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ARTICLE

Immunolocalization of Sonic Hedgehog (Shh) in Developing Mouse Lung

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SUMMARY Expression of sonic hedgehog (Shh) is required for normal development of the lung during embryogenesis. Loss of Shh expression in mice results in tracheoesophageal fistula, lung hypoplasia, and abnormal lung lobulation. To determine whether Shh may play a role later in lung morphogenesis, immunostaining for Shh was performed in mouse lung from embryonic day (E) 10.5 to postnatal day (PD) 24. Shh was detected in the distal epithelium of the developing mouse lung from E10.5 to E16.5. From E16.5 until PD15, Shh was present in epithelial cells in both the peripheral and conducting airways. Although all cells of the developing epithelium uniformly expressed Shh at E10.5, Shh expression was restricted to subsets of epithelial cells by E16.5. Between E16.5 and PD15, non-uniform Shh staining of epithelial cells was observed in the conducting airways in a pattern consistent with the distribution of non-ciliated bronchiolar cells (i.e., Clara cells) and the Clara cell marker CCSP. Shh did not co-localize with hepatocyte nuclear factor/forkhead homologue-4 (HFH-4), β-tubulin, or with the presence of cilia. These results support the concept that Shh plays a distinct regulatory role in the lung later in morphogenesis, when it may influence formation or cytodifferentiation of the conducting airways.

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Morphogenesis of the mammalian lung begins as a ventral outpouching of the foregut endoderm into the surrounding splanchnic mesoderm. Dichotomous branching of the lung primordium forms the left and right lung buds, which undergo subsequent generations of dichotomous branching to generate the lobes of the lung, the bronchopulmonary segments, and the respiratory tubules that will form the mature lung. Branching morphogenesis of the lung buds is dictated by the underlying mesenchyme, which provides signals that induce unique genetic programs at different locations in the lung (see Hogan 1999 for review).

One of the signaling molecules implicated in this complex process is sonic hedgehog (Shh), the vertebrate homologue of the *Drosophila* hedgehog gene (Hh). In the lung, Shh mRNA is expressed in the distal epithelium during early embryonic stages. The proposed Shh receptor, patched (Ptc), is expressed in the juxtaposed mesenchyme. Shh is autoproteolytically cleaved to produce N-terminal and C-terminal peptides. The N-terminal peptide is secreted and attaches to the cell membrane of the expressing cells, whereas the C-terminal remains unattached after secretion. The N-terminal fragment is believed to mediate signal transduction. At present, no biological function has been attributed to the C-terminal peptide. In the absence of Shh, the Ptc receptor prevents transcription of Shh-responsive genes by actively inhibiting smoothened (Smo), a transmembrane protein involved in relaying the Shh signal to the Gli family of transcriptional regulators. Secreted Shh binds to Ptc, relieving its inhibition of Smo, thereby allowing activating-Gli to translocate into the nucleus and bind to its consensus sequence in Shh-responsive genes (for review see Ingham 1995; McMahon 2000).

Several mouse models have been developed to examine the role of Shh during development. Lung-specific overexpression of Shh using the human 3.7-kD surfactant protein C (SP-C) enhancer/promoter leads

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to overexpression of Shh in the distal epithelium (Bellusci et al. 1997). Compared to wild-type lungs, the transgenic lungs exhibited increased epithelial and mesenchymal cell proliferation, leading to the formation of lungs with overly abundant mesenchyme and no functional alveoli (Bellusci et al. 1997). Transgenic mice died at birth, probably due to respiratory failure. Interestingly, Shh-dependent proliferation was not detected until embryonic stage 16.5 (Bellusci et al. 1997). Using an array of molecular markers, no difference in cell differentiation was found between wild-type and transgenic lungs at E17.5 and at birth (Bellusci et al. 1997).

Homozygous null mice for Shh exhibited tracheoesophageal fistulas, tracheal or esophageal atresia, lung hypoplasia, fistula-like fusion of the alimentary and respiratory tract, and abnormal digit formation, demonstrating its importance early in branching morphogenesis (Pepicelli et al. 1998). Further examination of the lungs from the null mutant mice showed that after formation of the primary lung buds subsequent branching did not occur, resulting in the formation of rudimentary respiratory organs with a few large, poorly vascularized airways (Pepicelli et al. 1998). These gross manifestations of the null Shh phenotype are similar to those described in humans possessing mutations in the SHH gene (for review see Dean 1996) and support the concept that Shh pathways play important roles in lung morphogenesis by influencing proliferation of both epithelial and mesenchymal components of the lung. Previous studies demonstrated the presence of Shh mRNA during the embryonic and early canalicular stages of mouse lung development, consistent with a proposed role for Shh in early lung branching morphogenesis (Urase et al. 1996; Bellusci et al. 1997; Litingtung et al. 1998; Pepicelli et al. 1998). The possibility that Shh plays a role in lung morphogenesis later in development has not been assessed. In the present study, immunohistochemistry was used to localize Shh protein in epithelial cells of the respiratory and conducting airways during fetal and postnatal stages of lung development. Our results indicate that Shh may play a regulatory role during both early and late lung morphogenesis.

Materials and Methods

Animal Maintenance

FVBN mice were housed under pathogen-free conditions in accordance with institutional guidelines. Embryonic day (E) 1 was defined as the day when formation of a vaginal plug was first detected.

Antibodies

A rabbit polyclonal antibody to rat thyroid transcription factor-1 (Nkx2.1) was generated as described previously (Guazzi et al. 1990) and used at a concentration of 1:1000 on fetal and postnatal mouse lung sections. A goat polyclonal antibody was generated to the N-terminal Shh peptide (Research Diagnostics; Flanders, NJ) and was used at a concentration of 1:100. A rabbit polyclonal antibody against recombinant rat Clara cell secretory protein (CCSP) was generated and used at a concentration of 1:3000. Specificity of the antibody for CCSP recombinant peptide and fetal lung homogenate was determined using Western blotting analysis (not shown). Sections of lung from wild-type mice and CCSP null mice were incubated in this antibody. Only those sections from the wild-type mouse lung exhibited specific staining, whereas no specific staining was detected on the sections from the CCSP null mouse lung (not shown). A mouse monoclonal antibody to β-tubulin IV was obtained from BioGenex (San Ramon, CA) and was used at a concentration of 1:160. A mouse monoclonal antibody raised against amino acids 1-101 of mouse hepatocyte nuclear factor/forkhead homologue-4 (HFH-4) antigen was used at a concentration of 1:1000. Specificity of the antibody for HFH-4 recombinant peptide was determined using a Western blotting analysis (not shown).

Immunohistochemistry

Immunohistochemical staining for Nkx2.1 was carried out using 5-μm paraffin sections that were deparaffinized, rehydrated, and heated at 90C in 0.1 M citric acid in 0.1 M sodium citrate (pH 6.0) for an initial 15 min and three successive 5-min periods (Zhou et al. 1996). After cooling, the sections were treated with 3% hydrogen peroxide in methanol for 15 min (for quenching of endogenous peroxidase), blocked in 2% goat serum in 0.1% TBS for 2 hr at room temperature, and incubated in Nkx2.1 primary antibody at 4C overnight. Control sections were incubated overnight at 4C in blocking serum alone. After application of primary antibody, sections were developed with a biotinylated goat anti-rabbit secondary antibody and a Vector Elite ABC kit (Vector Laboratories; Burlingame, CA). Development in NiDAB was followed by incubation in Tris-cobalt, which enhanced antigen localization, and by counterstaining with nuclear fast red. The sections were then dehydrated in an ascending series of ethanols, washed in three changes of xylene, and mounted under coverslips.

The Shh immunolabeling procedure was similar to that described above, consisting of deparaffinization and rehydration of 5-μm sections, incubation in 3% hydrogen peroxide and methanol, heating in 0.1 M citric acid in 0.1 M sodium citrate, and blocking in 2% horse serum with subsequent overnight incubation with the primary antibody. Control tissues were incubated overnight at 4C in blocking serum only.

For the competition experiments, three applications were used. One set of slides from different timepoints was treated with Shh primary antibody (1:100 dilution) as described above. The second set was treated as a control with no primary antibody application. The third application consisted of incubating the primary antibody (1:100 dilution) for 15 min with Shh recombinant peptide such that the concentration of the peptide was 100-fold that of the antibody. After incubation, the antibody/peptide solu-

tion was applied to the slides similarly to the primary antibody application. Thereafter, the slides were treated as described above.

Immunolabeling using monoclonal antibodies against HFH-4 and β-tubulin was carried out using the Vector M.O.M. Immunodetection Kit (Vector Laboratories) according to the manufacturer's directions, as previously described (Tichelaar et al. 1999a,b). Immunostaining for CCSP was carried out as described above without using the antigen retrieval procedure.

Results

Developmental Pattern of Shh Staining in Mouse Lung

The distribution of Shh in the mouse lung was determined from E10.5 to PD15 by immunohistochemistry. At E10.5, distribution of the Shh peptide was detected in all epithelial cells of the mouse lung buds and was co-localized with Nkx2.1 expression (Figure 1). Shh staining was detected in the epithelial cells of the distal primordial tubules on E13.5, at which time cells from the proximal regions of the tubules were clearly devoid of expression (Figure 2). This proximal–distal difference in Shh expression was maintained through E14.5 and E15.5 (Figure 3). Low-level staining of Shh was first detected in the bronchial epithelium on E16.5 at the end of the pseudoglandular stage (Figure 3). Unlike the uniform staining detected earlier in lung development, staining of Shh during these latter stages of development was detected only in subsets of cells (Figure 3). Localization of Shh in the bronchiolar and bronchial epithelium on E16.5, E17.0/17.5, and E18.0 was consistent with sites and abundance of non-ciliated epithelial cells (Figure 3). The intensity of Shh immunostaining peaked in the bronchiolar and bronchial epithelium on E17.0/17.5 and was maintained at this level on E18.0 (Figure 4). Shh staining remained intense in the distal respiratory epithelium (i.e., terminal saccules) during E17.0/17.5 and E18.0 (Figure 4). However, at PD1, when high levels of Shh were detected in the epithelium of the conducting airways, there was a marked decrease in Shh immunostaining in the peripheral respiratory epithelium (Figure 4). Low-level Shh staining was detected in the alveolar epithelium during the postnatal period (PD5–PD15; not shown) and was

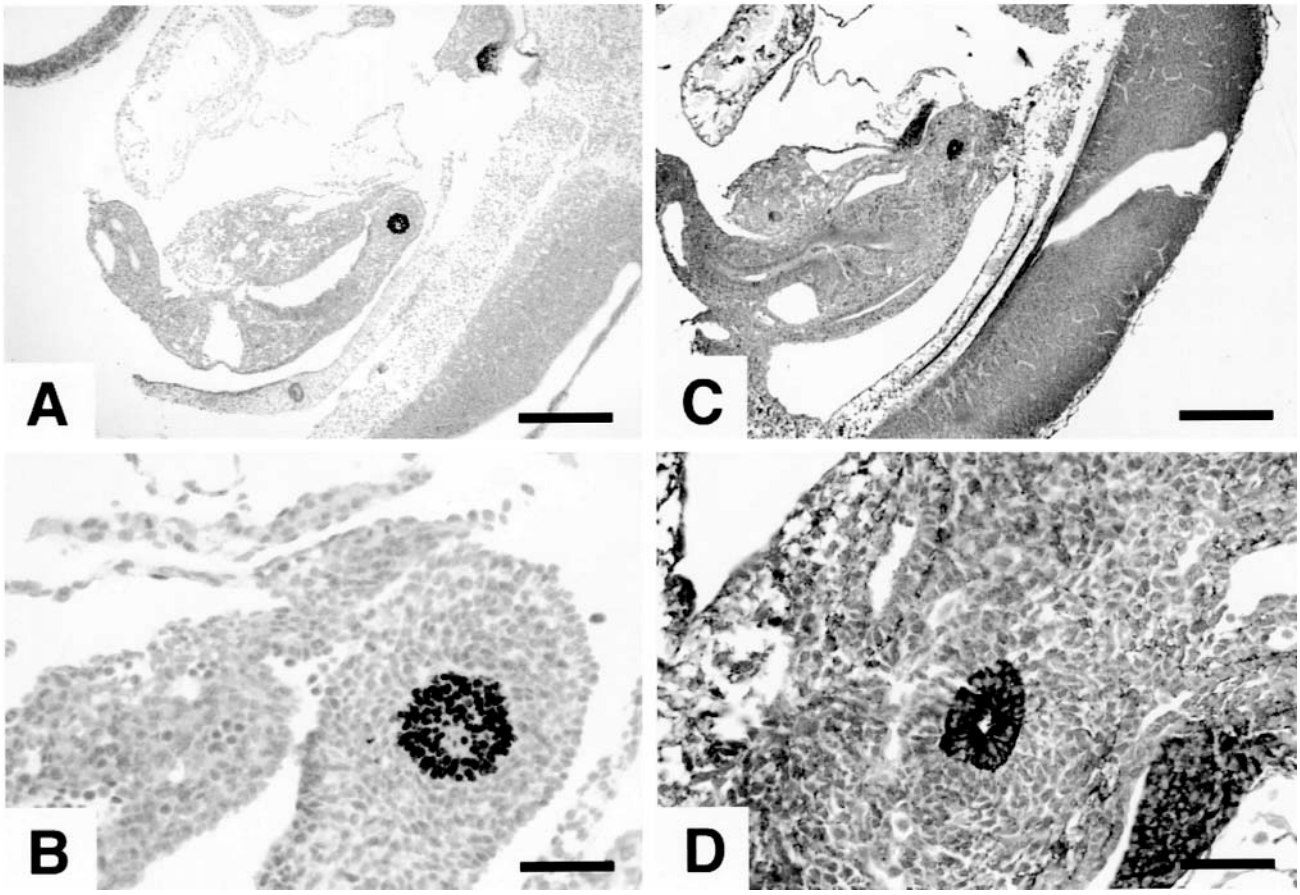

Figure 1 Immunolocalization of Nkx2.1 and Shh in E10.5 mouse lung. Nuclei of epithelial cells in the primordial lung bud were positive for Nkx2.1 staining (**A,B**). Epithelial cells of the primordial lung bud also stained positively for Shh protein (**C,D**). Bars: **A,B,D** = 250 μm; **C** = 50 μm.

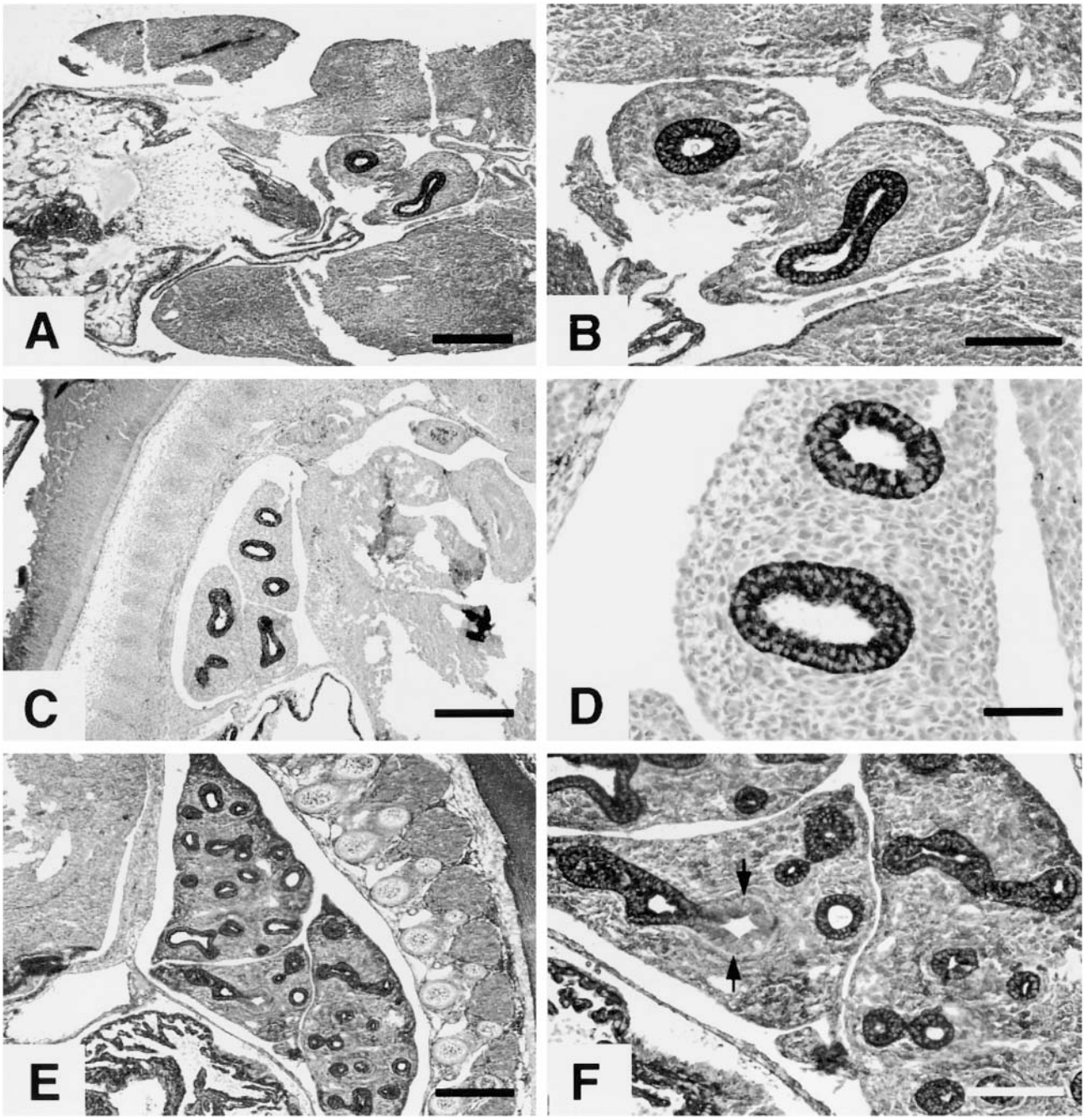

Figure 2 Shh expression during the early pseudoglandular stage of lung development (E11.5–E13.5). Staining for Shh was detected in epithelial cells of the primordial tubules on E11.5 (**A,B**) and throughout the pulmonary epithelium at E12.5 (**C,D**). Shh staining was restricted to epithelial cells in the distal region of the primordial tubules on E13.5 (**E,F**). Note that proximal epithelial cells of the primordial tubules were not immunostained for Shh (**F**, arrows). Bars: **A,C,E** = 250 μm; **B,F** = 125 μm; **D** = 50 μm.

maintained at detectable levels in the bronchial epithelium until PD15. By PD24, the alveolar and bronchial epithelia were devoid of Shh staining (not shown).

Specificity of Shh Staining in the Mouse Lung

To determine the specificity of the immunostaining, competition binding studies using the N-terminal Shh peptide were carried out with lung sections from E15.5, E17.0/17.5, E18.0, and PD1 (Figure 5). At all time points, the presence of the N-terminal peptide prevented staining in the lung epithelium, indicating that the immunostaining was specific for Shh.

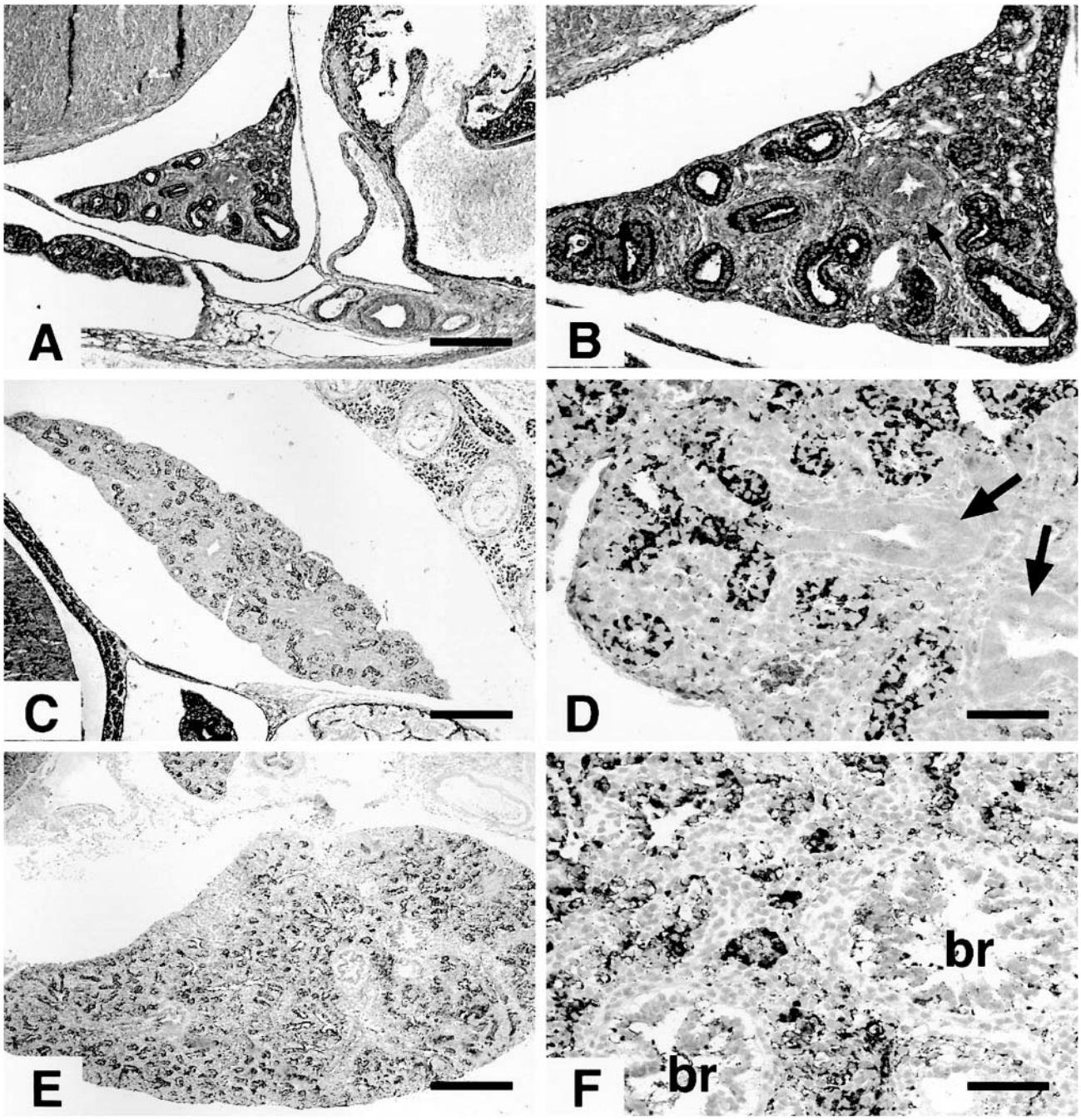

Figure 3 Shh expression during late pseudoglandular stage of lung development (E14.5–E16.5). Staining for Shh was detected in the respiratory epithelium of the distal acinar tubules and buds on E14.5 (**A,B**) and E15.5 (**C,D**). Shh staining was detected primarily in the epithelium of the acinar tubules, with some staining seen in the conducting epithelium of the bronchiolar tubules (br) on E16.5 (**E,F**). Again, the proximal epithelial cells of the primordial tubules were not immunostained for Shh (**B,D**, arrows). Bars: **A,C,E** = 250 μm; **B,F** = 50 μm; **D** = 100 μm.

Correlation of Shh Expression with Cell-specific Molecular Markers

To determine the epithelial cell type in the conducting airway that expressed Shh, serial sections of mouse lung at E18.0 and PD1 were taken and immunostained for Shh, β-tubulin, HFH-4, and CCSP (Figure 6). Expression of Shh was consistently detected in subsets of cells in the conducting epithelium that corresponded to non-ciliated cells, which protrude out into the lumen of the conducting tubules (Figure 6D). These dome-shaped cells stained for CCSP, a Clara cell marker

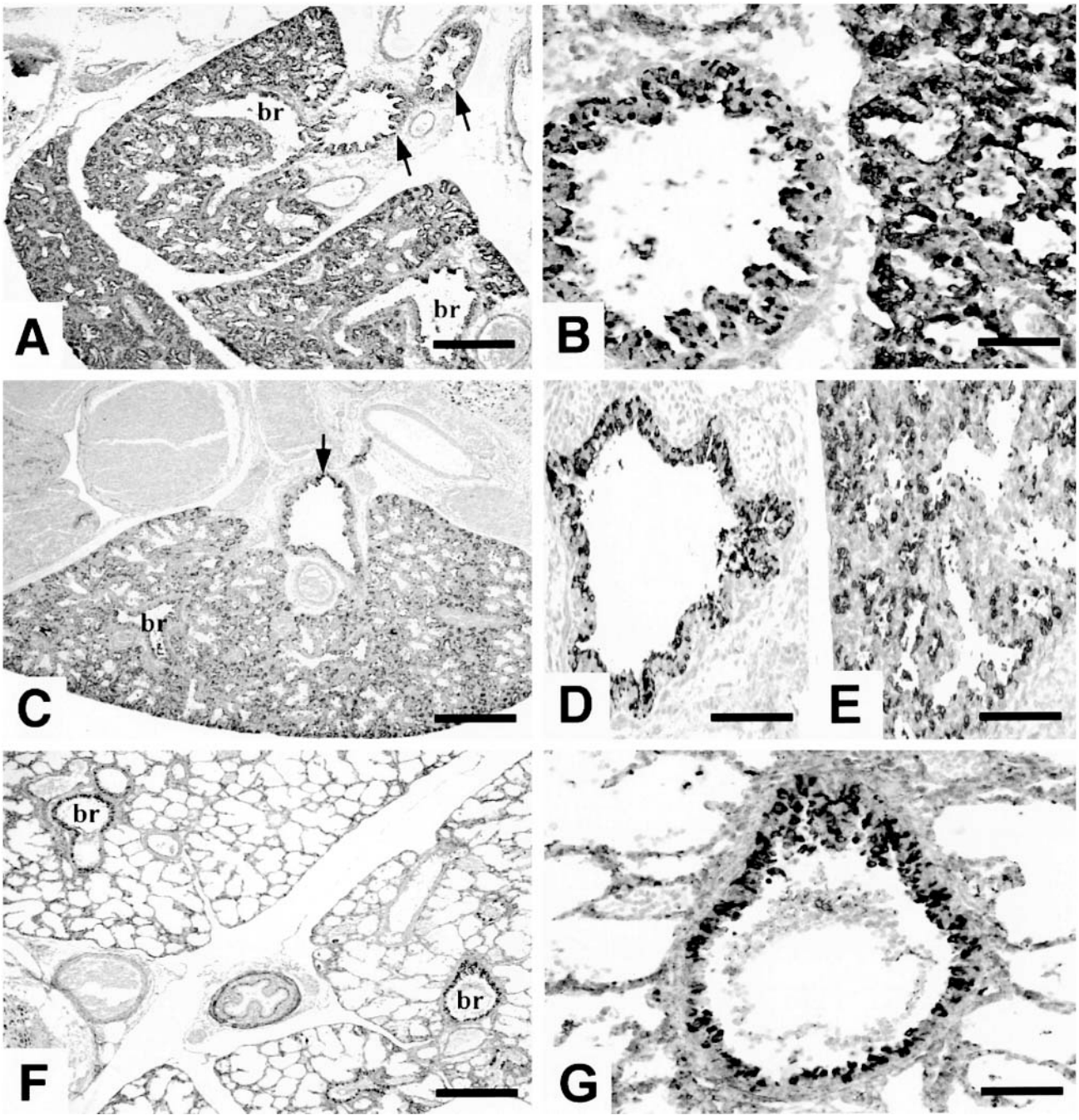

Figure 4 Shh expression during the saccular stage of lung development (E17.0–E18.0 and PD1). Staining for Shh was detected in the respiratory epithelium and at elevated levels in the bronchiolar (br) and bronchial (arrows) epithelium on E17.0/17.5 (**A,B**). Staining for Shh was also detected on E18.0 in both respiratory and conducting airways (**C–E**). Shh staining was detected in the respiratory epithelium, where it was markedly reduced. Shh staining was intense in the bronchiolar epithelium on PD1 (**F,G**). Bars: **A,C,F** = 250 μm; **B,G** = 50 μm; **D,E** = 100 μm.

(Figure 6B). The nuclei of ciliated cells stained with the HFH-4 antibody, which co-localized with β-tubulin immunostaining (Figure 6E and 6F) and the presence of cilia. The expression pattern of Shh was distinct from that of HFH-4 (Figure 6F) or β-tubulin (Figure 6E) and did not co-localize with the ciliated cells (Figure 6C). Sites of Shh staining were similar to that of CCSP (Figure 6B), indicating that Shh was expressed in non-ciliated epithelial cells in the conducting airways (i.e., Clara cells).

Discussion

Shh has been proposed to play a mitogenic role in sev-

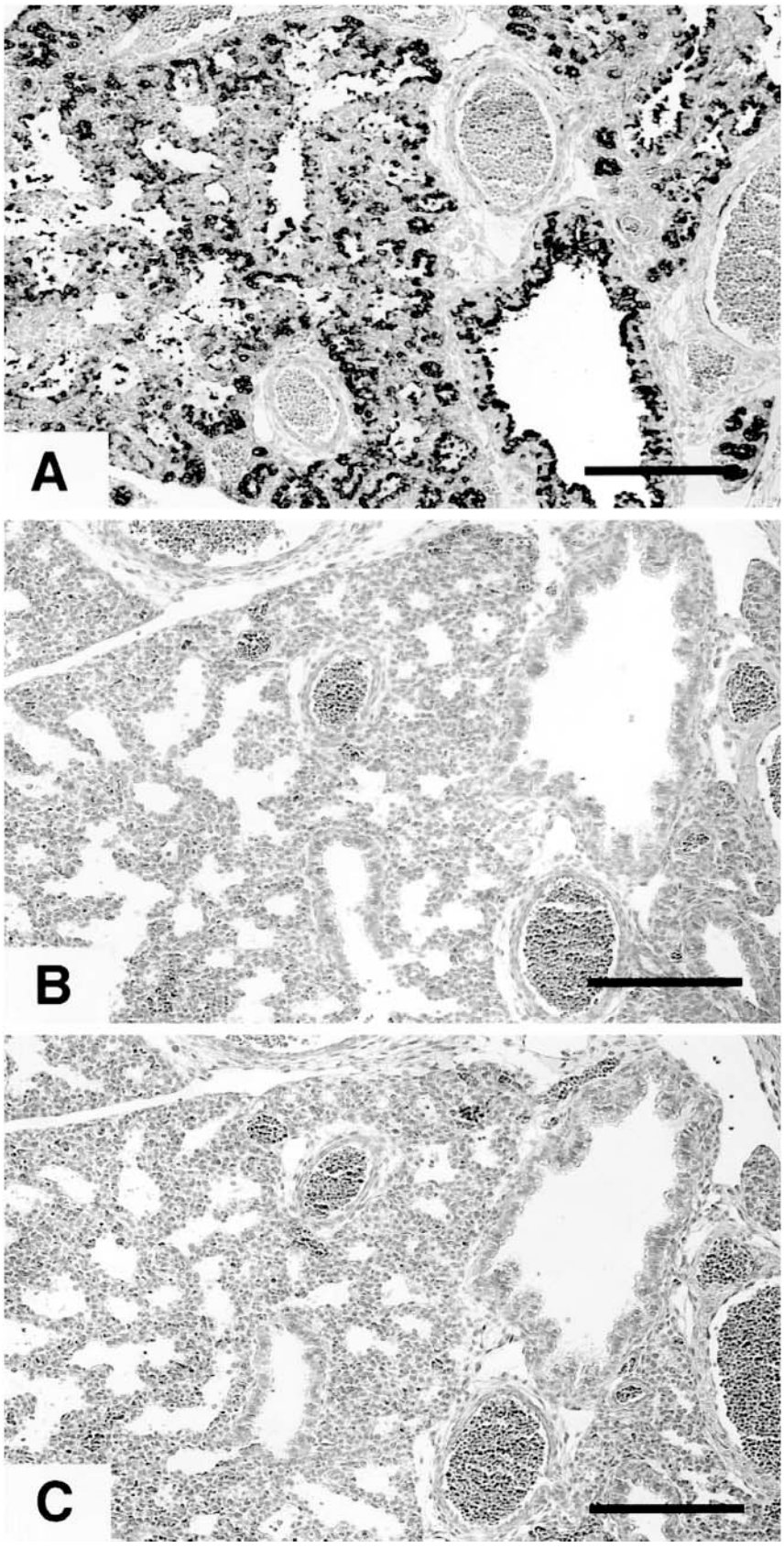

Figure 5 Specific binding of Shh antibody to mouse E18.0 lung tissue. Staining for Shh in both the peripheral respiratory and bronchiolar epithelium was detected on a serial sections incubated with Shh primary antibody (**A**). When the primary antibody was eliminated from the procedure, no specific staining for Shh was detected (**B**). Staining for Shh was undetectable on a serial lung section incubated in a pre-mixed solution containing Shh primary antibody and Shh recombinant protein (**C**). Bars = 125 μm.

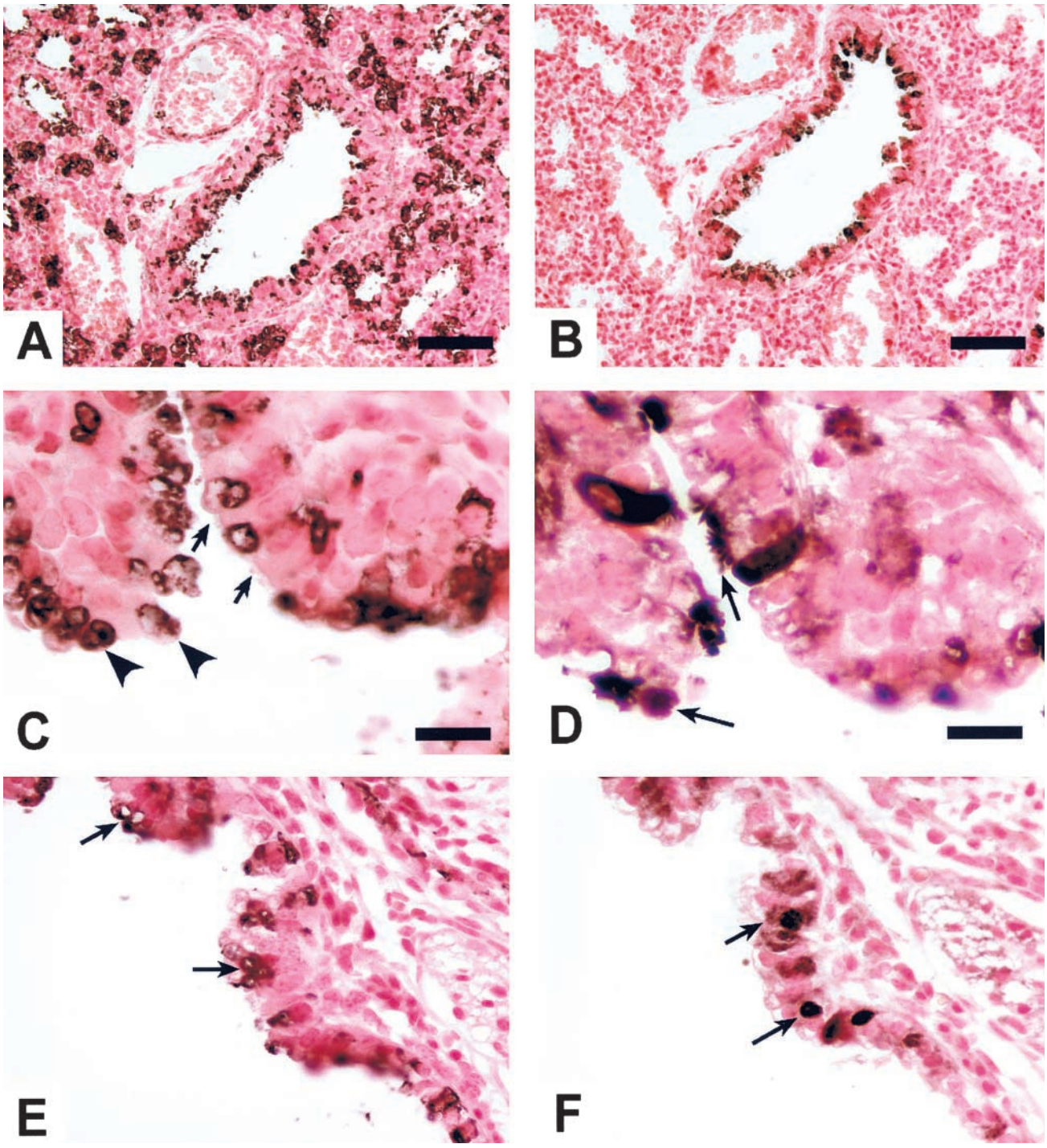

Figure 6 Immunostaining of Shh, CC10, HFH-4, and β-tubulin. On serial sections of E18.0 mouse lung, staining for Shh was detected in epithelial cells of the peripheral respiratory and conducting (bronchiolar) airways (**A**), whereas CCSP staining was detected only in the conducting airways (**B**). In the conducting airways, Shh staining was detected in the dome-shaped, protruding cells (arrowheads), consistent with Clara cells but not ciliated cells (arrows, **C**). Staining for β-tubulin (**D**) and HFH-4 (**F**) was detected in ciliated cells of the bronchial epithelium on serial sections of E18.0 lung in a pattern that did not correlate to that for Shh (**C,E**). Bars: **A,B** = 50 μm; **C,E,F** = 25 μm; **D** = 16 μm.

eral systems during embryonic development of several organs. In the vertebrate limb, Shh is required for patterning along the anterioposterior (A/P) axis and distal outgrowth of the limb bud (Riddle et al. 1993). The presence of Shh in the notochord directs neuronal differentiation of the ventral neural tube via a dose-dependent mechanism (for review see Briscoe and Ericson 1999). It may also be involved in the proliferation of precursor cells in the CNS (Echelard et al. 1993; Rowitch et al. 1999) and granular cell precursors in the cerebellum (Wallace 1999; Wechsler–Reya and Scott 1999), as well as retinal precursor cells in the neural retina (Jensen and Wallace 1997). Overexpression of Shh in the lungs leads to a significant increase in epithelial and mesenchymal cell proliferation (Bellusci et al. 1997), whereas loss of Shh expression results in enhanced cell death and decreased cell proliferation in the mesenchyme (Pepicelli et al. 1998). Mutations in regulatory genes leading to constitutive activation of Shh-responsive genes results in a wide variety of tumors (Wicking et al. 1999) and further implicates Shh as a mitogen.

Shh mRNA has been detected as early as E9.5 in the tracheal diverticulum (Litingtung et al. 1998) and throughout the lung bud epithelium at E10.5. It is during the embryonic stage of lung morphogenesis that epithelial cells from the primitive foregut endoderm invade the splanchnic mesenchyme forming the primordial tubules. At this time, epithelial cells lining the primordial tubules are relatively undifferentiated and undergo rapid proliferation. This proliferation results in continued invasion of the mesenchyme, with subsequent dichotomous branching of the growing tubules. The mesenchyme is also relatively undifferentiated at this stage, consisting of the cellular precursors of the pulmonary vasculature, smooth muscle, and cartilage, which form later in morphogenesis.

During early lung development (around E10), Shh is expressed in the epithelium of the primordial tubules along with Nkx2.1 and BMP-4 (for review see Perl and Whitsett 1999; Warburton et al. 1999; Cardoso 2000). All of these molecules are associated with proliferation and cytodifferentiation of the respiratory epithelium. Later in lung development, Shh expression is detected in the proximal airways where GATA-6 and HNF-3β are also expressed (Zhou et al. 1996; Keijzer et al. 2001). The transcription factors TTF-1, HNF-3β, and GATA-6 influence transcription of each other and of downstream genes critical to postnatal lung function, including CCSP, SP-A, SP-B, and SP-C. Previously, it has been proposed that Shh may not play a role in cytodifferentiation of the respiratory epithelium during lung morphogenesis. Analysis of the cell differentiation markers CCSP and SP-C indicated that epithelial differentiation along the P/D axis was not perturbed in transgenic SP-C/Shh and Shh null mutant mice (Bellusci et al. 1997; Pepicelli et al. 1998). However, because localization of Shh was investigated only during early lung morphogenesis, before extensive cytodifferentiation of the conducting airways, it is possible that Shh plays a role in cell proliferation and cytodifferentiation during later stages of lung morphogenesis.

The Shh receptor Ptc is expressed at E11.5 in the mesenchyme surrounding the distal region of the primordial tubules (Bellusci et al. 1997). The Gli genes, transcription factors that act downstream of Shh, are also expressed in the mesenchyme beginning at E11.5 (Grindley et al. 1997). From E15.5 to PD1 there is a coincident decrease in expression of Shh, Ptc, and all three Gli genes (Bellusci et al. 1997; Grindley et al. 1997). Because overexpression of Shh results in upregulation of Ptc and Gli1 in the lung mesenchyme (Bellusci et al. 1997; Grindley et al. 1997), Shh is believed to signal the mesenchyme directly in a paracrine fashion.

At E16.5, Shh was detected at low levels in a subset of epithelial cells in the bronchiolar tubules, the non-ciliated bronchiolar epithelial cells. The appearance of Shh in non-ciliated cells of the conducting airways occurs in association with cytodifferentiation of ciliated and non-ciliated cells in the conducting airways. Expression of HFH-4 is required for formation of ciliated cells in the conducting airways (Hackett et al. 1995; Pelletier et al. 1998; Tichelaar et al. 1999a,b). Mice deficient in either Shh (Tsukui et al. 1999) or HFH-4 (Chen et al. 1998) exhibit defects in left–right asymmetry. In addition, during this stage of lung morphogenesis, formation of the capillary bed and differentiation of cartilage and smooth muscle occur in the lung mesenchyme. It is possible that Shh plays a role in the morphogenesis of vascular and other non-epithelial components of the lung via activation of Ptc and in subsequent signal transduction via the Gli genes.

Postnatally, there was a significant decrease in Shh staining in the alveolar epithelium, whereas strong staining was maintained in the non-ciliated cells of the bronchiolar epithelium. This pattern of Shh staining persisted until PD15, when staining of the respiratory epithelium was not detectable and that detected in the bronchiolar epithelium was significantly reduced. Shh staining was not detected in either the respiratory or bronchiolar epithelium at PD24. The saccular stage of lung morphogenesis begins at E17.4 and continues until PD5. During this stage, there is a decrease in cell proliferation and further development of the respiratory and bronchiolar systems with the differentiation of Clara cells, ciliated cells, and Type I alveolar cells (Ten Have–Opbroek 1991). Clara and ciliated cell maturation begins during the canalicular stage (E16.6-17.4), consistent with the expression of CCSP and HFH-4. However, maturation of the alveolar regions and bronchial airways continues postnatally, being com-

pleted by 3–4 weeks of age (Ten Have–Opbroek 1991). In mice, alveolar development occurs in the postnatal period and is marked by the formation of secondary alveolar septa that allow formation of true alveolar ducts and alveoli by partitioning the terminal ducts and saccules (Ten Have–Opbroek 1991). This extensive remodeling of the lung architecture requires proliferation, migration, and differentiation of both epithelial and mesenchymal cell types. Therefore, the expression of Shh in postnatal lungs (up to PD15) indicates that it may be involved in later stages of lung development and maturation.

The retinoic acid (RA) signaling pathway has recently been shown to be both spatially and temporally regulated (Malpel et al. 2000). This dynamic expression pattern of RA indicates an involvement in both early and late lung morphogenesis (Malpel et al. 2000), which parallels our suggestion that Shh may undergo temporal and/or regional regulation. Furthermore, lung explants exposed to high concentrations of RA early in lung development exhibit abrogated branching morphogenesis with decreased formation of distal lung structures and an overall immature lung phenotype (Cardoso et al. 1995,1996). Because overexpression of Shh also results in an abrogation of branching morphogenesis and RA is known to induce Shh expression in the lung (Cardoso et al. 1996), it is possible that RA acts upstream of the Shh pathway during lung development.

Ongoing expression of Shh in the respiratory epithelium, with induction of Shh expression in the bronchiolar and bronchial epithelium during the later stages of development, suggests that Shh plays a role in lung morphogenesis or cytodifferentiation throughout lung development.

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