Sonic hedgehog is essential to foregut development

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Congenital malformation of the foregut is common in humans, with an estimated incidence of 1 in 3000 live births[1], although its aetiology remains largely unknown. Mice with a targeted deletion of Sonic hedgehog (*Shh*) have foregut defects that are apparent as early as embryonic day 9.5, when the tracheal diverticulum begins to outgrow. Homozygous *Shh*-null mutant mice show oesophageal atresia/stenosis, tracheo-oesophageal fistula and tracheal and lung anomalies, features similar to those observed in humans with foregut defects. The lung mesenchyme shows enhanced cell death, decreased cell proliferation and downregulation of *Shh* target genes. These results indicate that *Shh* is required for the growth and differentiation of the oesophagus, trachea and lung, and suggest that mutations in *SHH* and its signalling components may be involved in foregut defects in humans.

The respiratory and digestive tubes are both derived from the foregut primordium, which is surrounded by splanchnic mesoderm. Experiments in which *Shh* was either misexpressed in the chick hindgut[2] or overexpressed in the mouse lung endoderm[3] suggested a potential role for *Shh* in gut and lung morphogenesis. At 9.5 days post coitum (dpc), *Shh* is prominently expressed in the tracheal diverticulum (Fig. 1*a*), an endodermal outgrowth from the ventral wall of the foregut at the border of the pharyngeal endoderm. *Shh* expression is spatially restricted to the ventral aspect of the foregut, with a defined boundary between *Shh*-expressing and non-expressing cells (Fig. 1*a*, arrows). At 11.5 dpc, when the oesophagus is clearly separated from the ventrally positioned trachea (Fig. 1*b*), *Shh* expression is detected in the endoderm of the developing oesophagus, but is not apparent in the trachea of sectioned or whole-mount embryos (Fig. 1*b*). *Shh* continues to be expressed in the developing oesophagus at 13.5 and 15.5 dpc, whereas expression in the trachea, although detectable at 13.5 dpc, becomes more evident at 15.5 dpc (Fig. 1*c*,*d*). In addition, *Shh* is also expressed in the endoderm of the developing lung (refs 3–5; Fig 2*b*, inset). We used an antibody for the endodermal marker HNF-3β to examine the development of the foregut and its derivatives in both wild-type and $Shh^{-/-}$ mutant embryos. At 9.5 dpc, outgrowth of the primary buds of the lung, liver and pancreas from the foregut is apparent in wild-type embryos (Fig. 1*e*). Although hepatic buds are formed in $Shh^{-/-}$ embryos, development of the lung buds is delayed for at least half a day, appearing by 10.5 dpc (Fig. 1*h*,*i*). In the wild-type embryo, the trachea begins to partition from the oesophagus at 10.5 dpc, separated by a tracheoesophageal septum (Fig. 1*f*, arrowhead). In the $Shh^{-/-}$ mutant, the tracheoesophageal septum is not established, and instead, the two tubes are juxtaposed (Fig. 1*i*, double arrows). By 11.5 dpc, the wild-type trachea and oesophagus are completely separated (Fig. 1*g*), whereas the $Shh^{-/-}$ mutant trachea and oesophagus have not separated into distinct tubes. In the region just anterior to the stomach, the mutant oesophagus and trachea are morphologically difficult to distinguish and apparently merge as a single tracheo-oesophageal tube that connects to the stomach. Furthermore, this tracheo-oesophageal tube, which seems to end as a swelling (Fig. 1*j*, arrowheads), also makes contact with the

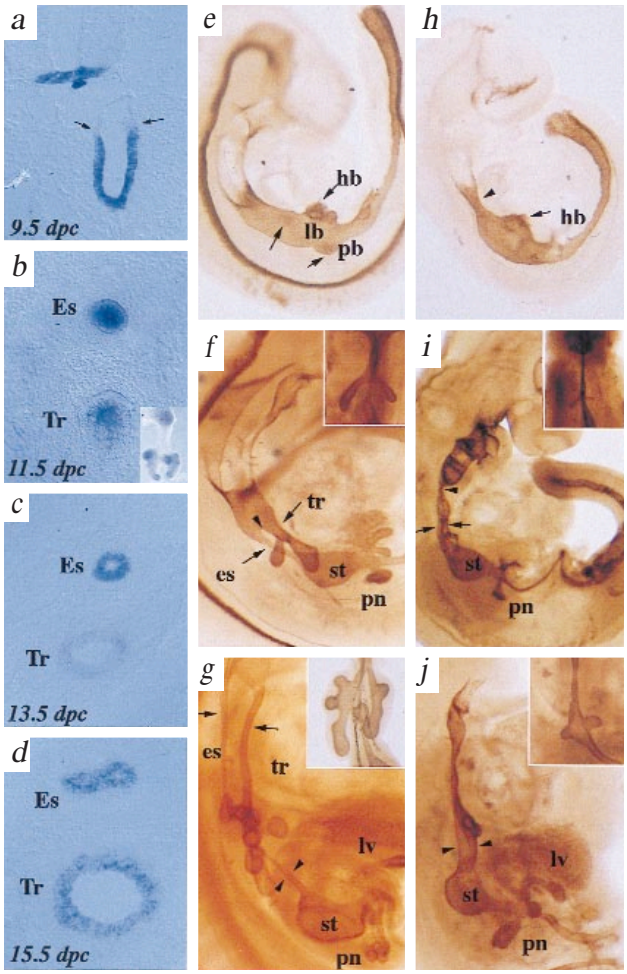

Fig. 1 Foregut defects and lung hypoplasia in $Shh^{-/-}$ mutant. *In situ* hybridization on cross-sections of wild-type embryos showing the tracheal diverticulum (***a***) and the foregut region at different embryonic stages (***b***,***c***,***d***) using *Shh* digoxygenin-labelled riboprobe. Arrows in (*a*) indicate a sharp *Shh* expression boundary at 9.5 dpc. The tracheal lumen (*b*) shows non-specific staining that is absent in the whole-mount embryo (inset). Whole-mount immunohistochemistry on 9.5 dpc (***e***,***h***), 10.5 dpc (***f***,***i***) and 11.5 dpc (***g***,***j***) wild-type and $Shh^{-/-}$ mutant embryos using an antibody specific for HNF3β. Note the abnormal constriction in the upper foregut of the mutant at this stage (*h*, arrowhead). At 10.5 dpc, the oesophagus is clearly separated from the trachea by the tracheo-oesophageal septum (*f*, arrowhead). In the mutant, the tracheo-oesophageal septum is not obvious, although there is a distinguishable line of separation between the oesophagus and trachea (*i*, double arrows). The constriction in the mutant (*i*, arrowhead) reduces the diameter of the upper foregut region. At 11.5 dpc, the posterior end of the mutant oesophagus appears as a swollen structure (*j*, arrowheads) that makes contact with the lung and the stomach. Insets in (*f*) and (*g*) show the wild-type lung lobes at higher magnification. Insets in (*i*) and (*j*) show the mutant lung buds. es, oesophagus; hb, hepatic bud; lv, liver; lb, lung bud; pn, pancreas; pb, pancreatic bud; st, stomach; tr, trachea.

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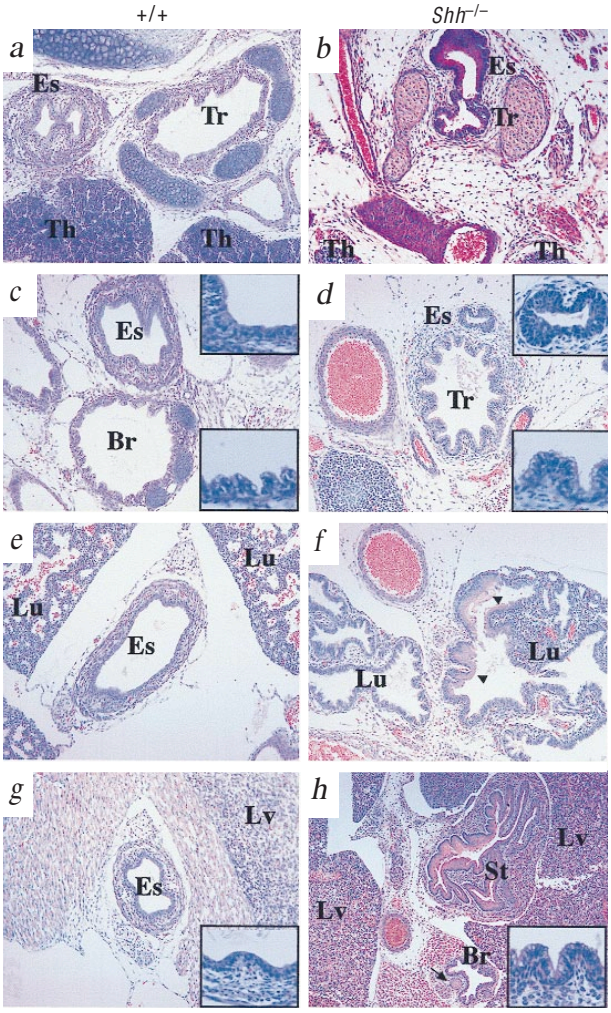

Fig. 2 Oesophageal atresia/stenosis and tracheo-oesophageal fistula in 17.5 dpc *Shh*$^{-/-}$ mutant. Sections at the thymus (Th) level showing the oesophagus (Es) and trachea (Tr) in wild-type and mutant (***a***,***b***) embryos. Note that the mutant oesophagus and trachea are in close contact (*b*). Sections at the bronchus level, showing oesophagus and bronchus (Br) in wild type (***c***). Insets show a higher magnification view of the oesophageal (top) and bronchial epithelial lining (bottom). Higher magnification insets in (***d***) show the oesophageal lining (top) and tracheal lining indicating the presence of goblet cells (bottom). Sections at the lung level showing oesophagus and lung (Lu) in the wild-type (***e***) and a gut-derived epithelium (arrowheads) fused with the lung epithelium in the mutant (***f***). Sections at the liver (Lv) and stomach (St) level showing the oesophagus in the wild-type (***g***) and a bronchus-like lumen (Br) with surrounding cartilage (arrow) in the mutant (***h***). Insets in (*g*) and (*h*) show higher magnification views of the oesophageal and bronchial linings respectively. All sections were stained with H&E and photographed at a magnification of ×10.

lung buds and subsequently fuses with them later in development (Fig. 2*j*). The *Shh*$^{-/-}$ mutant lung is also hypoplastic as compared with wild-type lung (Fig. 1*f*–*j*, inset), indicating that epithelial branching is affected (Fig. 1*j*).

To obtain more information on the mutant foregut at a cellular level, we performed histologic analysis on sections of 17.5 dpc mutant and wild-type embryos. In wild type, at the level of the thymus glands, the oesophagus and trachea are clearly distinct tubes (Fig. 2*a*). In the mutant, however, the trachea and oesophagus are still in close contact and the mutant trachea is surrounded by a characteristic cartilaginous structure (Fig. 2*b*). At the level of the heart, the mutant oesophagus shows a reduction in the size of its lumen (Fig. 2*a*,*d*). In more posterior sections at the level of the lungs, the wild-type oesophagus, which connects to the stomach, is clearly a separate organ from the lungs (Fig. 2*e*). In the mutant, a structurally distinct oesophagus is absent; instead, a region of thick mucosa more similar to the lining of the stomach than the oesophagus shows continuity with the lung epithelium (Fig. 2*f*, arrowheads). These data indicate that the mutant oesophagus merges with the lung epithelium (Fig. 2*f*). The tube emanating from the lung that connects to the stomach has tracheal/bronchial characteristics, shown by its characteristic histology and the presence of surrounding cartilage (Fig. 2*h*, arrow). Therefore, in the *Shh*$^{-/-}$ mutant, the stomach is connected at its anterior end not to the oesophagus but to a tracheal-like tube. Our results indicate that *Shh* is essential for the formation of the septum, and hence the proper partitioning of the respiratory tube from the digestive tube.

At 14.5 dpc, the wild-type lung exhibits an elaborate epithelial branching pattern (Fig. 3*a*,*c*). In the *Shh*$^{-/-}$ mutant, the asymmetry of the left and right lung lobes is completely absent, and the lung appears as a single lobe (Fig. 3*b*,*d*). Haematoxylin and eosin (H&E) staining of mutant lung sections reveals a few enlarged epithelial-lined lumena characteristic of the bronchus, indicative of an arrest in epithelial branching, and a sparse and loose mesenchyme structure (Fig. 3*d*). At 18.5 dpc, the wild-type lung is filled with an extensive network of epithelium-lined air sacs (Fig. 3*g*), whereas *Shh*$^{-/-}$ mutant lung at 18.5 dpc is reduced to a simple sac (Fig. 3*f*,*h*) occasionally lined with a small network of air sacs located at the periphery (Fig. 3*h*). These cellular defects in the lung suggest that mesenchyme proliferation is affected. TUNEL and BrdU labelling experiments show that the mutant lung exhibits enhanced cell death in the mesenchyme (data not shown) and decreased cell proliferation (Fig. 3*i*), with an overall percentage of dividing cells, both in the epithelium and the mesenchyme of *Shh*$^{-/-}$ mutant lung, 1.4- and 1.7-fold less than that of wild-type lung, respectively. Our results indicate that the mutant lung mesenchyme is preferentially affected by the lack of Shh function. The importance of *Shh* on the lung mesenchyme was further evaluated in culture. Wild-type and *Shh*$^{-/-}$ mutant lung at 11.0 dpc were cultured in the absence of exogenous growth factors. On the fifth day of culture, the wild-type lung showed an elaborate network of epithelial branching surrounded by a dense layer of mesenchymal cells (data not shown). The mutant lung, however, showed no sign of epithelial branching or mesenchymal proliferation (Fig. 3*j*,*l*). In the presence of Shh, proliferation of the mesenchyme was significantly enhanced and epithelial branching was clearly evident (Fig. 3*k*,*m*, arrows). This indicates that *Shh* expressed in the endoderm of the lung is essential for branching morphogenesis.

To determine whether defective lung development observed in the *Shh*$^{-/-}$ mutant is the result of selective downregulation of genes expressed in the lung mesenchyme and epithelium, we examined the expression of a number of genes in the lung of 11.5 dpc and 12.5 dpc mutant embryos. Most of these genes have been implicated directly or indirectly in the hedgehog signalling cascade (for review, see refs 6,7). Two known *Shh* effector genes, *Ptch* and *Gli*, are upregulated by *Shh* in response to overexpression of *Shh* in the endoderm[2,3]. At 11.5 dpc, *Ptch* and *Gli* are expressed at high levels in the mesenchyme adjacent to the terminal buds where *Shh* is normally expressed (Fig. 4*a*,*c*). In *Shh*$^{-/-}$ mutants, *Ptch* expression is downregulated in the mesenchyme (Fig. 4*b*) and *Gli* expression is undetectable (Fig. 4*d*). Similarly, *Bmp4* expression in the mesenchyme is downregulated, whereas epithelial expression is maintained (Fig. 4*f*), consistent with the ability of *Shh* to upregulate *Bmp4* expression in the chick hindgut[2]. *Gli3* expression in the mesenchyme also appears to be downregulated in the mutant (Fig. 4*j*). On the other hand, *Fgf10* expression in the distal mes-

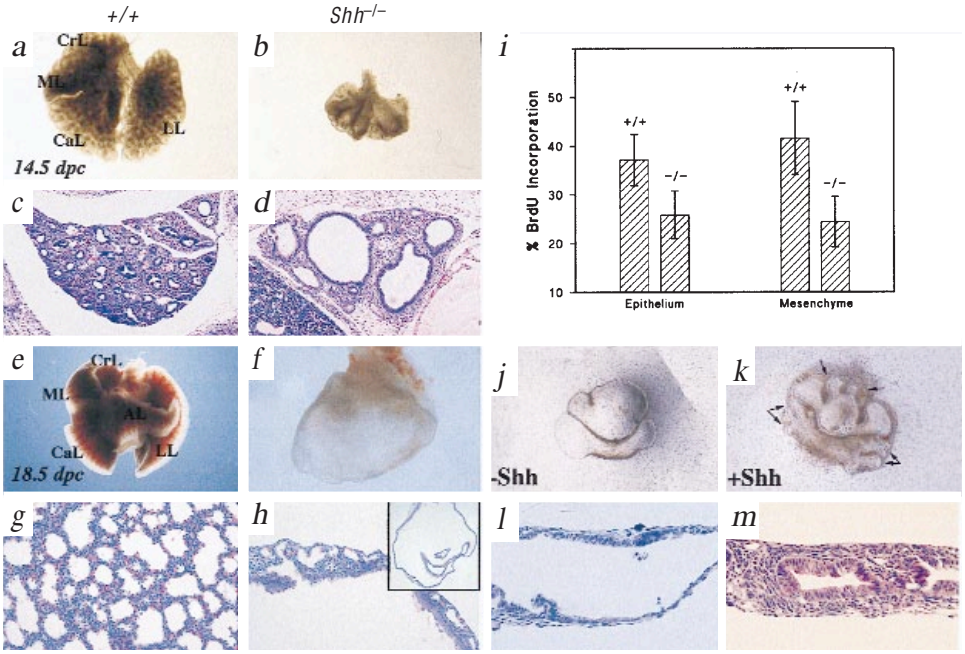

Fig. 3 Shh is essential in lung development. *a,b,e,f*, Whole mount view of wild-type (***a***,***e***) and *Shh*$^{-/-}$ mutant (***b***,***f***) lungs at 14.5 dpc (*a,b*) and 18.5 dpc (*e,f*). Wild-type lungs show characteristic lobulation (CrL, cranial lobe; ML, middle lobe; CaL, caudal lobe; LL, left lobe), which is not present in the mutant. Note that the mutant lung at 18.5 dpc is reduced to a dilated sac (*h*, inset). ***c***,***d***,***g***,***h***, H&E-stained sections of wild-type (*c,g*) and mutant (*d,h*) lungs at 14.5 dpc (*c,d*) and 18.5 dpc (*g,h*). ***i***, Histogram showing the percentage of BrdU-labelled cells in the epithelium and mesenchyme of 13.5 dpc wild-type and mutant lung. ***j***,***k***, *Shh*$^{-/-}$ lung at 11.0 dpc, cultured in serum-free medium in the absence (*j*) or presence (*k*) of Shh for 5 d. ***l***,***m***, H&E-stained sections of the cultured lungs in (*j*) and (*k*).

enchyme is not affected in the *Shh*$^{-/-}$ mutant lung (Fig. 4*g,h*). The genes expressed in the endoderm, including *Hnf3b*, *Fgfr2* and *Ttf1*, are not significantly affected in the *Shh*$^{-/-}$ mutant lung (Fig. 4*k–p*). The results presented here are consistent with the fact that Shh is a secreted signalling molecule that selectively induces gene expression and proliferation of the adjacent mesenchymal target cells in the lung.

The splanchnic mesoderm has been shown to induce a new developmental program in the gut endoderm[8,9]. Grafting experiments *in vitro* have demonstrated the inductive properties of the mesenchyme on endodermal differentiation[9–13]. We propose that *Shh* expressed in the endoderm is essential for the proliferation and differentiation of the splanchnic mesoderm, which in turn is required for proper differentiation of the foregut, consistent with the ability of Shh to rescue mutant mesenchyme proliferation in culture (Fig. 3*k,m*). The observation that *Gli2*$^{-/-}$ mutants and *Gli2*$^{-/-}$ and *Gli3*$^{+/-}$ double mutants have overlapping phenotypes with *Shh*$^{-/-}$ mutants (ref. 21) suggests that the Shh signal is mediated in part through the *Gli* gene family in the mesoderm.

Mutations in *SHH* have been associated with a dominant form of a human congenital malformation known as holoprosencephaly, with characteristic facial anomalies ranging from extreme cyclopia to mild ocular hypotelorism[14,15]. Targeted ablation of *Shh* in mice also revealed a similar phenotype[16]. Association of holoprosencephaly with foregut anomalies has been observed in some human patients[17,18], suggesting that common signals operate in both the forebrain and the foregut during development. Our studies have provided a genetic model for a detailed embryological evaluation of foregut-associated disorders.

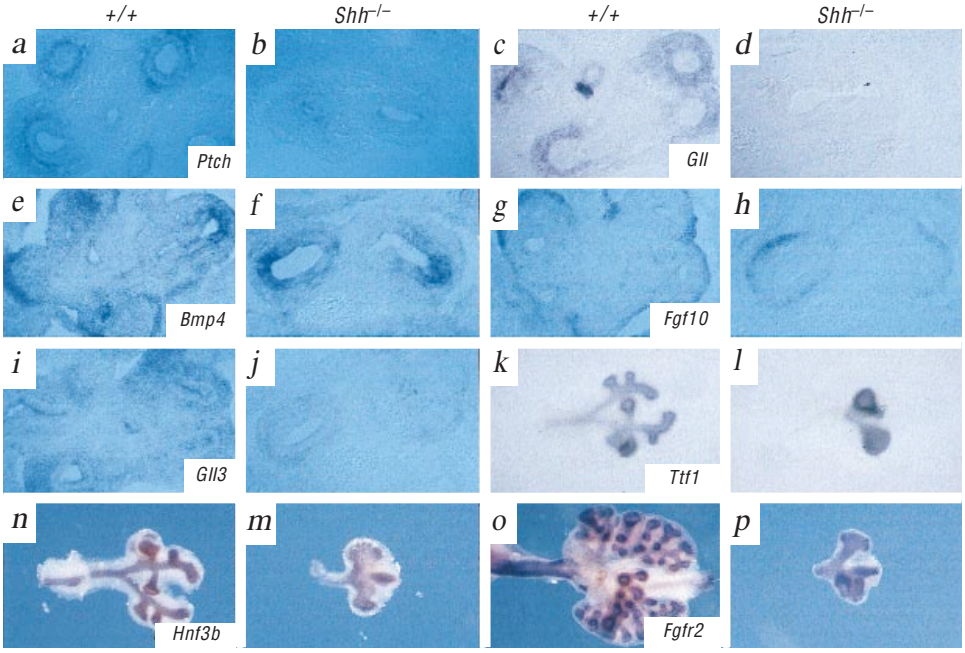

Fig. 4 Epithelial and mesenchymal gene expression in *Shh*$^{-/-}$ mutant lungs. Section (*a–j*) and whole-mount (*n–p*) *in situ* hybridization of 11.5 dpc (*a–m*) and 12.5 dpc (*o,p*) lungs using digoxygenin-labelled riboprobes: *Ptch* (***a***,***b***), *Gli* (***c***,***d***), *Bmp4* (***e***,***f***), *Fgf10* (***g***,***h***), *Gli3* (***i***,***j***), *Ttf1* (***k***,***l***), *Hnf3b* (***m***,***n***) and *Fgfr2* (***o***,***p***).

Methods

Embryos. The generation and identification of *Shh* heterozygous and homozygous mutant mice and embryos are as described[18].

In situ hybridization and immunohistochemistry. Whole-mount and cryosection *in situ* hybridization protocols were performed as described[16]. The following cDNA were used as templates for synthesizing digoxygenin-labelled riboprobes: *Shh*, *Hnf3b* (H. Sasaki and B. Hogan), *Fgfr2* (C. Deng), *Gli1* and *Gli3* (C-c. Hui), *Bmp4* (S-J. Lee), *Ptch* (M. Scott), *Fgf10* (S. Bellusci and B. Hogan) and *Ttf1* (S. Kimura). Whole-mount immunohistochemistry was performed on 9.5, 10.5 and 11.5 dpc wild-type and $Shh^{-/-}$ mutant embryos using anti-HNF-3β (gift of B. Hogan) as described[19].

Lung organ culture. Lungs were dissected out at 11.0 dpc and cultured on Nucleopore polycarbonate filters (8 μm pore size; Millipore) in serum-free medium (400 μl; Improved MEM Zinc option; Gibco) containing transferrin (10 μg/ml). The medium was changed every other day. In the rescue experiment, Shh (150 nM) was added to the culture medium. The recombinant Shh was isolated as described[20]. On the fifth day of culture, the lungs were photographed and fixed for sectioning and staining.

Analysis of cell proliferation and cell death in $Shh^{-/-}$ mutant lung *in vivo*. Females at 13.5 dpc, generated by $Shh^{+/-}$ heterozygous matings, were injected intraperitoneally with 5-bromodeoxyuridine (BrdU; 50 mg per kg body weight) in sterile PBS (0.5 ml). After 1 h, the embryos were collected, fixed in 4% paraformaldehyde for 4 h, washed several times in PBS, dehydrated in a graded series of ethanol, embedded in paraffin and sectioned. Sections were dewaxed in xylene and stained as described[3]. Briefly, sections were treated with pepsin and HCl and stained with mouse anti-BrdU antibody (Boehringer Mannheim) diluted in PBS/horse serum (1:10). Sections were treated with a biotinylated anti-mouse IgG antibody and detected with the avidin-biotin-peroxidase complex (Vector labs) and DAB/hydrogen peroxide. Cells in eight and eleven different photomicrographs representing random portions of the wild-type and *Shh* mutant lung respectively, at a magnification ×40, were counted and a student t-test performed. TUNEL staining for detection of apoptotic cells in the lung was performed using the apoptosis detection kit (Trevigen).

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