Hedgehog Signaling in Neonatal and Adult Lung

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Sonic Hedgehog (Shh) signaling is essential during embryonic lung development, but its role in postnatal lung development and adult lung are not known. Using *Gli1^{nlacZ}* reporter mice to identify cells with active Hh signaling, we found that *Gli1^{nlacZ}*-positive mesenchymal cells are densely and diffusely present up to 2 weeks after birth and decline in number thereafter. In adult mice, *Gli1^{nlacZ}*-positive cells are present around large airways and vessels and are sparse in alveolar septa. Hh-stimulated cells are mostly fibroblasts; only 10% of *Gli1^{nlacZ}*-positive cells are smooth muscle cells, and most smooth muscle cells do not have activation of Hh signaling. To assess its functional relevance, we influenced Hh signaling in the developing postnatal lung and adult injured lung. Inhibition of Hh signaling during early postnatal lung development causes airspace enlargement without diminished alveolar septation. After bleomycin injury in the adult lung, there are abundant *Gli1^{nlacZ}*-positive mesenchymal cells in fibrotic lesions and increased numbers of *Gli1^{nlacZ}*-positive cells in preserved alveolar septa. Inhibition of Hh signaling with an antibody against all Hedgehog isoforms does not reduce bleomycin-induced fibrosis, but adenovirus-mediated overexpression of Shh increases collagen production in this model. Our data provide strong evidence that Hh signaling can regulate lung stromal cell function in two critical scenarios: normal development in postnatal lung and lung fibrosis in adult lung.

Keywords: Hedgehog; lung fibrosis; bleomycin; fibroblast; alveolarization

The Hedgehog (Hh) family members control a wide range of actions during embryonic development (1) by regulating cell differentiation in a concentration-dependent manner, acting as mitogens, and influencing cell survival and cell death decisions. In lung, epithelium-derived Sonic Hedgehog (Shh) signals to mesenchyme and is required for branching morphogenesis (2), in large part by regulating Fgf10 expression (3). Overexpression of Shh in mice causes hyperproliferation of mesenchymal cells and respiratory failure at birth (4).

Canonical Hh signaling involves the 12-transmembrane receptor Patched-1 (Ptc1), the 7-transmembrane protein Smoothened (Smo), and the transcription factors Gli1, -2, and -3 (5). In the absence of Hh ligand, Ptc1 catalytically inhibits Smo. Binding of Hh ligand to Ptc1 disinhibits Smo, which then promotes the

nuclear accumulation of an activator form of Gli2 (Gli2-A). One of the direct downstream targets of Gli2-A is the *Gli1* gene; Gli1 is a transcriptional activator and amplifies the effects of Gli2-A. Although Ptc1 expression is a prerequisite for receiving Hh signals via the canonical pathway, *Ptc1* is also a transcriptional target of Hh signaling, and it is hypothesized that Hh-mediated induction of Ptc1 acts as a negative feedback on the Hh signaling pathway. The third member of the Gli family, Gli3, typically functions as a repressor (Gli3-R). However, activation of Smo promotes the elimination of Gli3-R protein and formation of an activator form of Gli3. In the lung, Gli2 is the key Gli transcription factor responsible for Shh-induced lung growth; overexpression of Gli2 in lung mesenchyme during development induces *Ptc1* and *Gli1* gene expression and increases proliferation of lung cells (6).

Whereas Hh signaling is indispensable during embryonic development, it has more restricted roles after birth. Postnatal development of the small intestine depends upon Hh signaling (7). In the adult human, Hh signaling maintains CNS and hair follicle stem cells (8, 9), the blood-brain barrier (10), and intestinal smooth muscle (11). Adult humans treated with a Smo inhibitor suffer side effects such as hair loss and weight loss (12). Certain cancers (e.g., basal cell carcinoma, medulloblastoma, pancreatic cancer, and non-small cell lung cancer) are associated with increased Hh signaling.

Fibrotic reactions in liver and kidney and in the tumor microenvironment are promoted by Hh signaling (13–16). Shh and/or Ihh are reexpressed and functional in experimental lung injury (17, 18). In idiopathic pulmonary fibrosis, Shh is expressed by epithelial cells (19), and microarray studies reveal evidence of Hh-dependent signaling (20); these observations raise the possibility that epithelium-derived Shh contributes to the pathologic processes that occur in interstitial lung diseases.

Genetic reporter mice in which critical sequences of *Gli1* have been replaced with the *nlacZ* gene (encoding β -galactosidase fused to a nuclear localization tag) have proved useful for the identification of cells responding to Hh signals (21). To determine the status of Hh signaling in normal adult lung epithelial and mesenchymal cells, we conducted an extensive characterization of normal adult *Gli1^{nlacZ/+}* lungs. We then performed functional experiments to determine whether Hh signaling influences a normal process, postnatal lung development (22), or a pathologic process, the pulmonary fibrotic response to bleomycin injury (23, 24).

MATERIALS AND METHODS

Animals

Mouse protocols were approved by the Institutional Animal Care and Use Committee at NYU Langone Medical Center. Mice carrying *Gli1^{lki}*, *Gli2^{lki}*, or *Ptc1^{lacZ}* knock-in alleles (Swiss Webster background) were genotyped as described (21, 25, 26). C57BL/6J mice were from Jackson Laboratories (Bar Harbor, ME).

Experimental Treatments

Bleomycin-induced fibrosis. Mice (8–10 wk old) were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (6 mg/kg). For

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Gli1^{nlacZ/+} mice, bleomycin (Sigma-Aldrich, St. Louis, MO) (5 U/kg in 50 μ l normal saline [NS]) or NS was administered retropharyngeally using a 200- μ l pipette tip. For C57BL/6J mice, bleomycin (1.3 U/kg in 50 μ l NS) or NS was instilled into the exposed trachea with a 28-gauge needle. Hydroxyproline assay is described in the online supplement.

Antibody treatments. Mice were injected intraperitoneally with 5E1 or isotype-matched IgG at Day -1 (60 mg/kg) and with 30 mg/kg doses at Days 7 and 14. Neonates received 5E1 or IgG (30 mg/kg intraperitoneally) at postnatal day 3 (P3) based on body weights at P7 (4 g for C57BL/6J mice and 5 g for *Gli1^{nlacZ/+}* mice). Further details are provided in the online supplement.

Adenovirus treatments. Mice were anesthetized intraperitoneally with ketamine/xylazine (doses described above) and held in a vertical position. Shh-adenovirus or control virus (10^9 plaque-forming units in 50 μ l PBS) was administered slowly into the nostrils while monitoring breathing pattern.

Lung Histology

After animals were killed and diaphragms were removed, a 24-gauge cannula was inserted into the exposed trachea and secured with sutures. For morphometric analysis, lungs were inflated to a pressure of 25 cm H₂O with 4% paraformaldehyde-PBS for 10 minutes. After fixation, the trachea was ligated. Lungs were immersed in 4% paraformaldehyde-PBS for 24 hours at 4°C. After fixation and heart removal, lungs were embedded in paraffin. Sections (5 μ m) were stained with H&E, dehydrated, and mounted with VectaMount.

Immunohistochemistry and Cytochemistry

Mice were anesthetized with pentobarbital sodium (120 mg/kg intraperitoneally). Bronchoalveolar lavage was performed if indicated, frozen lung sections were generated, and X-gal staining was performed by standard methods as described in the online supplement. Sections were counterstained with H&E, nuclear fast red (Vector Laboratories, Burlingame, CA), resorcin-fuchsin for elastin stainings, or picrosirius red (Electron Microscopy Sciences, Hatfield, PA) according to manufacturer's instructions. Collagen fibers were visualized by birefringence microscopy. Immunofluorescent (IF) staining was performed on 5- μ m lung cryosections (further details are provided in the online supplement).

Morphometric Analyses

Colocalization measurements. Sections of *Gli1^{nlacZ/+}* mice (untreated, age 9–16 wk or 4 wk after bleomycin treatment) were costained for β -gal and other antigens (SPC, CD-31, CD45, α smooth muscle actin [α -SMA] or Col1; see the online supplement).

Count of *Gli1*- or *Gli2*-expressing cells. Untreated *Gli1^{nlacZ/+}* and *Gli2^{nlacZ/+}* mice and *Gli1^{nlacZ/+}* mice 4 weeks after bleomycin treatment were used ($n = 3$ per group). For each mouse, total nuclei and β -gal+ cells were counted in multiple (range, 5–15) 20 $\times$ images of X-gal-stained areas of normal alveoli (excluding large airways and fibroblast areas).

Mean linear intercept measurements. Details are provided in the online supplement.

Construction of Shh-Expressing Adenovirus

Adenoviral plasmids were constructed using mouse Shh full-length cDNA (from A. McMahon, Harvard University) and the adenoviral expression system RAPAd (Gene Transfer Vector Core Service, University of Iowa). Further details are provided in the online supplement.

RESULTS

Persistent Hh Pathway Activation in Normal Neonatal and Adult Lung

To assess Hh pathway activation we used mice carrying a copy of the *Gli1^{nlacZ}* (*Gli1^{lki}*) reporter allele. The *Gli1^{lki}* allele provides a reliable readout of transcriptional activator response downstream of Hh signals (21, 27). *nlacZ* encodes a fusion protein

consisting of the enzyme β -galactosidase (β -gal) and a nuclear localization signal. Mice heterozygous for this allele have normal development and lifespan (21). We refer to mice heterozygous for the *Gli1^{lki}* allele as *Gli1^{nlacZ/+}* mice and to cells expressing β -gal from the *Gli1^{nlacZ}* allele as *Gli1⁺* cells. Recent studies suggest *Gli1* expression might be influenced by cross-talk from non-Hh signaling pathways (28–30). However, in embryos lacking *Gli2* and *Gli3*, *Gli1^{nlacZ}* is not expressed (27), and in *Shh*-null mutant embryos, *Gli1^{nlacZ}* is only expressed in cells near sources of *Ihh* and *Dhh* (21). Therefore, it appears that *Gli1^{nlacZ}* expression is indicative of cells undergoing Hh signaling, dependent on Gli-A transcription factor activity.

Histochemical staining for β -gal activity revealed *Gli1⁺* lung cells densely present during the first week of postnatal life (Figure 1). During this time period, *Gli1⁺* cells are present in all alveolar wall structures. By postnatal Day 14 (P14), there is a reduction in the number of *Gli1⁺* cells in the sense that many individual alveolar wall segments lack such cells, and this reduction is more marked at P21. This reduction in the number of the *Gli1⁺* cells likely reflects the reduction in *Shh* expression that occurs just before this time period (4) and/or fibroblast apoptosis. In addition, some of the loss of *Gli1⁺* cells at later time points is likely due to the loss of mesenchymal cells that occurs during the later phase of postnatal lung development in rodents (22, 31) as the alveolar septal walls mature.

We also assessed *Gli1^{nlacZ}* expression in normal adult (9–16 wk old) *Gli1^{nlacZ/+}* lungs. Histochemical staining for β -gal activity revealed *Gli1⁺* cells around large airways and vessels (Figures 2A and 2B). Most of the *Gli1⁺* peribronchiolar and perivascular cells are within collagen-rich adventitial zones; some are immediately adjacent to the smooth muscle layers surrounding vessels and airways. *Gli1⁺* cells are also present within the visceral pleura. *Gli1⁺* cells are not present within the epithelial layer of large airways. The alveolar walls of normal lung parenchyma contain only occasional *Gli1⁺* cells (Figures 2A–2C).

Distribution of *lacZ⁺* Cells in *Gli1^{nlacZ}*, *Gli2^{nlacZ}*, and *Ptch1^{lacZ}* Lungs Is Similar

Our findings contrast with a study that found evidence of Hh signaling in adult mouse airway epithelial cells (17). We sought further evidence that adult lung mesenchymal cells, but not epithelial cells, retain the capacity to respond to Hh signals by examining two additional reporter mice, *Gli2^{nlacZ}* (25) and *Ptch1^{lacZ}* (26).

Gli2 is not a transcriptional target of Hh signaling, but its expression is typically a precondition for a Gli activator response to Hh ligands (21, 25, 27). β -gal staining of lung sections from *Gli2^{nlacZ/+}* mice demonstrated *Gli2⁺* cells in a peribronchiolar, perivascular, and pleural distribution (Figures 2D–2F), identical to that of *Gli1⁺* cells. In the alveolar septa, however, *Gli2^{nlacZ}* mice have approximately 25 times more *lacZ⁺* cells than do *Gli1^{nlacZ}* mice (compare Figures 2C and 2F; quantification shown in Figure 3E). The results are consistent with the idea that alveolar septa contain *Gli2⁺/Gli1⁻* cells, which are seen in all embryonic tissues and are likely cells capable of responding to aHh signal but not currently doing so.

Ptch1 is the primary Hh receptor and is required for Hh signal transduction. Moreover, *Ptch1* transcription is up-regulated in response to Hh signaling. Watkins and colleagues used the *Ptch1^{lacZ}* reporter gene to demonstrate Hh pathway activation in neuroendocrine progenitors within lung epithelium, but not mesenchyme, after naphthalene injury (17). In contrast, by extending histochemical developing times, we were able to observe *lacZ⁺* cells in lung sections from *Ptch1^{lacZ/+}* mice only in

Figure 1. Hedgehog (Hh) signaling occurs during postnatal development. *Gli1^{nlacZ/+}* lung sections from postnatal day (P)0, P2, P7, P14, and P21 were stained for β -gal activity (blue). (A–E) Low power images show *Gli1*⁺ cells are initially present in alveolar walls and around bronchi but decrease significantly in the alveolar compartment by P14. At P21, *Gli1*⁺ cells are mostly seen around airways and vessels. (F–H) High-power images reveal that *Gli1*⁺ cells, present in alveolar walls and septa (arrowheads), decrease over time.

a peribronchial and perivascular distribution, mirroring the *Gli1*⁺ cell localization (data not shown).

Most *Gli1*⁺ Cells in Adult Lungs Are Adventitial Fibroblasts

To establish the identity of the *Gli1*⁺ adventitial cells, we performed IF microscopy on lung sections from *Gli1^{nlacZ/+}* mice using an antibody specific for β -gal and a second antibody recognizing one of several cell-type markers (Figures 2G–2J and Figures E1A–E1C in the online supplement). Approximately one third of *Gli1*⁺ cells colocalize with collagen type 1 (Col1) (Figures 2G and 2H); a similar fraction of Col1⁺ cells coexpress β -gal (23 $\pm$ 19%; *n* = 3 mice). Although many *Gli1*⁺ cells are closely apposed to vascular and bronchiolar smooth muscle layers, they are generally distinct from smooth muscle; careful

quantification revealed that fewer than 10% of *Gli1*⁺ cells coexpress α -SMA, and confocal imaging also showed colocalization with α -SMA only in occasional β -gal⁺ cells (Figures 2I and 2J).

The adventitial location of *Gli1*⁺ cells is similar to that of some lung dendritic cells, but no *Gli1*⁺ cells coexpress the hematopoietic marker CD45 (Figure E1B). Also, β -gal⁺ cells do not coexpress the endothelial marker CD31 or the type II pneumocyte marker surfactant protein C (SPC) (Figures E1A and E1C). β -gal⁺ cells also do not react with antikeratin antibodies, including AE1 and AE3 (data not shown), supporting the conclusion that Hh-responding cells in normal lung are not epithelial.

To confirm these results, in particular the α -SMA-negative status of most of the *Gli1*⁺ cells despite their close apposition to smooth muscle zones, we generated single-cell suspensions of lung cells from normal adult *Gli1^{nlacZ/+}* mice for IF staining

Figure 2. *Gli1^{nlacZ}* and *Gli2^{nlacZ}* reporter expression patterns reveal Hh pathway activation in adult lung fibroblasts. (A–C) *Gli1^{nlacZ/+}* lung sections and (D–F) *Gli2^{nlacZ/+}* lung sections were stained for β -gal activity (blue). (A, D) Low-power images demonstrate prominent distribution of *Gli1*⁺ (A) and *Gli2*⁺ (D) cells around large airways and accompanying vessels. (B, E) High-power images show the absence of *Gli1*⁺ cells (B) and *Gli2*⁺ cells (E) in airway epithelia and large vessel endothelia. (C, F) High-power images of lung periphery show *Gli1*⁺ (C) and *Gli2*⁺ (F) cells adjacent to the pleural boundary and in more proximal alveolar parenchyma. In the alveolar compartment, *Gli2*⁺ cells are more numerous than are *Gli1*⁺ cells. (G–J) The majority of *Gli1*⁺ adult lung cells are collagen-producing fibroblasts. Merged immunofluorescent images of immunostained *Gli1^{nlacZ/+}* lung sections are shown (β -gal: green, other markers: red; DAPI-stained DNA: blue). (G, H) Most β -gal⁺ cells are embedded in a Col1⁺ matrix (red). (I, J) β -gal⁺ cells closely surround the smooth muscle layers of vessels and large airways, but only a few coexpress α smooth muscle actin. b = bronchus; p = pleura; v = vessel.

(Figures E1G–E1K). Only $6.1 \pm 1.1\%$ of β -gal⁺ cells are α -SMA positive ($n = 3$ isolations), in good agreement with the results obtained with tissue sections. Again, no β -gal⁺ cells coreacted with antibodies against SPC or CD45. Over 90% of β -gal⁺ cells costained for Col1 ($n = 2$ isolations; data not shown) when a 1:100 concentration of the primary antibody was used (the concentration used for tissue staining), but only 45% costained for Col1 when the concentration was decreased to 1:300 ($n = 1$ isolation). These results suggest that the sensitivity of the assay is greater for cytopun cells and/or that extracellular collagen fragments derived from the isolation are adherent to the cells. Approximately 90% of β -gal⁺ cells costain for the mesenchymal marker vimentin ($n = 2$ isolations; Figure E1I).

Distribution of *Gli1*⁺ Cells after Bleomycin-Induced Lung Injury

We used the bleomycin-induced lung fibrosis model to assess the Hh pathway in lung fibrosis. Because we maintain the *Gli1*^{nlacZ/+} mice and other targeted genes on an outbred Swiss Webster background, we first determined whether *Gli1*^{nlacZ/+} mice develop fibrosis after bleomycin injury in a manner similar to fibrosis-prone strains such as C57BL/6 (24). A single retropharyngeal dose of bleomycin (5 U/kg) results in an acute inflammatory

injury around the bronchioles and blood vessels within the first week that is followed by fibrosis formation, as assessed by picrosirius red birefringence and trichrome staining, that peaks 3 to 5 weeks after bleomycin injury (data not shown).

Histological analysis of bleomycin-treated *Gli1*^{nlacZ/+} lungs revealed a striking spatiotemporal correlation between the localized development of fibrosis and the number and distribution of *Gli1*⁺ cells. One and two weeks after bleomycin exposure, the distribution and numbers of *Gli1*⁺ cells are essentially unchanged compared with nontreated and NS-treated lungs (data not shown). By 4 weeks, large numbers of *Gli1*⁺ cells are present in areas of fibrosis (Figure 3A). The distribution of *Gli1*⁺ cells is heterogeneous: some lesion areas display a high density of *Gli1*⁺ cells, whereas others are largely devoid of *Gli1*⁺ cells. *Gli1*⁺ nuclei within fibrotic zones are often elongated and arranged in parallel, suggesting that they are aligned with collagen fibrils (Figure 3B). *Gli1*⁺ cells are mainly found within areas of collagen deposition (as revealed by picrosirius red staining; Figure 3C).

We also observed an increase in the number of *Gli1*⁺ cells in histologically uninvolved *Gli1*^{nlacZ/+} alveolar septa after bleomycin treatment (Figure 3D). Quantification showed that the number of septal *Gli1*⁺ nuclei, expressed as a fraction of total nuclei in areas of preserved alveolar architecture, was significantly higher than in untreated *Gli1*^{nlacZ/+} mice, reaching almost half of the frequency

Figure 3. Bleomycin-induced fibrosis is characterized by increased numbers of *Gli1*⁺ fibroblasts and myofibroblasts. (A–D) *Gli1*^{nlacZ/+} lung sections, 4 weeks after bleomycin exposure, were stained for β -gal activity (blue) and counter-stained with H&E (A, B, D) or picrosirius red (C). (A) Low-power image illustrates high number and heterogeneous distribution of *Gli1*⁺ cells in fibrotic zones of bleomycin-treated lungs. (B) High-power image shows the clustered, linear arrangement of β -gal⁺ nuclei (arrows), characteristic of fibrotic regions. β -gal⁺ cells extend along eosin-stained fiber tracks. (C) β -gal⁺ cells are largely located within areas of yellow birefringence (arrows) produced by picrosirius-stained collagen fibrils. (D) High-power image shows *Gli1*⁺ cells (arrowheads) in the alveolar septa of histologically normal distal lung parenchyma in lungs exposed to bleomycin 4 weeks earlier. (E) Graph shows comparison of the number of *Gli1*⁺ cells and *Gli2*⁺ cells in alveolar regions of normal lungs with the number of *Gli1*⁺ cells in areas of preserved architecture 4 weeks after bleomycin exposure ($n = 3$ /group). Data are shown as mean and SD of each group. BLM = bleomycin. (F–I) *Gli1*⁺ cells in bleomycin-induced fibrotic lesions are fibroblasts and myofibroblasts. (F–I) Merged immunofluorescent images of *Gli1*^{nlacZ/+} lung sections, 4 weeks after bleomycin exposure, were costained with antibodies against β -gal protein (green) and either Col1 (F, G) or α smooth muscle actin (α -SMA) (H, I) (both red). Nuclei are stained with DAPI (blue); β -gal⁺ nuclei appear blue-green in the merged photographs. Col1⁺/ β -gal⁺ (G, arrow) and Col1[−]/ β -gal⁺ cells (G, arrowhead) can be identified. Many β -gal⁺ cells in fibrotic zones also express α -SMA (I, arrow); β -gal⁺/ α -SMA[−] cells are also present (I, arrowhead).

of *Gli2*⁺ cells in normal *Gli2*^{nlacZ/+} lung alveolar walls (Figure 3E). This observation is consistent with the possibility that *Gli2*⁺/*Gli1*⁺ cells present in normal lungs are activated after bleomycin injury by Hh or other signals to initiate *Gli1* expression through a Gli2-A-dependent mechanism (compare Figures 2C and 2F with Figure 3D).

Gli1⁺ Cells in Fibrotic Lesions Are Mostly Fibroblasts and Myofibroblasts

Gli1⁺ cells in fibrotic lesions appear to be fibroblasts or myofibroblasts based upon their cellular morphology, tendency to form elongated parallel tracks along matrix bundles, and marker expression. Approximately half of β -gal⁺ cells in fibrotic lesions colocalize with Col1 and one third with the myofibroblast marker α -SMA (Figures 3F–3I; quantitative data not shown).

β -gal expression in bleomycin-treated *Gli1*^{nlacZ/+} lungs is not detected in epithelial cells, as assessed by anatomical location or immunostaining. β -gal⁺ cells do not react with antikeratins or anti-SPC antibodies. Also, as in normal lungs, β -gal⁺ cells in fibrotic areas do not coexpress the endothelial marker CD31 or the hematopoietic marker CD45 (Figures E1D and E1E).

Effect of Shh Inhibition or Overexpression on Bleomycin-Induced Fibrosis

To determine the functional role of Shh signaling in fibrosis, we inhibited and overexpressed Shh in the bleomycin model. To inhibit Hh signaling, we treated mice with the anti-Hh mAb 5E1 (32) 1 day before intratracheal bleomycin administration and weekly thereafter. Mice were killed 3 weeks after bleomycin for analysis. Preliminary experiments in *Gli1*^{nlacZ/+} mice revealed that the antibody doses used eliminated reporter expression in cells of normal lungs (Figures E2A and E2B) and within fibrotic regions after bleomycin-induced injury (data not shown). Measurement of lung hydroxyproline showed that bleomycin treatment increased collagen content by more than 50% compared with saline-treated control, but 5E1 treatment had no further effect on collagen increase (Figure 4A). Treatment of uninjured mice

with 5E1 antibody did not alter lung histology over 6 weeks (data not shown).

To overexpress murine Shh, we created an adenoviral vector encoding full-length murine *Shh* (Ad-Shh; see the online supplement for details). The activity of the Ad-Shh was confirmed by culturing *Gli1*^{nlacZ/+} fibroblasts with HEK293 cells infected with virus (Figure 4B). These experiments were performed using cells in nonadherent conditions, where cells self-assemble into aggregates with mesenchymal cells on the interior and epithelial cells on the outside (Figure E5). Reporter expression occurs only at the outer boundary of the fibroblast layer (Figure 4B), consistent with the expected limited range of the wild-type Shh molecule, which is modified by cholesterol and palmitoyl moieties and not freely diffusible. Mice were treated with intratracheal bleomycin as before and then with Ad-Shh or control adenovirus 3 weeks later when fibrosis formation is most prominent. Mice were killed 4 weeks after bleomycin treatment because lung adenoviral vector expression is expected to persist for about a week. Under these conditions, Ad-Shh resulted in a further 25% increase in collagen content compared with control-treated mice (Figure 4A).

To determine whether Hh affects adult lung fibroblast phenotype, we examined the effect of recombinant soluble Shh on fibroblast survival in culture under conditions that favor fibroblast apoptosis. *Gli1*^{nlacZ/+} fibroblasts were cultured on an agarose surface to produce aggregates and treated with or without soluble recombinant Shh over 6 days. Shh treatment significantly increased the number of viable fibroblasts remaining at later time points (Figure 4C). These results, together with the bleomycin data and a recent publication showing that Shh regulates important fibroblast functions and prevents apoptosis (33), support the hypothesis that Hh signaling contributes to the maintenance of fibroblasts during wound resolution and/or stimulates proliferation of fibroblasts.

Effect of Hh Inhibition on Postnatal Lung Growth

The role of Hh signaling in postnatal lung development is not known. To address this issue, we treated mice on postnatal

Figure 4. Epithelial Sonic Hedgehog (Shh) overexpression increases bleomycin-induced lung fibrosis, whereas inhibition of Shh does not affect lung fibrosis. (A) C57BL/6J mice, exposed to bleomycin (1.3 U/kg), received treatment with Shh neutralizing antibody 5E1 or IgG isotype at Day -1 (60 mg/kg), Day 7 (30 mg/kg), Day 14 (30 mg/kg) or treatment with Shh-expressing adenovirus (Ad-Shh, 10^9 plaque-forming units per mouse) or control adenovirus (Ad-ctl) at Day 21. Lungs harvested on Day 21 or 28, respectively, were measured for collagen content (μ g/lung) using hydroxyproline assay ($n = 13$ per group for 5E1 experiment; $n = 8$ per group for Ad-Shh experiment; $n = 2$ for normal saline [NS]-treated control lungs). (B) HEK293 cells (HEK), infected with Ad-Shh or Ad-ctl, were placed in nonadherent coculture with primary *Gli1*^{nlacZ/+} lung fibroblasts (*Gli1*^{nlacZ} fb). X-gal staining after 72 hours shows β -gal⁺ cells only in coculture sphere containing Ad-Shh infected HEK cells (lower panel). Recombinant Shh (rShh) was used to show Shh responsiveness of *Gli1*^{nlacZ/+} fibroblasts (upper panel). (C) Primary lung fibroblasts were cultured as nonadherent spheres (35,000 cells/sphere) and stimulated with rShh for a maximum of 6 days. Trypsinized cells at later time points show significantly more viable cells in rShh-treated spheres (red line) than in the control spheres (blue line). Data are shown as mean and SD of each group. * $P < 0.05$.

Day 3 (P3) with the anti-Hh mAb 5E1 or with isotype-control IgG and assessed the effect on lung structure. Inhibition of Hh signaling in neonatal mice impairs intestinal absorption and leads to runting and death before weaning (7). In preliminary experiments, we found that mice treated with 5E1 maintained normal weight gain until at least P9 (Figure 5F) but began to lose weight relative to control mice after this time point. Values of standard tests of renal, liver, and endocrine status are normal at P9 in 5E1-treated mice (Figure E4A).

We examined lungs of P9 mice injected with 5E1 or control IgG at P3. Lungs were inflated and fixed at a constant pressure of 25 cm H₂O for measurement of mean linear intercept (MLI), a measure of airspace size. Control experiments showed that our inflation and fixation protocol detects anticipated differences in MLI resulting from inflation pressures of 10, 15, 20 and 25 cm H₂O (Figure 5E). Hh inhibition causes marked airspace enlargement with an increase in MLI of over 35% (Figures 5A, 5B, and 5E). Similar results were observed in mice treated on P3 and killed on P8 (data not shown). Despite the airspace enlargement, there is a normal number of alveolar septal tips in 5E1-treated mice (Figure 5A), suggesting that alveolar septation is not affected by Hh inhibition. Therefore, it appears that the measured airspace enlargement occurs as the result of increased lung compliance. Stains for the endothelial marker CD31 (not shown) or elastin (Figures E4B and E4C) were not affected by 5E1 treatment.

DISCUSSION

During branching morphogenesis of the lung, Shh is released by endoderm and influences mesenchyme, as evidenced by the observations that genetic deletion of *Shh* causes failed branching morphogenesis and overexpression of Shh in lung endoderm

causes overexpansion of lung mesenchyme (2, 4, 34–36). Recent studies have suggested that Hh signaling is reactivated in lung injury and fibrosis (18–20, 37, 38). In this work we characterized Hh-responding cells during postnatal lung development and in adult lungs with and without a fibrosis-inducing injury.

In postnatal lung development, a period of bulk alveolarization with formation of secondary septa is followed by a period of microvascular maturation. In rats, alveolarization occurs from approximately P4 to P13, and microvascular maturation is largely finished by the end of the third week (22, 31). During the second phase, the number of fibroblasts decreases by 10 to 20% despite an overall lung volume increase of 25%, and the resulting attenuation of the septal walls coincides with the fusion of two capillary layers to create a single capillary layer.

Shh gene expression decreases markedly near the end of embryonic lung development (4). In contrast, Hh signaling, defined by expression of the *Gli1* reporter, persists at a high level during the alveolar septation phase (Figure 1). Only during the microvascular maturation phase do Hh-responsive cells become markedly less prominent within septal walls, although they persist around large airways and vessels. These results suggest that Hh signaling promotes alveolar septation.

To test these hypotheses, we inhibited Hh signaling during the alveolarization phase. Hh inhibition did not inhibit alveolar septation, but airspaces were significantly enlarged (Figure 5), with preserved elastin and grossly intact vasculature assessed by CD31 staining. Therefore, Hh signaling is dispensable for alveolar septation, in contrast to other signaling and structural molecules, such as PDGF-A, fibrillin-1, TGF- β , ephrinB2, Notch, NeuroD, PECAM-1, and LTBP-3 (39–45). Instead, Hh signaling appears to affect the thickness and distensibility of the alveolar walls. Our working hypothesis is that early abrogation of Hh signaling results in fewer

Figure 5. Inhibition of Hh signaling affects postnatal lung development. C57BL/6J mice or *Gli1^{nlacZ/+}* mice were treated with 5E1 antibody (30 mg/kg) or isotype IgG at P3. Lungs were inflated at P9 with 4% paraformaldehyde at 25 cm H₂O pressure for 10 minutes (A, B) or with OCT/sucrose at the same volumes (C, D) then stained with H&E for morphometric analysis (A, B) or X-gal and Nuclear Acid Fast stain (C, D). (A, B) Low-power images of 5E1-treated lungs show enlarged alveolar airspaces when compared with IgG controls. High-power images (*insets*) show the presence of septa in 5E1 and IgG. (C, D) Low-power images show *Gli1⁺* cells are almost absent in the alveolar compartment in 5E1-treated mice when compared with IgG control. Some *Gli1⁺* cells remain present around bronchi and vessels. Higher-power images (*insets*) show the absence of *Gli1⁺* cells at septal tips. (E) Mean linear intercept (MLI) of mice treated with 5E1 or IgG at P3 and analyzed at P9 ($n = 8$ per group). MLI of control mice inflated at different inflation pressures show increasing MLI with higher pressures. (F) Weights of 5E1- and IgG-treated mice are not different from P3 to P9 ($n = 8$ per group). Data are shown as mean and SD of each group. b = bronchus; v = vessel.

mesenchymal cells (either through reduced proliferation or increased death) and that the phenotype observed in 5E1-treated mice is essentially that of accelerated maturation of the septal walls. Our results suggest the possibility that normal physiologic reduction of Hh signaling during the third week contributes to reduced numbers of septal fibroblasts through reduced proliferation or increased apoptosis and thereby acts to promote the microvascular maturation phase.

In the adult mouse, *Gli1*⁺ cells are nearly absent in the distal lung but remain numerous around large airways and vessels and in the visceral pleura. The facts that *Gli2*⁺ cells are present in higher numbers in distal lung and that there are increased numbers of *Gli1*⁺ cells in intact septa after lung injury suggest that there is a pool of septal mesenchymal cells capable of responding to Hh under appropriate conditions. *Gli1*⁺ cells are most numerous around airways, most likely due to Shh released by airway epithelium. Consistent with this, adult proximal lung epithelium expresses Shh as determined by detection of reporter expression in *Shh*^{lacZ} mice (not shown).

The lung contains a variety of stromal cells including smooth muscle cells, pericytes, lipocytes, myofibroblasts, and fibroblasts, and fibroblasts themselves are heterogeneous (46, 47). We show here that Hh signaling is another feature that defines a subset of stromal cells. The characteristics of these cells in normal adult lung (expressing collagen and vimentin but not α -SMA) indicate that they are fibroblasts and not myofibroblasts or smooth muscle cells. It remains to be determined whether *Gli1*⁺ fibroblasts comprise a distinct functional subset of fibroblasts and, if so, whether Hh signaling mediates this phenotype or is merely a marker. Our results indicate that lung fibroblasts have altered phenotypes in response to exogenous Hh *in vitro* and *in vivo*, but the physiologic effect of endogenous Hh signaling in adult lung remains to be determined. Our data clearly indicate, however, that Hh signaling mediates critical aspects of stromal cell function during early postnatal lung development.

Gli1 expression might occur independent of Hh signaling, although this does not appear to be the case during development (21). Supporting the role of Hh in downstream *Gli1* activation, treatment of neonatal mice with 5E1 nearly abolishes *Gli1* reporter expression (Figures 5C and 5D), although some *Gli1*⁺ cells persist around large airways and vessels. Similarly, 5E1 treatment of adult mice causes marked reduction in reporter expression, but again a smaller number of *Gli1*⁺ cells persist around large airways and vessels (Figures E2A and E2B). In the adult, however, treatment times of greater than 2 weeks are required to see this effect, perhaps due to prolonged perdurance of the β -gal protein in non-dividing cells.

There is emerging evidence that Hh signaling promotes fibrosis under some circumstances. For example, paracrine Hh signaling causes fibrotic reactions in zebrafish pancreas and in pancreatic carcinoma (48, 49). Hh signaling also promotes liver fibrosis and is involved in scleroderma models of fibrosis (13, 50). In renal fibrosis models, paracrine Hh signaling is increased, but the reported effects of Hh signaling inhibition are mixed (15, 16).

Stewart and colleagues, using immunohistochemistry, found that Shh is expressed in lung epithelium in human idiopathic pulmonary fibrosis (IPF) and in murine lung inflammation and fibrosis induced by FITC (19). The same group detected Shh protein, again by immunohistochemistry, around airways in mice 6 months after FITC instillation (38). Coon and colleagues showed, by *in situ* hybridization, that *Shh* is expressed in the epithelium of cysts within the IPF lung (37). Lozano-Bolanos and colleagues showed in a recent paper that the Shh pathway is activated in human IPF (33). They also provided extensive *in vitro* data indicating that Shh increases the proliferation, migration, extracellular matrix production, and survival of fibroblasts.

Our results indicate that Hh signaling persists during bleomycin-induced lung fibrosis (Figure 3). Fibrotic lungs contain an increased number of fibroblasts and myofibroblasts derived from endogenous cells, epithelial-mesenchymal transition, and/or recruitment of circulating marrow-derived fibrocytes. Among these fibroblasts and myofibroblasts in fibrotic lesions are numerous *Gli1*⁺ cells. In addition, in regions of grossly intact distal lung, there is an increase in the fraction of alveolar wall cells that are *Gli1*⁺. This level of endogenous Hh signaling does not appear to be critical for the bleomycin-induced fibrotic response because 5E1 treatment does not reduce elevated collagen levels (Figure 4A). However, adenovirus-mediated overexpression of Shh beginning 3 weeks after bleomycin administration results in a further increase in collagen deposition (Figure 4A). Thus, Shh, expressed at a sufficient level, is capable of increasing lung fibrosis. Preliminary adenoviral experiments in which adenovirus-encoded GFP expression was assessed by immunofluorescence visualization on lung sections indicated only patchy expression in distal lung, so our experiments may have underestimated the potential effect of Shh overexpression on distal lung fibrosis. Hh signaling has been reported to increase collagen production by fibroblasts and to trigger fibroblast-to-myofibroblast transformation (50). Further work is needed to determine whether Hh signaling is of sufficient extent to promote lung fibrosis in other experimental models or in human disease.

In conclusion, our results stress the importance of Hh signaling in the lung beyond embryogenesis. Not only do we provide evidence for a previously undescribed population of mesenchymal cells with activation of Hh signaling during postnatal lung development and reactivation during experimental lung injury, we also, for the first time to our knowledge, present mechanistic data implying that this cell population critically regulates normal postnatal lung maturation and has the potential to promote adult lung fibrosis.

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