 binding to the Gli binding consensus in the α -SMA gene promoter in intact cells, chromatin immunoprecipitation assay was performed with anti-Gli1 antibody and formaldehyde-fixed human lung fibroblast cell lysates. The results using control and IPF lung fibroblasts showed that the anti-Gli1 antibody precipitated a complex that, upon PCR amplification of the bound DNA and agarose gel electrophoresis, yielded a band $\sim$ 253 base pair (bp) in size (Figure 2B). Binding specificity of the antibody was confirmed by the absence of a band in samples incubated with nonimmune IgG. These findings confirmed that Gli1 could bind to the Gli binding consensus in the α -SMA promoter of human lung fibroblasts.

To ascertain that this binding of Gli to its cognate binding consensus in the α -SMA promoter was functionally important, site-directed mutagenesis was conducted to

mutate the Gli binding consensus or at a control site in the human α -SMA gene promoter. The WT and mutant α -SMA promoters were then inserted into promoterless luciferase reporter vector pGL420 to drive the expression of luciferase (Figure 2C, upper panel). After transfection into human lung fibroblasts, the promoter activity of these constructs was evaluated by luciferase assay after treating the cells without or with SHH. The results showed the expected activation of promoter activity by SHH of the WT construct, but mutation of the Gli binding consensus caused a $> 50\%$ decrease in α -SMA gene promoter activity even in cells treated with SHH (Figure 2C, lower panel). This was not unexpected because mutation at this site also inhibited Gli1 binding (Figure 2A). However, SHH was still able to stimulate the mutant construct, indicating that the Gli1 effect was additive to other factor(s) regulating this promoter. An unrelated mutation outside the Gli binding consensus had little effect on α -SMA gene promoter activity. Thus, Gli1 binding to its binding consensus played a significant role in the direct regulation of α -SMA gene expression, a marker of myofibroblast differentiation. Taken together, these *in vitro* findings indicated the ability of SHH to induce myofibroblast differentiation in a Smo- and Gli1-dependent manner. To further determine if such a mechanism would be important *in vivo*, the role of SHH in the bleomycin-induced model of pulmonary fibrosis was evaluated.

Reemergence of Hedgehog in Bleomycin-Injured Lung

Hedgehog signaling plays essential roles in lung development but can be reactivated in certain adult tissues (19, 20). To investigate if this also occurred in bleomycin-induced pulmonary fibrosis, murine lung tissue samples at Day 14 after bleomycin injury were analyzed for expression of Hedgehog ligands by quantitative PCR (qPCR). The results showed that SHH and IHH were the predominant isoforms expressed in the

Figure 2. (Continued). analyzed with a chromatin immunoprecipitation assay using anti-Gli1 antibodies or control IgG (IgG). The precipitated DNA and the unfractonated DNA (Input DNA) were analyzed by PCR using primers spanning the Gli1 binding consensus in the human α -SMA gene promoter region. The PCR products were then separated in a 1.3% agarose gel. Lad, DNA ladder. (C) *Upper panel:* Diagram showing the relative position to transcriptional start site (TSS) of the partial sequence of the WT Gli binding consensus mutant (GBEm) and control mutant (ConM) in the human α -SMA promoter constructs as indicated. After transfection, the promoter activity of these constructs was analyzed in IPF cells with or without SHH (500 ng/ml) treatment. *Statistically significant difference when compared with the respective WT promoter activity.

Figure 3. Expression of Hedgehog (HH) ligands in the lung. (A) RNA samples from saline (SAL)-injured or bleomycin (BLM)-injured mouse lungs ($n = 5$) were analyzed for the Hedgehog ligands SHH, Desert Hedgehog (DHH), and Indian Hedgehog (IHH) by qPCR after saline/bleomycin instillation 14 days after treatment. *Statistically significant difference versus the respective saline-treated group. (B) Protein samples from saline- or bleomycin-injured mouse lungs were analyzed for the Hedgehog ligand SHH by Western blotting. (C) Lung airway epithelial cells (AECs) from saline- or bleomycin-treated surfactant protein C–green fluorescent protein (SPC–GFP) mice were isolated by sorting for GFP. The cells were then analyzed by qPCR for SHH mRNA. *Significant difference from the saline group. (D) Lung AECs from saline- or bleomycin-treated SPC–GFP mice were isolated 14 days after treatment by sorting for GFP and analyzed by Western blot for SHH. The membrane was reblotted for GAPDH as loading control. (E) Human small AECs were treated with 4 ng/ml of TGF- β , 10 ng/ml IL-1 β , or 10 ng/ml TNF- α , and analyzed for SHH mRNA by qPCR. *Significant difference from respective untreated control. (F) Lung fibroblasts were treated as indicated and analyzed for TGF- β and Smad3 mRNA by qPCR.

adult murine lung, whereas DHH appeared to be a minor isoform (Figure 3A). Neither IHH nor DHH was significantly induced by bleomycin treatment, which was in contrast to the significant induction of SHH gene expression in bleomycin-treated mice. The

elevated SHH expression induced by bleomycin treatment was further confirmed at the protein level by Western blot analysis of whole lung tissue protein extracts (Figure 3B). The source of the reemergence of SHH was found to be in alveolar

epithelial cells, as evidenced by their induction in isolated AECII from bleomycin-treated mice (Figures 3C and 3D) but not in lung fibroblasts or alveolar macrophages (data not shown). SHH was also detected in human small airway epithelial cells. Of several known fibrogenic cytokines tested, TGF- β had the greatest effect on SHH expression (Figure 3E). IL-1 β and TNF- α had smaller effects but were statistically significant (Figure 3E). An apparent source of TGF- β for this induction of SHH in epithelial cells was identified in lung fibroblasts, which, in addition to undergoing myofibroblast differentiation in response to SHH, were found to significantly up-regulate TGF- β expression without significantly affecting Smad3 expression (Figure 3F). These findings suggest a potential crosstalk between epithelial cells and fibroblasts/myofibroblasts involving induced SHH and TGF- β in a positive feedback loop, which could result in amplification/propagation of the fibrogenic response. Moreover, these results suggest a role for SHH via activation of Smo- and Gli1-dependent myofibroblast differentiation in fibrosis. To evaluate this possibility, the effect of mesenchymal cell-specific Smo deficiency on fibrosis was analyzed using the bleomycin model.

Effects of Smo Deficiency on Bleomycin-Induced Pulmonary Fibrosis

To further pinpoint the role of Hedgehog signaling in fibrosis, the effects of selective Smo deficiency only in type I collagen-expressing (mesenchymal) cells were evaluated. To produce this cell-specific conditional Smo, knockout mice required the use of mice bearing the *Cre-ER(T)* gene driven by a far upstream enhancer of the $\alpha 2(I)$ collagen gene (35, 37). When appropriately crossed with mice bearing the loxP flanked Smo gene, the progeny mice became deficient in Smo only in collagen I-expressing (“mesenchymal”) cells (smo CKO mice) upon treatment with tamoxifen (35, 37). PCR analysis of tail genomic DNA confirmed the correct genotypes for parental, WT control, and smo CKO strains (Figure 4A). Thus, the presence of the *creER(T)* gene in the appropriately crossed mice was confirmed by detection of a ~ 500 bp DNA band (Figure 4A, left panel). The ~ 280 bp DNA band corresponding to the floxed Smo was

Figure 4. Effects of selective mesenchymal cell *Smo* deficiency on bleomycin-induced pulmonary fibrosis. (A) Representative genotypic analysis of the various strains of mice used to create the mesenchymal cell-specific *smo* conditional knockout mice (*smo* CKO mice) is shown. *Left panel:* The presence of the Cre recombinase and a mutated form of the ligand-binding domain of the estrogen receptor fusion protein gene (*Cre-ER(T)*) gene (~500 base pair band) was confirmed and shown as Cre in the genotypes. *Right panel:* The ~160 base pair DNA band expected for the *smo*⁺ allele [*Smo* (WT)] and a ~280 base pair DNA band expected for the *smo*^{fl} allele [*Smo* (FL)] are indicated with arrows. The “WT” or “FL” added after the *Smo* indicates the corresponding wild-type or floxed genes, respectively. (B) The *smo* CKO mice with genotype [*smo*^{fl/fl}, *Col1α2-CreER(T)*^{+/0}] and control (WT) mice with genotype [*smo*^{fl/fl}] were pretreated for 8 days with tamoxifen and injected endotracheally with SAL or BLM as indicated. Lung fibroblasts isolated from these mice were analyzed for *Smo*, α-SMA, and collagen I proteins by Western blotting. Four mice from each group were tested, and GAPDH was used as loading control. The blots were scanned, and the results are shown. *Significant difference relative to corresponding SAL control group. †Significant difference between the two indicated groups. (C) Effect of mesenchymal *smo* deficiency on bleomycin-induced lung histopathology. Representative sections from saline- or bleomycin-treated WT

detected in samples from homozygous and heterozygous mice bearing the *smo^{fl}* allele, and the WT ~ 160 bp DNA band was also seen in the heterozygotes (Figure 4A, right panel). When tamoxifen was injected into progeny with the [*col1a2-CreER(T)*^{+/-}, *smo^{fl/fl}*] genotype, the expression of Smo in lung fibroblasts was undetectable by Western blotting even in cells isolated from bleomycin-treated *smo* CKO mice (Figure 4B), thus confirming the loss of Smo in the mesenchymal compartment. In contrast, expression of Smo in epithelial cells, T cells, B cells, and macrophages was essentially unaffected in these mice when tested by flow cytometry with cell-specific markers (Figure E3). Smo expression was intact in lung fibroblasts from WT controls, which expressed the *loxP* flanked *smo* allele but did not bear the *creER(T)* gene. Moreover, fibroblasts from the *smo* CKO mice exhibited lower expression of α -SMA and type I collagen even in cells from bleomycin-treated mice, suggestive of reduced myofibroblast differentiation.

Further investigation revealed a reduction in morphological evidence of bleomycin-induced fibrosis in the *smo* CKO mice (Figure 4C). Fibrotic lesions were much smaller and less extensive, leaving only patchy areas of collagen deposition with greater preservation of normal alveolar architecture. No significant morphological change was observed between the lungs from saline-treated WT and *smo* CKO mice. This reduction in fibrosis in *smo* CKO mice was confirmed by lung tissue hydroxyproline analysis (Figure 4D). Although bleomycin caused the expected significant increase (65%) in lung hydroxyproline content in WT mice, it was significantly less (29%) in the *smo* CKO mice. Lung tissue protein (Figure 4E) and mRNA (Figure 4F) analysis for type I collagen and α -SMA further confirmed the significant reduction in matrix deposition and myofibroblast differentiation, respectively, in the *smo* CKO mice.

Reduction of Myofibroblasts in *smo* CKO Mice

To investigate if the reduction of collagen deposition in the lung was related to reduction of myofibroblasts in the lung, single cell suspensions isolated from lung tissue samples were analyzed for α -SMA-positive cells by flow cytometry. The results showed that lung cell samples from WT mice exhibited a significant bleomycin-induced increase in α -SMA-positive cells (3.75–10.1% of total cells), which was significantly less (3.4–7.7% of total cells) in the *smo* CKO samples (Figure 5A). This reduction in α -SMA-positive cells in lung tissue single cell suspensions from *smo* CKO mice was also reflected in reduced α -SMA protein (Figure 4B) and mRNA (Figure 5B) in lung fibroblasts cultured from these mice. Thus, there was a significant increase in α -SMA expression in cells from bleomycin-treated WT mice, with a significantly lower level in *smo* CKO cells. A similar situation was observed with respect to collagen I protein and mRNA (Figures 4B and 5B, respectively). Because α -SMA and heightened collagen I gene expression are two key markers of myofibroblast differentiation, these results suggest that attenuated fibrosis was associated with reduction in lung myofibroblast differentiation in *smo* CKO mice.

Discussion

Hedgehog is found to be essential in lung development for normal patterning and branching morphogenesis and for airway smooth muscle differentiation (20). However, reemergence of SHH signaling has been shown in the FITC-induced murine model of pulmonary fibrosis (43) but not in nonfibrotic lung inflammation (44). Similar reemergence was noted in epithelia of IPF lung (22); in scleroderma-associated dermal fibrosis (27, 45), liver (46, 47), and renal fibrosis (48); and in hyperoxic lung injury (26), suggesting a potential role in pathogenesis of chronic fibrotic diseases. The mechanism of how

Figure 5. Effect of mesenchymal *smo* deficiency on bleomycin-induced lung myofibroblast differentiation. Mice of the indicated strains were treated with SAL ($n = 5$) or BLM ($n = 5$) as indicated. (A) Three weeks later, lung single cell suspensions were obtained and analyzed for α -SMA-positive cells by flow cytometry. The results were expressed as the percentage of α -SMA-positive cells in the total lung cell suspension. *Significant difference from the respective SAL-treated group. **Significant difference between the indicated two groups. (B) Lung fibroblasts were isolated from the same groups of mice as in (A) and analyzed for α -SMA and collagen I gene expression by qPCR. Data were shown as mean $\pm$ SE from triplicate samples. *Statistically significant difference relative to the respective SAL-treated group. †Significant difference between the indicated two groups.

SHH is involved in fibrosis is not fully understood. However, in some of these animal model studies, suppression of

Figure 4. (Continued). (a and c, respectively) and *smo* CKO (b and d, respectively) mice were stained with Masson's trichrome. Original magnification: $\times 100$. (D) Mice of the indicated strains ($n = 5$ per group) were treated with SAL or BLM, and 21 days later the lungs were analyzed for hydroxyproline content. *Significant difference from the respective SAL-treated control group. **Significant difference between the indicated two groups. (E) Lung tissue samples from the same groups of mice as in (D) were analyzed by Western blotting for collagen I and α -SMA proteins. Each lane represents a sample from a single animal. GAPDH was used as a loading control. The blots were scanned, and the results ($n = 5$) are shown. *Significant difference relative to corresponding SAL control group. (F) Lung RNA samples from the same groups of mice as in (D) were obtained at Day 7 after SAL or BLM instillation and analyzed for α -SMA and collagen I mRNA by qPCR. Data are shown as mean $\pm$ SE from triplicate samples. *Significant difference from the respective SAL-treated group. †Significant difference between the indicated two groups.

fibrosis using Smo inhibitors or as a result of its deficiency in α -SMA-expressing cells is accompanied by reduced myofibroblasts, suggesting the importance of Hedgehog signaling in myofibroblast differentiation. Indeed, *in vitro* studies in hepatic stellate cells (29, 30, 33) and in dermal (27, 45) and renal fibroblasts (48) suggest a role for SHH in Smo-dependent myofibroblast differentiation. In apparent conflict with these observations, some studies using human lung fibroblasts cannot demonstrate such an effect (23, 24). In addition to these conflicting reports, the molecular mechanism downstream of Hedgehog signaling in the regulation of myofibroblast differentiation is unknown. This study was designed to address these issues by first evaluating the direct role of Hedgehog signaling in regulation of α -SMA gene expression in the context of myofibroblast differentiation in human lung fibroblasts and then to evaluate its *in vivo* significance by investigating the reemergence of Hedgehog signaling and its role in myofibroblast differentiation in the context of bleomycin-induced pulmonary fibrosis.

The addition of SHH to certain fibroblasts or hepatic stellate cells has been shown to induce myofibroblast differentiation in a Smo-dependent manner, as manifested by up-regulation of α -SMA expression (33). In this study, treatment of human lung fibroblasts from control or IPF lung fibroblasts with SHH caused similar dose-dependent induction of myofibroblast differentiation, with a more potent effect noted in IPF cells. A speculative explanation would be that the IPF cells were “primed” by prior *in vivo* exposure to the milieu in IPF lung tissue and consisted of more activated/differentiated cells that could more readily respond to SHH (e.g., increased receptor expression and/or primed signaling pathway). These results are in contrast to the inability to demonstrate this effect in previous studies using human lung fibroblasts (23, 24). The basis for this discrepancy is unknown but could be related to potential differences in the SHH used and/or experimental methods. In this study, the recombinant human SHH N-terminus with two isoleucine residues added to Cys24 (C24II; R&D Systems, Minneapolis, MN) was used, which is over 10-fold more active than the unmodified molecule, as described in the product literature from the manufacturer.

The previous studies do not specify the type of SHH used; thus, it is uncertain if this could account for the apparent discrepancy in results. The *in vivo* relevance of these concentrations of recombinant SHH is uncertain because the precise range of concentrations of SHH that lung fibroblasts would encounter *in vivo* is unknown. However, based on the ability to detect SHH in mouse lung tissue homogenates (Figure 3B) by Western blotting, microgram amounts of SHH might be present in murine lungs, especially in bleomycin-injured lungs. Moreover, the activity of native (lipid-modified) SHH is severalfold higher than the recombinant (non-lipid modified) molecule used in these *in vitro* experiments, thus necessitating the higher concentrations of recombinant SHH described here to approximate the activity of the native molecule. The stimulatory effect of SHH on α -SMA gene expression in this study was found to be Smo and Gli1 dependent, but for the first time the effect was shown to involve transcriptional activation of the α -SMA gene. Furthermore, a Gli binding consensus conserved in mouse, rat, and human α -SMA gene promoters was identified in this study and was found to bind Gli1 in cell-free assays and in the intact cell. Mutation of this binding consensus sequence abrogated binding to Gli1 and significantly inhibited SHH-induced transcriptional activity of the α -SMA gene. These findings indicated that SHH could induce myofibroblast differentiation by direct regulation of α -SMA gene expression in a Smo- and Gli1-dependent manner.

In vivo relevance or significance of this Hedgehog regulation of myofibroblast differentiation was suggested by confirmation of the reemergence of SHH in the murine model of bleomycin-induced pulmonary fibrosis. The other two Hedgehog isoforms were not significantly induced in this model. The source of this reemergence, which was inducible by TGF- β , was primarily in epithelial cells. Although SHH was undetectable in lung fibroblasts, these cells expressed elevated levels of TGF- β upon treatment with SHH, thus suggesting epithelial-mesenchymal crosstalk mediated by these two molecules, resulting in a potential positive feedback loop to amplify and/or propagate the fibrotic response. The epithelial-mesenchymal communication is consistent with a previous report of

epithelial-derived SHH driving fibroblast responses, including myofibroblast differentiation, in renal fibrosis (48).

To further confirm the role of SHH in the regulation of myofibroblast differentiation and pulmonary fibrosis, mesenchymal compartment-specific, Smo-deficient mice were generated and used in the bleomycin-induced pulmonary fibrosis model. As with the Smo inhibitor study, a significant reduction of fibrosis was observed. However, this experiment using the mutant mice also showed reduction in myofibroblast differentiation. Thus, cell compartment-specific deficiency of Smo-dependent signaling in the mesenchymal compartment effectively suppressed myofibroblast differentiation *in vivo*. These findings are similar to those seen in the liver when Smo deficiency was restricted to α -SMA-expressing cells (33). However, because α -SMA expression is an indicator of myofibroblast differentiation, the liver study cannot provide insight into the role of Smo in the differentiation process itself or at least at the early stage of myofibroblast differentiation when α -SMA has not been expressed, although it does indicate the importance of Smo in myofibroblasts in liver repair/fibrosis.

In summary, the results of this study indicate the ability of SHH to induce myofibroblast differentiation and its *in vivo* significance in the bleomycin model of pulmonary fibrosis. The results also suggest that the reemergence of Hedgehog signaling might mediate its effects by participation in epithelial-mesenchymal crosstalk involving epithelial-derived SHH stimulation of mesenchymal TGF- β expression, which could activate SHH expression in the epithelial compartment. Given that the evidence of SHH induction of myofibroblast differentiation and the potential epithelial source of SHH was obtained using human lung cells, the applicability to human fibrotic lung disease is suggested. ■

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