Sonic hedgehog regulates branching morphogenesis in the mammalian lung

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The mammalian lung, like many other organs, develops by branching morphogenesis of an epithelium [1]. Development initiates with evagination of two ventral buds of foregut endoderm into the underlying splanchnic mesoderm. As the buds extend, they send out lateral branches at precise, invariant positions, establishing the primary airways and the lobes of each lung. Dichotomous branching leads to further extension of the airways. Grafting studies have demonstrated the importance of bronchial mesenchyme in inducing epithelial branching, but the significance of epithelial signaling has largely been unstudied. The morphogen *Sonic hedgehog* (*Shh*) is widely expressed in the foregut endoderm and is specifically upregulated in the distal epithelium of the lung where branching is occurring [2]. Ectopic expression of *Shh* disrupts branching and increases proliferation, suggesting that local *Shh* signaling regulates lung development [2]. We report here that *Shh* is essential for development of the respiratory system. In *Shh* null mutants, we found that the trachea and esophagus do not separate properly and the lungs form a rudimentary sac due to failure of branching and growth after formation of the primary lung buds. Interestingly, normal proximo-distal differentiation of the airway epithelium occurred, indicating that Shh is not needed for differentiation events. In addition, the transcription of several mesenchymally expressed downstream targets of Shh is abolished. These results highlight the importance of epithelially derived Shh in regulating branching morphogenesis of the lung.

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Results and discussion

To uncover the role of *Shh* in respiratory tract development, we examined the effects of a null mutation in the mouse *Shh* gene [3]. At 10.5 days *post coitum* (dpc), the lung of wild-type siblings consists of a left and right bud [1]. By 12.5 dpc, the tracheal epithelium has separated ventrally from the esophageal component of the foregut, and the two lung buds have formed several lateral branches which will give rise to primary airways of the lung lobes (Figure 1a–c). In contrast, the esophageal and tracheal tubes remained closely associated in *Shh* mutants (Figure 1d,e) and, although left and right buds formed, they either had not branched or possessed one abnormally positioned branch point (Figure 1f). Wild-type lungs undergo considerable growth and branching in organ culture. *Shh* mutant lungs, however, failed to grow or branch extensively (data not shown) and bronchial mesenchyme cells detached from the endodermal epithelium. We conclude that the defect in branching morphogenesis is independent of other *Shh*-expressing organs (i.e. the gut), and that the observed branching phenotype reflects an absence of Shh signaling, which is normally associated with the branching process.

To determine if branching was merely delayed and whether *Shh* plays a role in differentiation, we examined lungs removed at 15.5 dpc (data not shown) and 18.5 dpc (Figure 1g,h). At these times, five well-developed lobes are evident in the wild type (four right, one left), and highly branched airways form a ramifying epithelial network, the respiratory tree (Figure 1i,k,l). To mediate gas exchange in the alveolar sacs, the respiratory surface is well vascularized (Figure 1g). In contrast, *Shh* mutants formed only a rudimentary respiratory organ with a few large, poorly vascularized airways (Figure 1h). The trachea and esophagus were so closely juxtaposed that their tubes shared some common epithelia (Figure 1e), and a fistula-like fusion of the alimentary and respiratory tract was formed, mirroring a lethal anomaly well described in human pathology [4,5] (Figure 1j,m).

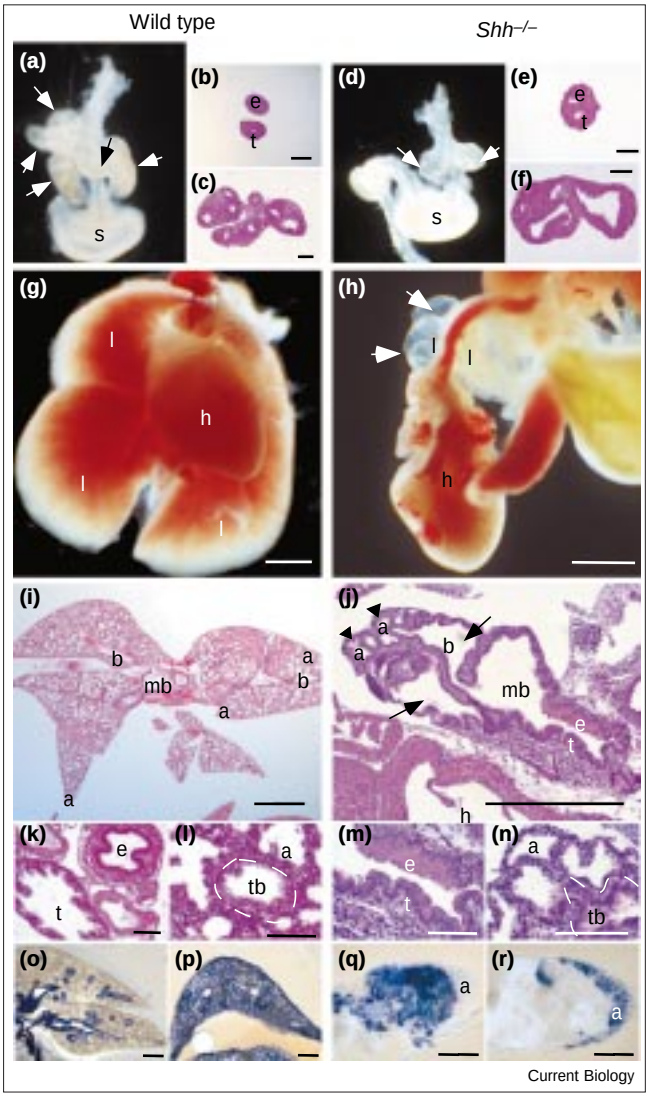
Remarkably, despite the absence of branching, evidence of normal proximo-distal epithelial differentiation could be observed. Most proximally, the pulmonary epithelium formed a columnar epithelium typical of the mainstem bronchi (Figure 1m) and expressed *CCSP* [6], a marker for terminally differentiated secretory Clara cells (Figure 1q). More distally, the epithelium consisted of a mixture of columnar and cuboidal epithelium as observed in the bronchioles (Figure 1n), and alveolar air sacs were formed, which correspondingly expressed *SP-C* [7], a type II pneumocyte marker (Figure 1r).

In summary, *Shh* is not required for proximo-distal differentiation of lung epithelium, but is essential for three different

events of regional morphogenesis of the foregut endoderm, formation of the tracheo–esophageal septum, lung lobation and generation of the respiratory tree, all of which are essential in forming a functional lung.

The exact role for *Shh* in branching processes remains to be determined. Grafting studies indicate that, whereas budding can be supported by mesenchyme from many different sources, only bronchial mesenchyme can induce organotypic branching morphogenesis [8]. The requirement for *Shh* in the epithelium suggests that regulation of its expression may be a reciprocal epithelial response to mesenchymal signaling.

To examine in more detail how Shh might regulate early branching of the lung epithelium, we performed *in situ* hybridization with digoxigenin-labeled probes recognizing general targets of Hedgehog signaling (Figure 2a–e and data not shown), or genes specifically implicated in lung morphogenesis (Figure 2f–k). As the *Shh* mutants were growth retarded and showed a general delay in lung budding, we compared expression of these markers at 12.5 dpc with wild-type embryos collected at 11.5 dpc and 12.5 dpc.



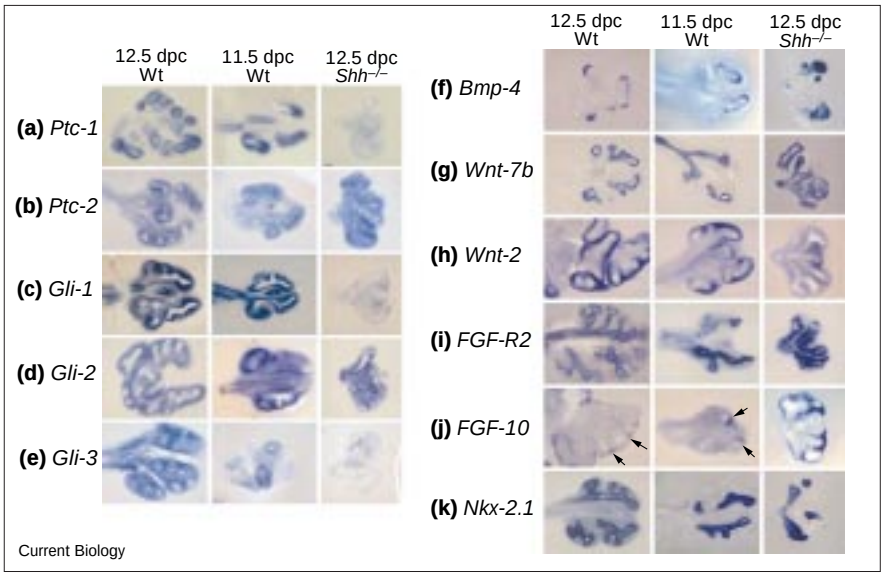
Patched genes encode proteins thought to be Hedgehog receptors, whereas *Gli* genes encode transcriptional mediators of Hedgehog signaling [9]. Both *Ptc-1* and *Gli-1* are upregulated when *Shh* is ectopically expressed in the lung, indicating that here, as elsewhere in the embryo, they are transcriptional targets of Shh signaling [9,10]. Consistent with this model, *Ptc-1* and *Gli-1* are normally expressed in the mesenchyme of wild-type embryos with highest levels at the distal branch points mirroring epithelial *Shh* expression [10] (Figure 2a,c). In *Shh* mutants, only basal levels of expression of both genes were detected (Figure 2a,c). *Gli-3*, which shows more widespread expression in the mesenchyme, was also downregulated (Figure 2e). In contrast, *Ptc-2*, which is expressed at higher levels in the epithelium, and *Gli-2*, which is normally expressed more uniformly in the mesenchyme, were not altered (Figure 2b,d). These data indicate that the lung mesenchyme, not the epithelium, is most likely the direct cellular target of Shh signaling. Further, they suggest that modulation of *Gli-1* and *Gli-3* transcription may be a critical aspect of lung morphogenesis. As *Gli-1* mutants do not

Figure 1

Morphology and epithelial phenotype of *Shh*$^{-/-}$ mouse lungs. **(a–c)** Wild-type mouse foregut at 12.5 dpc. (a) At this stage, the lung has branched several times to give rise to distinct lobes (arrows). (b) The trachea and esophagus are separate tubes. (c) Cross-section at the level of the lung shows branching and lobation. **(d–f)** Foregut from *Shh* mutant mice at 12.5 dpc. (d) *Shh*-deficient lungs have failed to undergo lobation or subsequent extensive branching. (e) The trachea and esophagus remain fused at the tracheo–esophageal septum. (f) Mutant lungs have branched only once. **(g,h)** At 18.5 dpc, in the wild-type lung (g), air sac formation is in progress and the respiratory surface is in tight association with blood vessels; in the poorly vascularized mutant lungs (h), there is little branching or growth, but air sac formation at the distal epithelial tips is apparent (arrows). **(i)** By 18.5 dpc, wild-type lungs have established the conducting airways and respiratory bronchioles, and alveolar formation is in progress. **(j)** In contrast, in a mutant lung of the same stage, branching is dramatically decreased. Only a few primary branches (arrows) and air sacs (arrowheads) are present. **(k,l)** In the wild type, the trachea and esophagus are separated (k); the trachea is lined by columnar cells, and the esophagus by stratified epithelium. The air sacs are made up of cuboidal cells (l). **(m,n)** In the mutant, the trachea and esophagus are fused to form a fistula and differentiation into columnar and stratified epithelium is apparent (m) as is the characteristic cuboidal epithelium of the air sacs (n). The demarcation lines between terminal bronchioles and the respiratory surface are indicated. **(o,p)** Wild-type lung at 18.5 dpc showing expression of (o) *CCSP* in Clara cells of the proximal lung epithelium, and (p) *SP-C* in type II pneumocytes of the distal epithelium. **(q,r)** Both (q) *CCSP* and (r) *SP-C* are expressed in the correct proximo-distal domain in the mutant. Bars denote 1 mm (g,h only) or 10 μm. Panels a,d,g and h are ventral views; all other panels show transverse sections. Abbreviations: a, air sac; b, bronchus; e, esophagus; h, heart; l, lung; mb, mainstem bronchus; s, stomach; t, trachea; tb, terminal bronchioles.

Figure 2

In situ hybridization analysis of gene expression in the lungs of *Shh* mutants. Expression of the indicated genes was investigated in whole-mount vibratome sections through lungs removed from wild-type (wt) embryos at 11.5 and 12.5 dpc, and *Shh* mutant embryos at 12.5 dpc.



have a lung phenotype, the *Shh* phenotype cannot simply be ascribed to a loss of *Gli-1* transcriptional activity [10]. Given that post-transcriptional processing regulates *Gli* (*Cubitus interruptus*) activity in invertebrates [11], we cannot rule out that Gli-2 is expressed, but post-transcriptionally inactivated. *Gli* genes are clearly involved in lung development, as shown by the relatively weak lobular hypoplasia observed in *Gli-3* mutants [10], but revealing the full extent of *Gli* action may require the generation of compound mutants.

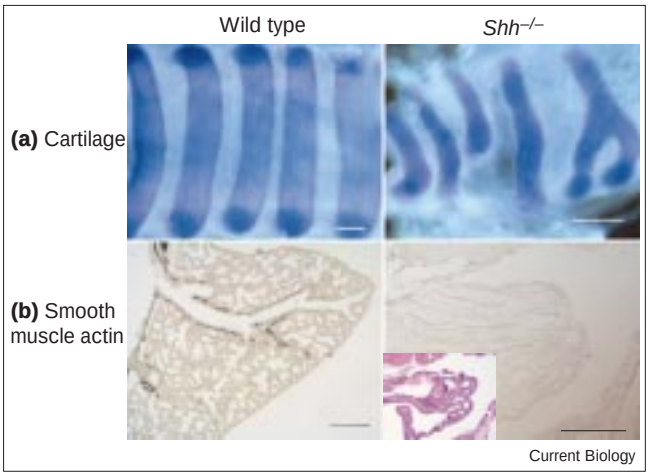
Hedgehog signaling regulates the expression of members of the bone morphogenetic protein (Bmp), Wnt and fibroblast growth factor (FGF) families [11]. In the lung, *Bmp-4* is strongly expressed in the distalmost tips of the epithelium. Ectopic expression results in decreased epithelial proliferation, disrupted branching and reduced differentiation of distal cell types in the airway [12]. In *Shh* mutants, *Bmp-4* was found to be expressed in the normal sites but at higher levels (Figure 2f), suggesting that enhanced *Bmp-4* signaling could contribute to the block in branching. *Wnt-7b* is normally expressed in the lung epithelium and is required for normal branching (S. Lee, W. Cardoso, B. Parr and A.M.; unpublished observations), whereas *Wnt-2* is expressed in the underlying mesenchyme suggesting a role in epithelial maintenance [2]. In *Shh* mutants, *Wnt-7b* expression was not altered (Figure 2g) but *Wnt-2* expression was downregulated (Figure 2h). This observation lends further support to the model that the lung mesenchyme is the primary target of Shh signaling and indicates that mesenchymal signaling is abnormal in *Shh* mutants. However, no role for Wnt-2 in lung development has been reported in *Wnt-2* mutants [13].

Ectopic expression of a dominant-negative form of the gene for an FGF receptor, *FGF-R2*, in the lung epithelium arrests branching after formation of left and right buds [14]. Although an arrest after initial budding is reminiscent of *Shh* mutants, subsequently the lungs grow caudally as tubes and differentiate into proximal epithelial structures. Interestingly, *Fgf10* is expressed in mesenchyme cells preceding branch formation and can induce branching of lung epithelium in culture [15]. In *Shh* mutants, expression of *FGF-R2* was unaltered (Figure 2i). In contrast, *Fgf10*, which in wild-type embryos is highly localized to small patches of mesenchyme at a distance from the lung epithelium (Figure 2j, arrows), was found to be expressed broadly in mesenchyme immediately adjacent to the epithelium in the mutant lung. Thus, *Shh* is not required for *Fgf10* expression but Shh signaling may spatially restrict *Fgf10* expression within the distal mesenchyme, as suggested by transgenic studies [15]. Whether the altered position of *Fgf10* expression then disrupts branching remains to be determined.

HNF-3β and *Nkx-2.1* are specific transcriptional effectors of Shh signaling in the neural tube. In the gut, *HNF-3β* is widely expressed in the epithelium, including the lung, whereas *Nkx-2.1* expression is specific to the lung epithelium and a few other endodermal derivatives [16]. Mice lacking *Nkx 2.1* develop cystic unbranched lungs indicating that it is essential for lung morphogenesis [16]. Expression of both genes was unaltered in *Shh* mutant lungs, suggesting that in this organ their expression is independent of the Shh signaling pathway (Figure 2k and data not shown).

As loss of *Shh* activity predominantly affects the expression of mesenchyme markers, we analyzed late mesenchyme

Figure 3



Mesenchyme differentiation at 18.5 dpc. (a) Both wild-type and *Shh* mutant lungs have cartilaginous rings around the trachea, as indicated by alcian blue staining. (b) Whereas in the wild-type lung a layer of smooth muscle surrounds the conducting epithelium, the mutant lung mesenchyme does not differentiate into muscle. The inset shows histological staining of an adjacent section from the mutant lung. Bars denote 10 mm.

differentiation. Formation of cartilage rings, albeit disorganized, occurred in the mutant (Figure 3a), while the layer of smooth muscle that normally lines the proximal epithelium was absent (Figure 3b). The observation that Shh is required for formation of smooth muscle is in agreement with previous studies [17].

In summary, the results reported here establish *Shh* as a regulator of foregut development and more specifically as a key factor in the control of branching morphogenesis in the mouse lung. They also indicate that the genetic control of growth and branching in the lung epithelium most likely involves both epithelial and mesenchymal interactions at the branch points, and that the downstream targets of Shh signaling in this organ are primarily mesenchymally expressed genes.

Materials and methods

Shh *mutants*

Generation of the *Shh* mutants has been described elsewhere [3].

*Histological/*in situ *hybridization analysis*

Tissue was processed for standard histology, or a modified *in situ* hybridization procedure [18].

Antibody staining

Antibody staining with a monoclonal antibody against smooth muscle actin (Sigma) was carried out according to the manufacturer's instructions.

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