Sonic Hedgehog Signaling Regulates Myofibroblast Function during Alveolar Septum Formation in Murine Postnatal Lung

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Abstract

Sonic Hedgehog (Shh) signaling regulates mesenchymal proliferation and differentiation during embryonic lung development. In the adult lung, Shh signaling maintains mesenchymal quiescence and is dysregulated in diseases such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. Our previous data implicated a role for Shh in postnatal lung development. Here, we report a detailed analysis of Shh signaling during murine postnatal lung development. We show that Shh pathway expression and activity during alveolarization (postnatal day [P] 0–P14) are distinct from those during maturation (P14–P24). This biphasic pattern is paralleled by the transient presence of Gli1⁺;α-smooth muscle actin (α-SMA)⁺ myofibroblasts in the growing alveolar septal tips. Carefully timed inhibition of Hedgehog (Hh) signaling during alveolarization defined mechanisms by which Shh influences the mesenchymal compartment. First, interruption of Hh signaling at earlier time points results in increased lung compliance and wall structure defects of increasing severity, ranging from moderately enlarged alveolar airspaces to markedly enlarged airspaces and fewer secondary septa. Second, Shh signaling is required for myofibroblast differentiation: Hh inhibition during early alveolarization almost completely eliminates Gli1⁺;α-SMA⁺ cells at the septal tips, and Gli1-lineage tracing revealed that Gli1⁺ cells do not undergo apoptosis after Hh inhibition but remain in the alveolar septa and are unable to express α-SMA. Third, Shh signaling is vital to mesenchymal proliferation during alveolarization, as Hh inhibition decreased

proliferation of Gli1⁺ cells and their progeny. Our study establishes Shh as a new alveolarization-promoting factor that might be affected in perinatal lung diseases that are associated with impaired alveolarization.

Keywords: Sonic hedgehog signaling; postnatal lung development; myofibroblast; fibroblast; α-smooth muscle actin

Clinical Relevance

Sonic Hedgehog (Shh) signaling controls embryonic mesenchyme and is dysregulated in adult lung diseases with altered mesenchymal function, such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. To determine whether and how Shh regulates the mesenchyme during postnatal lung development, we carried out a detailed temporal-spatial analysis of Shh signaling pathway expression and activity, and characterization of cell types involved during alveolarization and maturation phase. We detect temporally distinct structural defects altering lung compliance caused by Shh inhibition during secondary septum formation and uncover Shh-induced myofibroblast differentiation and proliferation of Gli1⁺ mesenchymal cells as two key components of normal postnatal lung development. Our study establishes Shh as a new alveolarization-promoting factor that might play a role in perinatal lung injury.

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Though most lung structures are generated during embryonic lung development, the resulting saccular lung requires the final alveolar stage of development (1). The two phases of the alveolar stage, *alveolarization* and *maturation*, expand the alveolar surface and thin the air–blood interface (2). During alveolarization (~ postnatal day [P]0–P14 in rodents), secondary septa form, thereby dividing the lung into an intricate structure of connected alveoli and ducts. The balanced deposition of collagen, elastin, and proteoglycans promotes structural support and elasticity of the growing septa, and is key to the lung's mechanical properties (3). One of the cellular septum components, the myofibroblasts, produce matrix and exert contractile forces that counteract respiration-induced lung stretching (4). During maturation (~P14–28 in rodents), the secondary alveolar septa become thinner as the mesenchymal cell number decreases (5, 6), and the initial double capillary layer fuses to a single layer (2). Perinatal and early postnatal injury profoundly affect these processes, as seen in the clinical syndrome of bronchopulmonary dysplasia (7). Defining the pathways that instruct epithelial and mesenchymal cells during these phases will provide insight into the underlying mechanisms of developmental lung disease. Important roles for mesenchyme-associated signaling pathways, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor (TGF), have been described in animal models of alveolar septation (8). Though no role for Sonic Hedgehog (Shh) signaling has been discovered, it is an excellent candidate pathway, because it communicates from lung epithelium to mesenchyme during embryonic development, contributing to the balanced proliferation and differentiation of mesenchyme essential for branching morphogenesis, and is active in the postnatal phase (9). We thus hypothesized that Shh is necessary for alveolarization and maturation of the postnatal lung.

Shh is a morphogen that regulates organogenesis of the lung and other organs (10). Canonical Hedgehog (Hh) pathway activity entails binding of Hh ligand to its receptor, Patched 1 (Ptch1), followed by release of Ptch1-mediated inhibition of Smoothened (Smo), leading to

post-translational activation of the transcription factors, Gli2 and Gli3, and downstream transcription of target genes, including *Gli1*, *Ptch1*, and *Hhip* (9). *Gli1* expression, though not required for Hh signaling in most contexts, provides positive feedback and serves as a useful reporter of active Hh signaling, because *Gli1* is a direct downstream target (11). Ptch1 and Hh-inhibitory protein (HHIP), on the other hand, contribute to negative-feedback loops: Ptch1 inhibits Smo and internalizes Shh for degradation; HHIP inhibits Shh by competing with Ptch1 at the cell surface. Polymorphisms in HHIP are linked to increased susceptibility to asthma and chronic obstructive pulmonary disease (12–14).

Shh is released from lung epithelium and signals to the mesenchyme. *Shh* germline knockout results in hypoplastic lungs with defective branching and decreased mesenchyme (15–17). The absence of α -smooth muscle actin (α -SMA)⁺ cells in the bronchial walls of *Shh* knockout mice suggests that absent Shh affects cells that normally differentiate into bronchial smooth muscle cells and myofibroblasts. Mesothelial cell movement into the lung is decreased in mice with mesothelium-restricted loss of Hh signaling (18). Fate mapping of Gli1⁺ cells from embryonic day [E] 12.5 shows that they give rise to myofibroblasts at the septal tips (19). These findings suggest that Shh signaling is required for mesenchymal cell differentiation into myofibroblasts and smooth muscle cells, but the postnatal role of Shh is not well defined.

Shh and *Ptch1* mRNA are present in lung at birth (15) and Shh protein is detected until P15 (17). Up-regulation of Shh and Ptch1 expression after hyperoxia-induced lung injury in neonatal rats raises the possibility that Hh signaling might play a role in bronchopulmonary dysplasia (20). We detected Hh-responding cells (Gli1⁺) throughout the postnatal period using the *Gli1*^{nlacZ} reporter (21) (22). Whereas peribronchial and perivascular Gli1⁺ cells persist, alveolar Gli1⁺ cells decrease in number after P14. The correlation of fewer Gli1⁺ cells with alveolar maturation suggests a functional link. After P14, the septal walls are reduced to a thin capillary-rich meshwork, with a decrease in fibroblasts (23) and an increase in fibroblast apoptosis (5). These changes are consistent with loss of Hh signaling.

To better understand the role of Shh signaling during postnatal lung development, we conducted experiments to: (1) establish the spatiotemporal relationship between Shh pathway expression and activity and the cellular events underlying the structural changes during postnatal lung development; and (2) test the hypothesis that timed postnatal abrogation of Shh pathway activity alters lung mesenchymal function.

Materials and Methods

Animal Experiments

All procedures were approved by the Institutional Animal Care and Use Committee at New York University Langone Medical Center (New York, NY). Heterozygous animals of the following genotypes were used: *Gli1*^{nlacZ} (22), *Shh*^{nlacZ} (24), *Gli1*^{creERT2} (25), and *R26R*^{tdTom} (26). For lineage-tracing experiments, *Gli1*^{creERT2} mice were cross-bred with *R26R*^{tdTom} to generate double-heterozygous mutants. Genotyping was done as previously described (21). *C57BL/6J* and *R26R*^{tdTom} mice were from Jackson Laboratories (Bar Harbor, ME).

For Hh inhibition experiments, neonatal animals were injected subcutaneously with 30 mg/kg of pan-Hh antibody 5E1 (ImmunePrecise, Victoria, BC, Canada) (27) or IgG isotype control (Lampire, Pipersville, PA) at P1, P3, P5, or P7. To activate Cre-recombinase for lineage-tracing experiments, mice received tamoxifen (250 μ g/g) or corn oil subcutaneously at P1. Recombination of *tdTom* was detected at P3 in tails by immunofluorescence (IF) microscopy. Recombined animals received one subcutaneous dose of 5E1 or IgG.

Lung Histology and Morphometry

Animals were killed using pentobarbital (120 mg/kg) at indicated time points. For IF-based localization studies and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining, lungs were fixed with 4% paraformaldehyde and optimal cutting temperature (OCT) medium embedded (21). For morphometric analysis, the lungs were inflated with 4% paraformaldehyde at 25 cm H₂O (21). Hematoxylin and eosin-stained lung sections (5 μ m) were analyzed, blinded to treatment group and

sample number. At least 10 random fields of the alveolar compartment were analyzed, including both right and left lung, using a Nikon Ni epifluorescence microscope and Nikon NIS-Elements software (Nikon, Melville, NY). To assess the alveolar airspace, the mean linear intercept (MLI) was measured (21). To assess secondary septum formation, we measured the alveolar septal tip count, calculated by dividing the number of septal tips per image by the number of complete alveolar spaces per image (see Figure E3D in the online supplement). We used Picrosirius red, Resorcin-Fuchsin and periodic acid-Schiff-Alcian blue staining to detect collagen, elastin, and proteoglycans, respectively.

Pressure-Volume Curves

Lung volumes were measured at different pressures through *ex vivo* inflation with PBS, following Shifren's protocol with minor modifications (see the online supplement) (28).

Lung Elastin, OH-Proline, and Total Protein Content

Lung elastin, OH-proline and total protein content were measured as described previously (28).

IF Staining

OCT-frozen lung sections (5 μ m) were stained with primary and IF-labeled secondary antibodies (see the online supplement) (21). Terminal deoxynucleotidyl transferase dUTP nick end labeling staining was done per the manufacturer's recommendations (Roche, Indianapolis, IN). IF-based colocalization quantification was performed on high-, medium-, and low-power images using an Ni epifluorescence microscope and Nikon NIS-Elements software (21), as well as a Vectra3 multispectral-imaging system (PerkinElmer, Waltham, MA) and InForm software (Chicago, IL).

Quantitative RT-PCR

Total RNA extraction from lung, reverse transcription and quantitative RT-PCR (qRT-PCR) were performed following standard protocols (see the online supplement). qRT-PCR was performed using a Step-One-Plus instrument (Applied Biosystems, Waltham, MA) and the Quantifast SYBR-green mix (Qiagen, Germantown, MD). Assays were

normalized to *Gapdh* and *Hprt*, and relative gene expression was calculated by the $2^{-\Delta(\Delta CT)}$ method. Gene expression changes were considered significant if the Student's *t* test indicated a *P* value less than 0.05 in both *Gapdh*- and *Hprt*-normalized samples.

Statistical Analysis

Data are presented as mean ($\pm$ SD). Student's *t* test was used for statistical analysis, with *P* less than 0.05 considered significant.

Results

Shh Pathway Expression and Activity during Alveolarization and Maturation

We first established the temporal relationship between Hh pathway genes and structural processes during secondary septum formation and maturation. Therefore, we measured mRNA expression of *Shh*, *Gli1*, *Ptch1*, and *Hhip* in *C57BL/6J* mice, and assessed Hh pathway activity in *Gli1^{nlacZ}* reporter mice (22). These data were related to morphometric and cell proliferation measurements (Figure 1). There is a marked increase in the expression of *Shh*, *Gli1*, and *Ptch1* by P2. *Shh* expression peaks between P2 and P14. Similarly, expression of the direct transcriptional target *Gli1* peaks between P2 and P10, whereas *Ptch1* and *Hhip* show more modest changes and remain elevated until P18 (Figures 1A and 1B). In *Gli1^{nlacZ/+}* reporter mice, the fraction of *Gli1*⁺ cells in the alveolar area decreases after birth to approximately 11% at P2, and thereafter increases to 30–32% at the peak around P8–P10 (Figure 1C). We also examined the spatial distribution of *Gli1*-expressing cells throughout the postnatal period (Figure E1). At P0, *Gli1*⁺ cells are localized around the airways and in primary septa. After a nadir of several days, in which *Gli1*⁺ cells are mostly located in peribronchial and perivascular locations, increased numbers of *Gli1*⁺ cells appear in the alveolar septa, including the tips, peaking around P8–P10 and declining thereafter, whereas peribronchial and perivascular *Gli1*⁺ cells remain constant. MLI measurements document the underlying process of alveolarization between P3 and P9 (Figure 1D). Beyond P9, the MLI stabilizes, consistent with reports that, during lung maturation,

the number and size of alveoli do not change significantly (29). The data reveal a biphasic pattern, where *Shh* signaling and expression is high during alveolarization and falls during maturation.

Given the proliferative effect of *Shh* signaling, we assessed cell proliferation in the alveolar compartment. The percentage of proliferating (Ki67⁺) cells increases from approximately 6% at P2 to approximately 18% at P6, and declines to approximately 5% at P14 (Figure 1E). We also measured proliferative indexes of cells positive or negative for reporters of *Shh* and *Gli1* expression (*Shh^{nlacZ}* and *Gli1^{nlacZ}* mice; Figure 1F). *Gli1*⁺ cell proliferation peaks during alveolarization at P6, whereas proliferation of *Shh*⁺ cells, which are *Gli1*[−] and should be unresponsive to Hh ligand, remains at similar levels from P2 to P6 and then declines. The magnitude of the proliferation index of *Gli1*⁺ cells indicates that they are a prominent component of the mesenchymal cell expansion during alveolarization (30).

Characterization of Cells Expressing Shh or Gli1 during Postnatal Lung Development

A large body of evidence supports the strict dichotomy between *Shh*-expressing epithelial cells and *Ptch1*-expressing mesenchymal cells during embryonic lung development (9, 31). To identify the cell types involved in *Shh* signaling in postnatal lung, and to confirm the persistence of this dichotomy in the postnatal period, we characterized *Shh*[−] and *Gli1*-expressing cells in appropriate reporter mice based on IF costaining with cell-specific markers and anatomic location (see summary in Table E1). Fluorescent cells were analyzed in both the peribronchial and the alveolar compartment.

Shh⁺ cells line the airway lumen and express the epithelial markers, E-cadherin (Figure 2A, *a*) and pan-cytokeratin (Figure E2A, *a*). These cells are distinct from the underlying layer of α -SMA⁺; *Shh*[−] cells (Figure 2A, *d*). In the alveolar compartment, *Shh*⁺ cells are E-cadherin⁺ and constitute 10–15% of all E-cadherin⁺ cells (Figure 2A, *b*). Approximately 70% of *Shh*⁺ cells express the alveolar type I cell marker, receptor for advanced glycation endproducts (RAGE), and approximately 30% of the *Shh*⁺ cells express the alveolar type 2 marker, surfactant protein (SP) C

Figure 1. Sonic Hedgehog (Shh) pathway expression and activity during alveolarization and maturation phase. (A and B) mRNA expression of Hedgehog (Hh) pathway genes was measured in *C57BL/6J* mice at indicated time points ($n = 3$ /time point). A biphasic pattern of Hh pathway gene expression is seen, high during alveolarization and low during maturation. Normalized gene expression is shown in fold change from postnatal day (P) 0 (A) and details of mean and SD for each time point (B). (C) Gli1⁺ cells in the alveolar compartment were detected in *Gli1^{lacZ/+}* mice by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining at indicated time points ($n = 3$ /time point). (D) Mean linear intercept (MLI) measurements of postnatal lungs inflated at 25 cm H₂O pressure are shown ($n = 3$ –6/time point). (E) Proliferative index of all alveolar lung cells was assessed at indicated time points by Ki67 immunofluorescence (IF) staining ($n = 3$ /time point). (F) Proliferative index in Shh⁺ and Gli1⁺ cells from *Shh^{lacZ/+}* and *Gli1^{lacZ/+}* lungs, respectively, was assessed at indicated time points by Ki67 IF staining ($n = 3$ /time point). For all experiments (A–F), data are shown as mean and SD for each time point.

Figure 2. Characterization of *Shh*- and *Gli1*-expressing cells during postnatal lung development. The peribronchial compartment (bronchus lumen, dotted lines) and the alveolar compartment of immunostained (A) *Shh*^{nlacZ/+} lung sections and (B and C) *Gli1*^{nlacZ/+} lung sections were evaluated by co-IF, and representative images from P6 are shown. Detected proteins by color are indicated on each image, and DAPI nuclear staining appears blue. (A) High-power images show *Shh*⁺;Ecad⁺ cells in bronchial epithelial cells (a) and dispersed in the alveolar walls (b). *Shh*⁺;SPC⁺ cells are present in the alveolar walls (c). *Shh*⁺ cells are α -SMA⁻ in the bronchi (d) and the alveoli (e). X-gal staining shows b-gal⁺ cells in the bronchial epithelial layer and in the alveolar spaces (f). (B) Peribronchial *Gli1*⁺ cells are vimentin (vim)⁺ (d), but mostly collagen-1 (col1)⁻ (a), α -SMA⁻ (b), fibroblast-specific protein (FSP)⁻ (e), and CD31⁻ (h). Alveolar *Gli1*⁺ cells are mostly col1⁺ (c) and vim⁺ (f). *Gli1*⁺;PDGFR α ⁺ cells are detected in both the peribronchial (g) and alveolar compartment (i). (C) A time course from P2 to P6 shows appearance of *Gli1*⁺; α -SMA⁺ cells at the tips of the growing secondary alveolar septa (arrows), which constitute approximately 25–30% of all alveolar *Gli1*⁺ cells. α -SMA, α -smooth muscle actin; b, bronchus; Ecad, E-cadherin; PDGFR, platelet-derived growth factor receptor; SPC, surfactant protein C; v, vessel; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Scale bars: 50 μ m.

(Figure 2A, c). *Shh*⁺ cells do not express the mesenchymal markers, α -SMA (Figure 2A, e) and collagen-1 (Figure E2A, b), the endothelial marker, CD31 (Figure E2A, c), or the hematopoietic marker CD45 (data not shown).

We also examined *Gli1*⁺ cells (Figures 2B and 2C). In the peribronchial areas, most *Gli1*⁺ cells are negative for the fibroblast marker, collagen-1 (Figure 2B, a), in contrast to adult lung (21). Conversely, in the alveolar compartment, almost all *Gli1*⁺ cells are collagen-1⁺ (Figure 2B, c). The majority of *Gli1*⁺ cells are vimentin⁺, but are fibroblast-specific protein 1⁻ (Figure 2B, d and e). Although adjacent to α -SMA⁺ bronchial smooth muscle cells, most of the peribronchial *Gli1*⁺ cells are α -SMA⁻ (Figures 2B, b, and Figure 5C).

Given the requirement of PDGFR α ⁺ fibroblasts for secondary septum formation (32), we also examined colocalization of PDGFR α with *Gli1*, and found that 20–25% of peribronchial and 10–15% of alveolar *Gli1*⁺ cells also express PDGF receptor α (PDGFR α) (Figure 2B, g and i). *Gli1*⁺ cells do not express E-cadherin, SPC, CD45 (Figure E2), or CD31 (Figure 2B, h). In the alveolar compartment, approximately 30% of collagen-1⁺; *Gli1*⁺ cells are also α -SMA⁺ (Figure 2C). Interestingly, most collagen-1⁺ and α -SMA⁺ cells are located at the septal tips of secondary septa, and are increasingly numerous during the beginning of alveolarization. The presence of 10–15% *Gli1*⁺;PDGFR α ⁺ cells in the alveolar septal walls and tips (Figure 2B, i) suggests that they are part of the population of α -SMA⁺;PDGFR α ⁺ fibroblasts described by others (32, 33). We performed triple IF for α -SMA, *Gli1*, and PDGFR α , and found that approximately 50% of all α -SMA⁺; *Gli1*⁺ cells are also PDGFR α ⁺, with many of the triply labeled cells located at the septal tips (Figure E2C).

Overall, these results indicate that *Gli1*⁺ cells in the alveolar compartment are interstitial fibroblasts and myofibroblasts, consistent with lineage-tracing experiments for myofibroblasts (19). In contrast, most peribronchial and perivascular *Gli1*⁺ cells are fibroblasts, and only few if any are peribronchial smooth muscle cells. In the peribronchial compartment, *Shh*⁺ cells are bronchial epithelial cells, and in the alveolar compartment, alveolar type 1 and 2 epithelial cells.

Inhibition of Hh Signaling during Alveolarization Affects Secondary Septum Initiation and Lung Compliance

We previously shown that Hedgehog (Hh) pathway inhibition at P3 with the anti-Hh monoclonal antibody, 5E1, causes increased MLI (21). To confirm that 5E1 affects Hh canonical signaling in this context, we examined several downstream target genes. As expected, 5E1 treatment at P3 decreases expression of *Gli1*, *Ptch1*, and *Hhip* (Figure E3A). *Gli1* expression is decreased by approximately 90%, whereas *Ptch1* and *Hhip* expression levels are decreased by approximately 55% and approximately 80%, respectively. In contrast, expression levels of *Smo* and the genes encoding Hh ligands (*Shh*, *Ihh*, and *Dhh*), which are not transcriptional Hh targets, are unchanged (Figure E3A). These data confirm that 5E1 blocks canonical Hh pathway activity and does not affect epithelial *Shh* expression.

We examined lung structure after Hh inhibition. A time course experiment shows that Hh inhibition initiated at P3 affects lung morphology as early as P6 (Figure E3B). Morphometric assessment was conducted on P6 and P8 lungs. To determine whether there is a decrease in numbers of secondary septa formed, we measured the alveolar septal tip count, defined as the ratio of the number of alveolar septal tips to the number of alveolar air spaces (Figure E3D). Hh blockade at P1 decreases the septal tip count by approximately 20%, along with increasing the MLI, at P6 and P8 (Figures 3A and 3B), indicating that Hh inhibition impaired septum initiation or, less likely, resulted in destruction of newly formed septa. In contrast, Hh inhibition at P3 increases MLI, but does not alter septal tip count at P6 and P8 (Figures 3A and 3B), suggesting that the critical *Shh* signaling period for alveolar septum initiation is before P3. We further tested at which time point Hh inhibition no longer affects MLI. We found that Hh inhibition at P5 results in subsequent increased MLI, but inhibition at P7 does not (Figure E3C). To test whether the MLI increase after Hh inhibition at P3 could at least in part be due to decreased compliance and increased size of the lung when inflated at 25 cm H₂O pressure, we measured wet lung weight and lung volume at different inflation pressures *ex vivo* in 5E1- and

IgG-treated lungs at P8 (Figure 3C). Wet lung weights and body weights were not different between the treatment groups. In contrast, the pressure–volume curves show 16–24% increased lung volumes in Hh-inhibited lungs at inflation pressures of 15–25 cm H₂O.

Inhibition of Hh Signaling Alters Gene Expression of Structurally Important Extracellular Matrix and Intracellular Molecules

In liquid-inflated lungs, compliance is a function of extracellular matrix (ECM) components, such as elastin, collagen, proteoglycans, and (probably to a lesser extent) the cell compartment. Therefore, we evaluated whether blockage of Hh signaling affects expression of important fibroblast and myofibroblast genes encoding the ECM molecules, collagen-1 (*Col1a1*), collagen-4 (*Col4a1*), elastin (*Eln*), fibrillin (*Fbn1*), fibronectin (*Fn1*), versican (*Vcan*), and the cellular molecules, vimentin (*Vim*), Desmin (*Des*), Transgelin (*Tagln*), and α -SMA (*Acta2*). We also assessed mesenchyme-associated signaling pathways that are essential to lung development and for which cross-talk with Hh signaling has been reported (9), such as PDGF (*Pdgfa*, *Pdgfra*, *Pdgfrb*), FGF (*Fgf10*), connective tissue growth factor (CTGF) (*Ctgf*), TGF- β /bone morphogenetic protein (BMP) (*Tgfb1–3*, *Ltbp1–4*, *Serpin1*, *Itgb6*, *Itgb8*) and wingless-related integration site (Wnt) (*axin*, *Lef1/Tcf7*, *Tcf7L2*, *Wif1*) (Figure E4A). qRT-PCR analyses from total lung extracts indicate that Hh inhibition does not alter expression of most of these genes and pathways. mRNA expression levels of *Col1a1*, *Col4a1*, *Eln*, *Fn1*, and *Fbn1*, encoding ECM proteins that are important components of primary and secondary septa, are not decreased after Hh inhibition. However, expression of *Vcan*, another ECM component, is significantly reduced. Furthermore, expression of *Vim* and *Acta2*, which is important to the function of bronchial smooth muscle cells and myofibroblasts (4), are also significantly altered in 5E1-treated lungs.

Hh Pathway Inhibition Decreases Expression of the Proteoglycan, Versican

We assessed the amount and distribution of collagen, elastin, and proteoglycans in

5E1-treated lungs. We measured total collagen-1 and elastin protein at P8 after Hh inhibition and found no significant differences (Figure E5). We also performed histochemical stainings at P8 to screen for expression of collagens (Picrosirius red), elastin (Resorcin-Fuchsin), and proteoglycans (periodic acid–Schiff–Alcian blue). Although we did not observe alterations in staining intensity or distribution for collagens and elastin (data not shown), we found decreased proteoglycan staining in the septal walls and tips (Figure 4A, *a* and *b*).

Versican, a proteoglycan secreted by fibroblasts, is involved in lung development and tissue remodeling in lung disease (34), and is induced by Hh signaling in the developing enteric nervous system (35). We found that, at P8, versican is expressed in the alveolar compartment (Figure 4A, *c* and *d*), but not in peribronchial areas (data not shown). Versican⁺ cells are present in the septal walls and tips. In *Gli1^{lacZ}* lungs, versican⁺;Gli1⁺ cells are present in the alveolar septal walls and tips. Approximately 50% of versican⁺ cells are also Gli1⁺. Septal versican⁺ cells are markedly decreased in 5E1-treated C57 and *Gli1^{lacZ}* lungs (Figures 4A and 4C). Quantification in *Gli1^{lacZ}* lungs shows that Hh inhibition decreases the fraction of septal versican⁺ cells from 30 to less than 5% (Figure 4D), consistent with our mRNA expression data (Figure 4B).

Inhibition of Hh Signaling Causes a Decrease in Myofibroblasts at the Septal Tips

As both myofibroblasts and peribronchial smooth muscle cells possess the contractile cell element, α -SMA we tested whether Hh inhibition affects the numbers of α -SMA⁺ cell at the septal tips or around bronchi in C57 and *Gli1^{lacZ}* lungs. Strikingly, after Hh inhibition, there is an almost complete absence of α -SMA⁺ cells at the septal tips at P6 (Figures 5B–5D). In contrast, peribronchial α -SMA⁺ cells are not affected by 5E1 treatment (Figures 5C, 6B, and 6C). A similar pattern is observed for Gli1⁺ cells, which are mostly absent in the septa, but remain around the airways in the 5E1-treated lungs. Inhibition of Hh signaling at both P3 and P1 equally abrogated α -SMA

Figure 3. Inhibition of Hh signaling during alveolarization affects both secondary septum initiation and septum composition. C57BL/6J mice ($n = 3$ – 6 /group) were treated with a single dose of either anti-Hh antibody 5E1 or isotype IgG (30 mg/kg) at P1 or P3. Lungs, harvested and inflated at 25 cm H₂O pressure at (A) P6 and (B) P8, were fixed, and cut sections were examined for morphologic (left panel images) and morphometric changes (MLI, alveolar septal count; middle and right panel). Lungs that were 5E1 treated at P3 show enlarged airspaces, but unchanged alveolar septal count at P6 and P8 (images and MLI). 5E1 treatment at P1 results in both increased MLI and decreased alveolar septal tip count. Scale bars: 50 μm . (C) Lungs were isolated from 5E1-treated ($n = 6$) and IgG-treated ($n = 5$) mice at P8. Pressure–volume curves show increased compliance of 5E1-treated lungs at 15, 20, and 25 cm inflation pressure (left panel). Body weight (middle panel) and wet lung weight (right panel) were not affected by 5E1 treatment. For all experiments, data are shown as mean and SD for each time point. * $P < 0.05$.

Figure 4. Inhibition of Hh signaling decreases expression of the extracellular matrix molecule versican (Vcan) in the alveolar septa. Mice were treated with 5E1 or IgG (30 mg/kg) at P3 and evaluated at indicated time points. (A) Lung sections of *C57BL/6J* mice at P6 were stained with periodic acid–Schiff–Alcian blue to detect proteoglycans (a and b). IF staining for versican in *C57BL/6J* lungs at P6 show decreased expression in the alveolar septa and septal tips (c and d). (B) *Vcan* mRNA expression is reduced in 5E1-treated lungs at P5 ($n = 6\text{--}7/\text{group}$, two litters). (C) Co-IF for Vcan and Gli1 in P6 *Gli1^{nlacZ/+}* shows the presence of Gli1⁺;Vcan⁺ cells at the septal tips, and Hh inhibition decreases versican expression in Gli1⁺ and Gli1[−] cells in the alveolar septa. DAPI-stained nuclei appear blue. Scale bars: 50 μm . (D) Quantification shows a significant decrease in both Gli1⁺ and Vcan⁺ cells at P6. Data are shown as mean and SD for each time point.

expression in cells at the septal tips, but had no gross effect on the numbers and appearance of bronchial smooth muscle cells or SPC⁺ epithelial cells. These findings show that the presence of myofibroblasts at the septal tips during alveolarization is dependent upon canonical Hh signaling and indicates alteration of a cellular septum component by Hh signaling.

Inhibition of Hh Signaling Blocks Gli1⁺ Cell Differentiation to Myofibroblasts

We next asked whether the absence of Gli1-expressing myofibroblasts in the alveolar

septa after Hh blockage is due to loss of the Gli1 lineage-labeled cells or failure to differentiate. To track the Gli1 lineage throughout postnatal lung development, we employed the *Gli1^{creERT2};R26^{tdTom}* reporter, which allows labeling of cells with active Hh signaling by tamoxifen-induced Cre-recombinase-mediated activation of *tdTomato* (*tdT*) expression (26). The progeny of P1-labeled Gli1-expressing cells are detectable at P6 and P10 as tdT⁺ cells in three locations: alveolar septa (including the tips), the visceral pleura, and the peribronchial smooth muscle layer (Figures

6B and 6C). tdT⁺ cells are at the alveolar septa tips, and many are also $\alpha\text{-SMA}^+$. They are in the same location as the Gli1⁺; $\alpha\text{-SMA}^+$ cells that we (Figure 2C) and others (19) described. Interestingly, there are also numerous tdT⁺; $\alpha\text{-SMA}^+$ cells within the smooth muscle cell layer of airway walls. These lineage data suggest that Gli1⁺ mesenchyme provides progenitor cells for myofibroblasts and bronchial smooth muscle cells during postnatal lung development, both of which presumably lose Gli1⁺ expression once differentiated.

Figure 5. Inhibition of Hh signaling causes a decrease in myofibroblasts at the septal tips. Mice were treated with 5E1 or IgG (30 mg/kg) at P3 and evaluated at indicated time points. (A) *Acta2* mRNA expression levels are decreased in 5E1-treated lungs that were harvested at P5 ($n = 6-7$ /group, two litters). (B and C) Lungs of *C57BL/6J* and *Gli1^{nlacZ}* mice were sectioned and immunostained at P6. (B) Representative images of *C57BL/6J* lungs show strong cellular staining in the alveolar septal tips, which is abolished by Hh inhibition. (C) Co-IF staining for α -SMA, and Gli1 in *Gli1^{nlacZ}* lungs shows that Gli1⁺; α -SMA⁺ cells are absent in the alveolar septal walls after Hh inhibition (a and b). In contrast, peribronchial Gli1⁺ cells, which are distinct from α -SMA⁺ bronchial smooth muscle cells, remain after Hh inhibition (c and d). DAPI-stained nuclei appear blue. b, bronchus. Scale bars: 50 μ m. (D) Quantification of Gli1⁺ cells in the alveolar compartment shows almost complete absence in the alveolar septa with 5E1 treatment. Data are shown as mean and SD for each time point.

We next assessed whether cells of the Gli1 lineage are present or absent in septal tips after Hh inhibition. We labeled Gli1 lineage at P1, followed by Hh inhibition at P3 (Figure 6A); tdT⁺ cells were present in the alveolar septal walls at P6 (Figure 6B), equal to the IgG control (Figure 6D). However, there are no α -SMA⁺;tdT⁺ cells in the septa in 5E1-treated lungs. These findings indicate that the P1-labeled Gli1 lineage survives to at least P6, despite Hh inhibition at P3. They and/or their progeny persist in the septal walls, but fail to differentiate into myofibroblasts. Lung evaluation at P10 in the same experimental setup shows that tdT⁺ cells are still

present in the alveolar septa with Hh inhibition, but are significantly decreased in number (Figures 6C and 6D), possibly due to changes in proliferation or apoptosis of these cells after P6. Of note, in the same experiments, there was no effect on numbers of α -SMA⁺;tdT⁺ bronchial smooth muscle cells.

Inhibition of Hh Signaling Blocks Gli1⁺ Cell Proliferation

Given the proliferative and antiapoptotic properties of Hh signaling, we tested whether cell proliferation or apoptosis is affected by Hh pathway inhibition. We evaluated 5E1-treated and control

lungs at P4, P5, and P6 for Ki67 expression (proliferation) and terminal deoxynucleotidyl transferase dUTP nick end labeling reactivity (apoptosis). Although there is no difference in the percentage of apoptotic cells in the alveolar compartment at P4, P5, and P6 after 5E1 treatment (data not shown), we found an approximately 30% decrease in the number of Ki67⁺ cells by P6 (Figure 7A). To test whether Gli1⁺ cells contribute to the decrease in proliferating cells, we assessed Ki67 staining in *Gli1^{nlacZ/+}* lungs at P6. In the 5E1-blocked lungs, overall proliferation, as well as Gli1⁺ cell proliferation, are decreased (Figure 7B). We also measured overall and

Figure 6. Inhibition of Hh signaling blocks differentiation of Gli1⁺ cells to myofibroblasts at P6. Representative images of immunostained lungs are shown. (A) Gli1 lineage was traced in the *Gli1^{creERT2};R26^{tdTom}* reporter, by tamoxifen (Tam) administration at P1, treated with 5E1 or IgG at P3, and evaluated at P6 and P10. (B and C) Co-IF for tdTomato (tdT) and α-SMA shows that Gli1 lineage (tdT⁺, red) is present in three different areas of postnatal lung at P6 and P10: the alveolar septal tips, the pleura mesothelium, and in and around the bronchial smooth muscle cell layer. α-SMA staining is seen in septal myofibroblasts, and in bronchial and vascular smooth cells. tdT⁺ cells in the alveolar compartment of 5E1-treated lungs are α-SMA⁻ at P6 and P10. Of note, peribronchial α-SMA⁺ cells remain present after Hh inhibition. DAPI-stained nuclei appear blue. b, bronchus. (B) Quantification of tdT⁺ cells at P6 and P10 shows a significant decrease at P10, but not at P6. Scale bars: 250 μm, 100 μm, and 50 μm. Data are shown as mean and SD for each time point.

Figure 7. Inhibition of Hh signaling decreases proliferation of Gli1⁺ cells and their progeny. Mice were treated with 5E1 or IgG (30 mg/kg) at P3 and evaluated at indicated time points. (A) Proliferative index of all alveolar cells in C57BL/6J lungs ($n = 4$ –6/group, two litters), evaluated with Ki67 staining at P4, P5, and P6, is significantly different at P6. (B) Proliferative index in treated Gli1^{nlacZ/+} lungs at P6 ($n = 3$ –4/group) shows a decrease in all alveolar cells and the Gli1⁺ cells, whereas it is unchanged in Gli1⁻ cells. (C) Proliferative index in 5E1-treated Gli1^{creERT2/+};R26^{tdTom} reporter ($n = 4$ /group) shows decrease in total alveolar cells and in the Gli1 lineage. Data are shown as mean and SD for each time point.

Gli1 lineage-specific proliferation in Gli1^{creERT2/+};R26^{tdTom} lungs treated with tamoxifen at P1 and with 5E1 or control at P3. Again, total proliferation, as well as proliferation of tdT⁺ cells, are decreased (Figure 7C), consistent with our lineage-tracing data showing a decrease of Gli1 lineage in 5E1-treated lungs at P10 (Figure 6D). These data, together with the phenotypic changes after Hh inhibition, indicate that Hh signaling provides a proliferative stimulus to the mesenchyme during alveolarization.

Discussion

In this study, we aimed to elucidate the role of Shh signaling during postnatal lung development. We established that the biphasic pattern of Hh pathway gene expression and activity parallels the temporal pattern of alveolarization (expression high) and maturation (expression low). We revealed four specific functions of Hh signaling during alveolarization with consequences on lung structure and the mesenchymal compartment: (1) Hh signaling is required for myofibroblast differentiation during growth of the secondary alveolar septa; (2) Hh signaling promotes mesenchymal

proliferation, especially in the Gli1 lineage; (3) Hh signaling is required for normal versican expression; and (4) very early inhibition of Hh signaling reduces the number of new septa formed.

In this study, we show that Shh signaling affects alveolar myofibroblasts, an essential component of the growing secondary septa, where they are most prominent during alveolarization and disappear during maturation (36). Myofibroblasts produce elastin and express contractile elements, such as α -SMA that may maintain tension of the alveolar entry rings (37). PDGFA signaling is required for myofibroblasts during secondary septum formation (32, 33). Other signaling pathways, such as FGF, CTGF, and TGF, also influence alveolarization; however, their link to myofibroblast function is less clear (8). Here, we identify an additional pathway, Shh signaling, that is required for alveolar septal myofibroblast differentiation. We show that approximately 30% of alveolar cells expressing the Shh target Gli1⁺ cells also coexpress α -SMA. In the absence of canonical Hh signaling, septa are almost totally devoid of α -SMA-expressing myofibroblasts. Furthermore, cells previously expressing Gli1 are not eliminated after Hh signaling is inhibited,

but are unable to continue or initiate α -SMA expression, suggesting that their differentiation to myofibroblasts is blocked.

Our data demonstrating that myofibroblast differentiation in postnatal lung is regulated by Hh signaling are corroborated by several observations made by others. First, α -SMA-expressing cells are absent in Shh^{-/-} lungs (38). Second, the embryonic Gli1 lineage gives rise to septal myofibroblasts in the secondary septa (19). Third, there is a Gli consensus site within the *Acta2* promoter, and Gli1-dependent *Acta2* expression is inducible by Hh pathway activation (39). Furthermore, we showed that *Acta2* expression throughout the postnatal period shows a peak during alveolarization and significantly lower levels thereafter, a time course similar to the one that we observed for Hh pathway genes. Though alveolar myofibroblasts are thought to be the major source of elastin, we were unable to demonstrate decreased elastin expression in total lung RNA and protein, or altered deposition, when Hh is blocked.

It is important to reconcile our findings with what is known of PDGFA signaling in postnatal lung development. Besides the vital role of PDGFA for the process of alveolarization, studies by others suggest

that both Hh and PDGF α affect fibroblasts and myofibroblasts in the growing alveolar septa. McGowan and colleagues (40) report that PDGF α^+ ; α -SMA $^+$ cells, which reside in the alveolar entry rings of alveolar ducts, accumulate at the septal tips around P4–P8, similar in timing and location to the Gli1 $^+$; α -SMA $^+$ cells we observe, suggesting that these two cell populations substantially overlap, or might even be the same. Their observed peak of *Ptch1* and *Gli1* expression at P8 in primary lung fibroblasts is consistent with our observations of Hh pathway target expression and pathway activity. The fact that 50% of alveolar α -SMA $^+$; Gli1 $^+$ cells are also PDGF α^+ further strengthens this idea. Based on their work, McGowan and McCoy (41) proposed a gradient along the septum between Hh and PDGF α signaling that guides mesenchymal cell migration from septum base to tip. Our inhibition data suggest that Hh signaling is required for normal septal composition, and at least in part for septum initiation, independent of the PDGF α -mediated differentiation signal, in that the number of PDGF α -expressing cells in the alveolar compartment was not decreased after Hh inhibition. The data also suggest that PDGF α pathway expression is not downstream of Hh signaling, as others have hypothesized (19). PDGF α is present throughout embryogenesis, and therefore the lung septation defect in PDGF α knockout mice is very severe. It is not known how inhibition of PDGF α signaling in the early postnatal period would affect septum initiation and elongation. It is possible that the observed lung phenotype after Hh inhibition is due to effects of Hh signaling on other signaling pathways involved in alveolarization, such as FGF, CTGF, and TGF (8). Our mRNA screening, however, did not show gene alterations for FGF10, CTGF, TGF, and Wnt signaling molecules. Therefore, we did not choose to explore these pathways further.

Our data provide evidence for differentiation- and proliferation-promoting roles for Shh in the postnatal lung. However, it is less clear when this particular role ends or switches to the opposite role of maintaining adult mesenchyme quiescent (42). We observe high numbers of Gli1 $^+$ proliferating cells during alveolarization, and a decrease in Gli1-lineage proliferation when Hh

signaling is blocked. During maturation, Gli1 $^+$ cells decrease substantially in the alveolar compartment (our data) and, at the same time, the mesenchymal space and its cell number decrease, accompanied by an apoptosis peak around P18 (5, 6). We did not find increased apoptosis when Shh signaling was inhibited during early alveolarization, suggesting that a Shh deficit alone may not account for the mesenchymal apoptosis peak that occurs during the later normal decline in Shh expression and signaling (5).

In adult lung, we and others observe very few Gli1 $^+$ cells in the alveolar compartment, whereas the peribronchial and perivascular pool remains prominent (21, 42, 43). It is possible that the few residual Gli1 $^+$ cells in the alveolar compartment are lipofibroblasts, which have been proposed as progenitors to myofibroblasts and are essential to realveolarization in adult lung (44, 45). Our time course data for *Acta2* expression, and the fact that α -SMA $^+$ cells in the alveolar compartment after P21 are restricted to the alveolar ducts (36), suggest that most of the alveolar Gli1 $^+$ cells only differentiate in the presence of Shh ligand in the clearly defined time window that we have established. In adult lung, during bleomycin injury, we showed an increase of Gli1 $^+$; α -SMA $^+$ myofibroblasts in the fibrosis regions (21), and others demonstrated that the Gli1 lineage gives rise to these cells, consistent with the myofibroblast differentiation-promoting role of Shh. Our studies in postnatal lung, therefore, might allow us to use this knowledge when investigating the role of Shh signaling in mesenchyme in pulmonary fibrosis.

We have shown here that inhibition of Hh signaling in the postnatal period increases lung compliance. Thus, quantitative and/or qualitative aspects of lung composition (cellular or extracellular) that contribute to compliance must be altered in a way that increases compliance. We evaluated several lung components that might contribute to compliance, and identified one ECM component, the proteoglycan, versican, and one cellular component, the interstitial myofibroblast, that are dramatically altered by Hh inhibition. However, we do not yet know if either, or

both, of these effects account for the change in compliance. Versican, a chondroitin-sulfate proteoglycan, is expressed by mesenchymal cells in many organs, including the lung (34). Together with other proteoglycans, it creates hydrated gels in the alveolar septal wall interstitium in which collagen and elastin are embedded (3). In adult lung, versican shows a patchy expression pattern in the alveolar septa, and is thought to be expressed by fibroblasts (46). Versican is the predominantly expressed mesenchymal proteoglycan during the development of terminal sacs and alveoli in late-gestation sheep lungs (47). High versican expression levels have also been found in human embryonic lung fibroblasts (48) and in multipotent mesenchymal stem cells during differentiation *in vitro* (49). In this work, we find significant versican expression in the alveolar septa, and our co-IF data suggest that versican is at least in part produced by Gli1 $^+$ fibroblasts. Hh inhibition decreased the number of versican $^+$ cells in the alveolar septa, consistent with other developmental studies in which epithelial Shh was found to induce mesenchymal versican expression (35, 50).

In summary, we demonstrate that Shh signaling regulates mesenchyme during alveolarization by stimulating mesenchymal proliferation as well as myofibroblast differentiation and function in the growing secondary septa, thereby affecting the mechanical properties of the lung. This regulation is important for normal postnatal lung development. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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