Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis

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SUMMARY

Branching morphogenesis of the embryonic lung requires interactions between the epithelium and the mesenchyme. Previously, we reported that Sonic hedgehog (Shh) transcripts are present in the epithelium of the developing mouse lung, with highest levels in the terminal buds. Here, we report that transcripts of mouse patched (Ptc), the homologue of a Drosophila gene encoding a putative transmembrane protein required for hedgehog signaling, are expressed at high levels in the mesenchyme adjacent to the end buds. To investigate the function of SHH in lung development, Shh was overexpressed throughout the distal epithelium, using the surfactant protein-C (SP-C)-enhancer/promoter. Beginning around 16.5 dpc, when Shh and Ptc RNA levels are normally both declining, this treatment caused an increase in the ratio of interstitial mesenchyme to epithelial tubules in transgenic compared to normal lungs. Transgenic newborn mice die soon after birth. Histological analysis of the lungs at the light and electron microscope level shows an abundance of mesenchyme and the absence of typical alveoli. In vivo BrdU labeling indicates that Shh overexpression results in increased mesenchymal and epithelial cell proliferation at 16.5 and 17.5 dpc. However, analysis of CC-10 and SP-C expression reveals no significant inhibition in the differentiation of proximal and distal epithelial cells. The expression of genes potentially regulated by SHH was also examined. No difference could be observed between transgenic and control lungs in either the level or distribution of Bmp4, Wnt2 and Fgf7 RNA. By contrast, Ptc is clearly upregulated in the transgenic lung. These results thus establish a role for SHH in lung morphogenesis, and suggest that SHH normally regulates lung mesenchymal cell proliferation in vivo.

Key words: Shh, Ptc, Bmp4, lung development, transgenic overexpression, mouse embryo

INTRODUCTION

Lung development in the mouse starts at 9.5 dpc with the evagination of a pair of endodermal epithelial buds from the ventral foregut into the surrounding splanchnic mesoderm. Subsequently, during the pseudoglandular stage (9.5-16 dpc), rapid growth and branching of the primitive lung epithelium occurs to form the conducting airways and the terminal acinar buds. During the canalicular (16-17 dpc) and the saccular (17 dpc to birth) stages, the terminal buds progressively dilate to form sac-like structures, the future alveoli, characterized by a thinning of the mesenchyme and a close apposition with blood capillaries (Ten Have-Opbroek, 1991).

A complex interplay between mesenchymal and epithelial cells takes place during lung development. Grafting experiments have shown that branching morphogenesis and differentiation of the proximal tracheal epithelium can be induced by interaction with distal mesenchyme, while the mesenchyme surrounding the trachea will inhibit the growth and branching of the distal epithelium (Alescio and Cassini, 1962; Wessels, 1970; Goldin and Wessels, 1979; Shannon, 1994). A major goal is to identify the components of the signaling pathways mediating these interactions.

At the molecular level, a number of different polypeptide growth factors have been shown to be important for lung development. These include fibroblast growth factors (FGFs), Transforming growth factor betas (TGFβs) and Bone morphogenetic proteins (BMPs). For example, transgenic misexpression of Fgf7/Kgf in the distal mouse lung epithelium using a surfactant protein-C (SP-C)-enhancer/promoter. Beginning around 16.5 dpc, when Shh and Ptc RNA levels are normally both declining, this treatment caused an increase in the ratio of interstitial mesenchyme to epithelial tubules in transgenic compared to normal lungs. Transgenic newborn mice die soon after birth. Histological analysis of the lungs at the light and electron microscope level shows an abundance of mesenchyme and the absence of typical alveoli. In vivo BrdU labeling indicates that Shh overexpression results in increased mesenchymal and epithelial cell proliferation at 16.5 and 17.5 dpc. However, analysis of CC-10 and SP-C expression reveals no significant inhibition in the differentiation of proximal and distal epithelial cells. The expression of genes potentially regulated by SHH was also examined. No difference could be observed between transgenic and control lungs in either the level or distribution of Bmp4, Wnt2 and Fgf7 RNA. By contrast, Ptc is clearly upregulated in the transgenic lung. These results thus establish a role for SHH in lung morphogenesis, and suggest that SHH normally regulates lung mesenchymal cell proliferation in vivo.

Key words: Shh, Ptc, Bmp4, lung development, transgenic overexpression, mouse embryo
development. Histological analysis of newborn lungs indicated an immature phenotype, with decreased expression of SP-C protein and abnormally dilated intrapulmonary veins (Kaartinen et al., 1995). By contrast, the precise role of TGFβ1 in lung morphogenesis is still unclear. Lung branching morphogenesis is inhibited by exogenous TGFβ1 in vitro (Serra et al., 1994) and the differentiation of transgenic lungs overexpressing TGFβ1 in the developing epithelium is blocked at the pseudoglandular stage (Zhou et al., 1996). However, TGFβ1 null mutant mice have normal lungs, probably due to compensation by other TGFβ isoforms expressed in the lung (Shull et al., 1992; Kulkarni et al., 1993).

Several members of the Bmp gene family are expressed in the mouse embryonic lung, including Bmp4, 5 and 7. Bmp5 is expressed in the lung mesenchyme from 10.5 dpc through at least 16.5 dpc (King et al., 1994). While the lungs of most Bmp5 homozygous null mutants are normal, on certain genetic backgrounds they contain fluid-filled cysts (Green, 1968). At the pseudoglandular stage, Bmp4 transcripts are detected at high levels in the epithelium of the distal end buds and at lower levels in the adjacent distal mesenchyme (Bellusci et al., 1996). Overexpression of Bmp4 throughout the developing epithelium with the SP-C enhancer/promoter results in abnormal lung morphogenesis, with cystic terminal sacs, and inhibition of epithelial proliferation and a reduction in the number of differentiated Type II epithelial cells.

We and others have previously shown expression of Shh in the developing mouse lung. Shh is a vertebrate homologue of Drosophila hedgehog (hh), which encodes a secreted signaling protein regulating Decapentaplegic (Dpp) and Wingless (Wg) in target cells (Herberlein et al., 1993; Ingham, 1993; Basler and Struhl, 1994; Capdevilla et al., 1994; Diaz-Benjumea et al., 1994). Low levels of Shh RNA are present throughout the epithelium but high levels are seen at the tips of the distal buds (Bitgood and McMahon, 1995; Bellusci et al., 1996; Urase et al., 1996). A mouse homologue of Patched (Ptc), a segment polarity gene in Drosophila, has been identified (Goodrich et al., 1996). This gene encodes a putative multipass transmembrane protein required for HH signaling, and is invariably upregulated in Drosophila cells in response to HH (Hooper and Scott, 1989; Nakano et al., 1989; Tabata and Kornberg, 1994). Indeed, Ptc upregulation is a consistent feature of cells responding to a HH signal, even though, paradoxically, HH is thought to repress PTC activity (Ingham et al., 1991; Lepage et al., 1995). Thus, ectopic expression of hh in the anterior of imaginal discs leads to upregulation of Ptc and to increased growth and duplications of adult structures. Recently, mutations in the human Ptc gene have been identified in patients with hereditary basal cell nevus syndrome, a condition associated with abnormal proliferation and patterning of many different tissues (Johnson et al., 1996). We report here that the mouse patched gene is expressed at high levels in the mesenchyme surrounding the terminal end buds of the lung, and possibly at much lower levels in the adjacent epithelium.

The observation that Bmp4, Wnt2 and Shh are expressed in the end buds suggested to us that these three secreted factors could interact during lung development (Bellusci et al., 1996). We proposed a model in which SHH, secreted by the distal epithelium of the lung, induces Bmp4 and Wnt2 expression in the surrounding mesenchyme. According to the model, WNT2 (and possibly a member of the FGF family) might help to maintain Shh expression in the epithelium, while BMP4 might act on the distal epithelium to activate Bmp4 expression, establishing an autoregulatory loop similar to that proposed in the developing mouse tooth bud (Vanio et al., 1993), and locally inhibiting proliferation.

In order to test this model we have overexpressed Shh throughout the distal epithelium, using the SP-C enhancer/promoter. Shh overexpression results in an increase in epithelial and mesenchymal cell proliferation and to a lung which contains an abundance of mesenchyme and no functional alveoli. At the molecular level, Ptc expression but not Bmp4, Wnt2 or Fgf7 is upregulated in the SP-C-Shh transgenic lungs from 17.5 dpc suggesting that the model previously proposed is not correct. By contrast, these results do support the idea that SHH normally plays a key role in branching morphogenesis, and acts as a mitogen for mesenchymal and possibly epithelial cells independently of major changes in Bmp4 and Wnt2 expression.

MATERIALS AND METHODS

Plasmid construction

The SPC-mouse Shh vector was constructed using a mouse Shh cDNA (gift from Dr Andrew McMahon, Harvard University). The 1900 bp cDNA was inserted into a vector containing the 3.7 kb human SP-C promoter region (Korfhagen et al., 1990). An SV40 small T intron and a 0.4 kb sequence containing a poly(A) addition site with stop codons in all three reading frames were present at the 3’-end of the cDNA. The expression cassette was excised with NdeI and NotI.

Generation and identification of transgenic mice

Transgenic mice were generated as described by Hogan et al. (1994). DNA was purified by low melting point agarose gel electrophoresis (GIBCO, Life Technologies Inc.) followed by gelase digestion (Epicentre Technology), phenol-chloroform extraction, ethanol precipitation and passage through a Qiaquick column (Qiagen). The transgene was injected into the pronuclei of (C57BL/6FDBA)F2 mouse eggs at a concentration of 3 ng/μL. Noon of the day of injection is 0.5 dpc.

The genotype of embryos was determined by PCR analysis of genomic DNA extracted from embryonic liver. The primers used were 5'-AGGAAACAAACAGGCTTCAAA-3' (SP-C-primer for 5') and 5'-GATGTGAAGCTTGGATTCCAT-3' (mouse Shh for 3'). The cDNA was amplified for 27 cycles at 61°C. Of a total of 76 embryos obtained between 15.5 dpc and birth after SP-C-Shh injection of eggs, 19 were transgenics. The number of transgenic embryos obtained at each stage and the number of lungs over-expressing the transgene (respectively in parentheses) were as follow; 15.5 dpc (6;2), 16.5 dpc (1;1), 17.5 dpc (6;1), 18.5 dpc (4;2) and newborn (2;2).

In situ hybridization

The whole-mount in situ hybridization protocol was based on one used previously (Winnier et al., 1995). The following murine cDNAs were used as templates for synthesizing digoxigenin-labeled riboprobes: 642 bp Shh (kindly provided by Dr Andrew McMahon) and 841 bp Ptc (kindly provided by Dr Lisa Goodrich). The non-radioactive in situ hybridization of tissue sections was based on a protocol used previously (Sasaki and Hogan, 1993). Digoxigenin-labeled antisense RNA probes were prepared for mouse surfactant protein-C (758 bp) and rat CC-10 (450bp) (Dr Jeffrey Whitsett). Radioactive in situ hybridization of lung sections was carried out essentially as described by Zhao et al. (1993). The following murine cDNAs were used as templates for synthesizing 35S-
labeled riboprobes: 474 bp mouse SP-A, 1.55 kb mouse SP-B, 2.5 kb mouse CFTR (Dr Jeffrey Whitsett), 630 bp Wnt2 and 642 bp Shh (Dr Andrew McMahon), 1.5 kb full length mouse Bmp4 (Winnier et al., 1995), 841 bp Ptc (Dr Lisa Goodrich) and 622 bp mouse fgf7 (kindly provided by Dr Ivor Mason). In order to provide a qualitative comparison of levels of gene expression between transgenic and normal lungs, six to nine sections (7 μm) of transgenic and normal lungs fixed and processed under the same conditions were placed on the same slide. The slide was then processed as described by Zhao et al. (1993) so that the sections were treated under exactly the same conditions with respect to probe concentration and specific activity, washed at the same temperature and stringency, exposed for the same time to the emulsion and developed under the same conditions. Photomicrographs (both bright and dark field) of control and transgenic sections to be compared were taken with the same exposure time.

Northern blot analysis
Total RNA was isolated using guanidine thiocyanate extraction and cesium chloride ultracentrifugation (Chirgwin et al., 1979). 20 μg total RNA samples were analyzed on a 2% formaldehyde-1.2% agarose gel and blotted onto a Magna Graph Nylon Transfer membrane (Micron Separations Inc., Westborough, MA). The same filter was hybridized successively with radiolabeled [32P]cDNA probes for Shh, Ptc and β-actin (Stratagene Prime-It II kit, Stratagene, La Jolla, CA). The filter was hybridized for 16 hours at 42°C in 50% formamide, 10% dextran sulfate and 10% SDS. The filter was washed at 65°C in 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and then in 0.1% SDS. The blot was exposed to Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) in the presence of an intensifying screen at −70°C overnight. The autoradiograms were scanned using a laser densitometer (BioRad Laboratories, Richmond, CA). The levels of Shh and Ptc mRNA were normalized to the level of β-actin to correct for differences in RNA loaded.

Analysis of lung proliferation in vivo
Females between 15.5 and 18.5 days of pregnancy were injected intraperitoneally with a mixture of 5-bromo 2′-deoxyuridine (BrdU) and 5-fluoro 2′-deoxyuridine (FUdR) (Sigma) (50 and 10 mg per kg body weight respectively) in 0.5 ml of sterile PBS. After 1 hour and 15 minutes, lungs were collected, fixed in 4% paraformaldehyde for 1 hour, washed 3 times with PBS, dehydrated in 25%, 50%, 75% and 100% ethanol (5 minutes each), embedded in paraffin and sectioned (7 μm). Sections were then dewaxed and stained as described previously (Sakai et al., 1994). Briefly, staining of trypsinized sections was carried out with a rat anti-BrdU antibody (Accurate Chemical, Westbury, New York) diluted 1:400 in PBS. Sections were then treated with peroxidase-coupled rabbit anti-rat IgG (Vector) diluted 1:100 in PBS using 3-amo,9-ethyl carbazole as the chromogen. Photomicrographs, at magnification ×40, of random portions of nine different sections of a control and transgenic lung were examined and all the cells were counted and scored as labeled or unlabeled. At 15.5 and 16.5 dpc it was possible to distinguish clearly between epithelial and mesenchymal cells. At 17.5 days, this distinction was not possible, so that only the overall percentages of labelled nuclei were compared between control and transgenic mice. Paired groups of data were analyzed by Student’s t-test using the SAS 6.10 program. Results were determined to be significant at P<0.05.

Electron microscopy
Tissue for electron microscopy was placed in 2% glutaraldehyde in phosphate buffer, postfixed in osmium tetroxide, stained en bloc with uranyl acetate, processed by standard dehydration in graded ethanols before infiltration and embedded in Spurr’s embedding medium (EM Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate-lead citrate before viewing in a Philips 300 electron microscope.

RESULTS
Gene expression in embryonic mouse lung
At 11.5 dpc, the lung consists of an outer layer of splanchnic mesenchyme surrounding a single tracheal tube and a small number of distal branching epithelial tubules. As shown previously (Belluscio et al., 1996), Shh is expressed at a low level throughout the epithelium and at a high level in the distal buds (Fig. 1A). This pattern appears to be maintained at 15.5, 16.5 and 17.5 dpc (Figs 3A,B,E,F, 10E,F) although at these
times transcripts cannot be localized with certainty to the distal buds. Northern analysis of total lung RNA shows that Shh expression slowly declines as development proceeds (Fig. 2).

In contrast to the epithelial expression of Shh, Ptc transcripts are detected at high levels in the mesenchyme around the end buds of 11.5 dpc lungs (Fig. 1B). Mesenchymal expression of Ptc was confirmed by sectioning after whole-mount in situ hybridization (Fig. 1C). In addition, we used a more sensitive technique, radioactive in situ hybridization, to detect transcripts in sectioned tissue (Fig. 1D-F). This clearly showed Ptc RNA in the mesenchyme, but also suggested that there is a low level of expression in the distal epithelium. Northern analysis shows a clear decline in Ptc RNA levels between 15.5 dpc and birth (Fig. 2).

Expression of Shh in transgenic lungs
As previously described, the 3.7 kb enhancer/promoter of the mouse SP-C gene drives expression of transgenes throughout the distal epithelium of the lung, but not in the proximal airway, starting at 10 dpc (Wert et al., 1993). A construct was therefore generated in which the mouse SHH protein was expressed under the control of the SP-C enhancer/promoter. Transgene expression was assayed by in situ hybridization using a mouse Shh riboprobe. Of 19 lungs positive for transgene integration according to PCR analysis of genomic DNA, only 8 overexpressed Shh RNA. This proportion is similar to that reported previously for the SP-C-Bmp4 construct (Bellusci et al., 1996). As shown in Fig. 3A-H, much higher levels of Shh transcript were typically present throughout the distal epithelium of these particular transgenic lungs compared to the controls, both at 15.5 and 17.5 dpc. Note that all the studies reported hereafter have been conducted with the transgenic lungs overexpressing SHH.

Abnormal development of SP-C-Shh transgenic lungs
No obvious macroscopic differences were seen at 15.5, 16.5, 17.5 and 18.5 dpc between transgenic and normal lungs. Both the size and the wet weight after fixation and dehydration of the normal and transgenic lungs were similar. For example two normal lungs at 16.5 dpc had an average wet weight of 19.5±0.1 mg compared with 19.6 mg for a single SP-C-Shh transgenic lung. However, a clear difference was seen at birth. The two transgenic lungs obtained were smaller than normal (Fig. 4) and about half of the wet weight (an average of 39.3±4 mg from four normal lungs compared with 18.6 mg for one of the SPC-Shh transgenic lungs).

On histological analysis, no phenotypic difference could be detected between transgenic and normal lungs at 15.5 dpc (Fig. 5A-D). However at 16.5 dpc, there appeared, at low magnification, to be an increase in the relative proportion of mesenchyme to epithelial tubules in sections taken at comparable longitudinal levels of SP-C-Shh versus control lungs (Fig. 5E,F). However, at high magnification, the organization of the epithelium in the transgenic lung appeared normal (Fig. 5G,H). Around 17.5 dpc, normal lung architecture undergoes major changes, leading to the formation of sac-like structures, the precursors of the alveoli. In the SP-C-Shh transgenic lung, the expansion of the epithelial tubules seems to occur, but the interstitial mesenchymal compartment is much thicker compared to that of the normal lung (Fig. 5I-L).

Two transgenic pups were born, but soon died. In both cases the lungs were hypercellular, with an absence of alveoli char-

Fig. 2. Expression of Shh and Ptc during development. 20 μg of total RNA from lungs isolated and pooled from litters at 15.5 dpc to birth was analyzed as described in Materials and Methods. The blot was hybridized with mouse Shh and mouse Ptc. β actin was used as a control for loading and normalizing between ages. Levels of transcripts for Ptc (approx. 8 kb) and Shh (approx. 3 kb) decline as development proceeds (25% and 29% decrease at 18.5 dpc compared to 17.5 dpc and 68% and 39% decrease at birth compared to 17.5 dpc for Ptc and Shh respectively).

Fig. 3. Shh expression in control and transgenic lung as revealed by in situ hybridization of sections with an 35S-labeled antisense mouse Shh riboprobe. Section of a 15.5 dpc (A,B) normal (N) or (C,D) transgenic (T) lung showing high expression in the distal epithelium (de) of the transgenic lung but no expression in the mesenchyme (m). Sections of a 17.5 dpc (E,F) normal and (G,H) transgenic lung. The levels of expression were not comparable between ages. Scale bar: 280 μm.
The abnormal phenotype of the SP-C-Shh transgenic lungs suggests that Shh overexpression results in increased cell proliferation and/or cell survival, particularly in the mesenchymal component of the lung. Cell proliferation was therefore examined by exposing 15.5, 16.5 and 17.5 dpc embryos in utero to BrdU for 1 hour. The relative proportion of cells that had entered or passed through S phase was then determined by immunocytochemistry of lung sections. The results are summarized in Table 1. At 15.5 dpc, when transgenic and normal lungs are histologically similar, no difference was observed in the proliferation rate of epithelial and mesenchymal cells between transgenic and control lungs (Fig. 7A,B) (32% versus 35% for the epithelium and 23% versus 24% for the mesenchyme, respectively). However, at 16.5 dpc, there is about a 2-fold increase in the rate of proliferation of both epithelial and mesenchymal cells in the transgenic lung (Fig. 7C,D) (28% versus 13% in the epithelium and 26% versus 15% in the mesenchyme, respectively). By 17.5 dpc, it was not possible to easily distinguish between epithelial and mesenchymal cells. Therefore, all the cells labeled were counted and compared to the total number of cells. The results show that there is more cell proliferation in the SP-C-Shh transgenic lung compared to the normal counterpart (9% versus 4% respectively).

**Expression of CFTR, SP-A, SP-B, SP-C and CC-10 in transgenic lungs**

In order to investigate if cell differentiation was altered in the SP-C-Shh transgenic lung, in situ hybridization on sections was carried out using riboprobes for five marker genes, Cystic fibrosis transmembrane conductance regulator gene (CFTR), SP-A, SP-B, SP-C and CC-10. Transcripts for CFTR encode a chloride channel expressed by epithelial cells in a large variety of organs including lung and intestinal tract (Snouwaert et al., 1992). Transcripts for SP-A and SP-B are normally detected from 14–15 dpc in the distal epithelium and expressed later in nonciliated bronchiolar epithelial (Clara) cells and in Type II but not Type I epithelial cells (Kalina et al., 1992). Transcripts for SP-C are detected from 10.5 dpc in the distal epithelium and later switched off in the precursors of type I cells and then restricted to the mature type II cells in the alveolar sacs (Wert et al., 1993). CC-10 expression is not detected before 17.5 dpc, and is present only in the epithelial Clara cells located in the terminal bronchioles (Singh et al., 1993).

The transcripts corresponding to these five genes are detected in the normal and transgenic lungs at 17.5 dpc and birth (data not shown for SP-A, SP-B and CFTR). Fig. 8A-D shows that the distribution of SP-C transcripts is similar in SP-C-Shh and control lungs at 17.5 dpc. At birth, however, because of the drastic difference in lung structure, it was difficult to compare directly the pattern of expression in transgenic and normal lungs (Fig. 8E-H). Although SP-C transcripts are detected, in the absence of a specific marker for type I cells we cannot say whether the differentiation of the respiratory epithelium is completely normal in the transgenic lung. However, CC-10 expression was comparable in the transgenic and control lung at both 17.5 dpc and birth (Fig. 8I-L).

**Table 1. Cell proliferation in SP-C-Shh transgenic lung**

<table>
<thead>
<tr>
<th>dpc</th>
<th>Normal (%)</th>
<th>Transgenic (%)</th>
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<tr>
<td>Epithelium</td>
<td>15.5</td>
<td>34.6±2.4</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>24.1±2.3</td>
<td>22.6±2.3</td>
</tr>
<tr>
<td>Epithelium</td>
<td>16.5</td>
<td>13.4±3.9</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>15.0±2.5</td>
<td>25.7±4.4</td>
</tr>
<tr>
<td>All cells</td>
<td>17.5</td>
<td>4.4±0.9</td>
</tr>
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Lung sections were processed for immunocytochemistry using an anti-BrdU antibody. Epithelial and mesenchymal cells (at 15.5 and 16.5 dpc) or all the cells (at 17.5 dpc) labeled were counted and compared to the total number of cells in each case. The ratio of the total number of cells (labeled and unlabeled) in the epithelium/total number of cells in the mesenchyme was as follow: 15.5 dpc 1544/4096 and 1339/3796 for the normal and the transgenic lung respectively. 16.5 dpc 1319/3743 and 1571/3284 for the normal and transgenic lung respectively. 17.5 dpc 5404 and 6440 for the normal and transgenic lung respectively. No statistical difference was observed between normal and transgenic lungs at 15.5 dpc (P>0.05). The P values, calculated at 16.5 and 17.5 dpc, indicate that the overproliferation observed in the transgenic lung, in the epithelium and in the mesenchyme, is statistically significant (P<0.001).
cating that there is no delay in the differentiation of the conducting airway. This is in contrast to the result observed for transgenic lungs overexpressing TGFβ1 under the control of the SP-C promoter/enhancer in which CC-10 expression was inhibited (Zhou et al., 1996).

**Effect of Shh overexpression on Ptc mRNA levels**

In *Drosophila*, HH upregulates the transcription of Ptc (Ingham, 1993; Tabata and Kornberg, 1994; Basler and Struhl, 1994). Similar results have been obtained in mouse and in chicken in response to the ectopic expression of Shh in the neural tube and limb bud, respectively (Goodrich et al., 1996; Marigo et al., 1996). To determine if Shh overexpression altered the expression of Ptc in the lung, sections of control and transgenic lungs at 15.5, 16.5, 17.5 dpc and newborn were hybridized with 35S-labeled antisense riboprobes for Ptc. Although this technique does not provide a quantitative measure of the levels of gene expression, standardized conditions between transgenic and control lung sections allow a qualitative comparison between samples (see Materials and Methods). The level of Ptc transcripts was clearly increased in the mesenchyme of the transgenic lung at 17.5, 18.5 dpc and newborn compared to the corresponding controls (Fig. 9E-F for 17.5 dpc and data not shown for 18.5 dpc and newborn). However, at 15.5 and 16.5 dpc, the level of Ptc expression in the transgenic lungs is similar to the corresponding controls (data not shown for 15.5 dpc and Fig. 9A-D for 16.5 dpc). Unfortunately, at 17.5 dpc it is difficult to distinguish epithelial from mesenchymal cells, especially in the transgenic lungs, so that it is not possible to say whether Ptc is upregulated in the epithelium as well as the mesenchyme.

**Effect of Shh overexpression on Bmp4, Wnt2 and Fgf7 mRNA levels**

In *Drosophila*, hh misexpression causes ectopic expression of Wg, Dpp and Ptc (Ingham, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994). In the chick embryo, ectopic SHH induces Bmp4 in hindgut mesenchyme (Roberts et al., 1995), and Bmp2 transcription is induced in limb bud in response to

![Image of histology](image-url)

**Fig. 5.** Histology of SP-C-Shh transgenic and control lungs at 15.5, 16.5 and 17.5 dpc and newborn. (A) Section of 15.5 dpc control lung. (B) Section of 15.5 dpc transgenic lung. (C) Higher magnification of the control lung in A shows multiple, small terminal buds and distal epithelium (de) surrounded by mesenchymal (m) cells. (D) Higher magnification of the transgenic lung in B shows no obvious abnormalities. (E) Section of control lung at 16.5 dpc reveals portion of bronchus (br) and blood vessel (bv). (F) Section of transgenic lung at 16.5 dpc shows an increase in the proportion of mesenchymal cells to epithelial tubules. (G) Higher magnification of control lung in E shows terminal buds lined by distal epithelium and surrounded by mesenchyme. (H) At higher magnification, the transgenic lung in F shows no obvious abnormalities. (I) Sections of 17.5 dpc control lung. The distal tubules have dilated and subsequently, the mesenchyme has thinned and septation appears to be progressing. (J) Section of 17.5 dpc transgenic lung showing a drastic increase in the mesenchymal component. (K) Higher magnification of the normal lung in I shows developing alveoli (a) and thinning mesenchyme (arrowheads). (L) Higher magnification of the lung in J shows the mesenchymal component which is thicker than in controls (arrowheads). (M) Section of newborn control lung shows a bronchiole and normal septation. (N) Section of newborn transgenic lung shows a hypercellularity, with the absence of alveoli. (O) Higher magnification of control lung in M shows normal septation. (P) Higher magnification of transgenic lung in N shows absence of septation and increase in cell density. Scale bar, A, B, E, F, I, J, M: 560 μm; C, D, G, H, K, L, O, P: 70 μm.
ectopic SHH and FGF4 (Laufer et al., 1994). We therefore examined by in situ hybridization the expression level of Bmp4, Wnt2 and Shh in SP-C-Shh and control lungs from 15.5 dpc to birth. The results obtained at these different times were similar. No upregulation of Wnt2 (data not shown) or Bmp4 could be observed. Fig. 10 shows that in the SP-C-Shh transgenic lung the expression of Bmp4 is virtually identical to the control whereas, as expected, Shh expression is much higher.

Thus, there appears to be no detectable upregulation of Bmp4 or Wnt2 expression level in the SP-C-Shh transgenic lung.

We further investigated if Fgf7 upregulation in the mesenchyme of the transgenic lung could account for the increase in cell proliferation in the transgenic lung. However, we failed to detect any upregulation of Fgf7 in the transgenic lung (data not shown), suggesting that SHH does not act through FGF-7 to induce cell proliferation.
DISCUSSION

Expression of Shh and Ptc during lung development

We report here that Shh and Ptc are both expressed at high levels in the end buds of the mouse embryonic lung. As shown previously (Bellusci et al., 1996) and in Fig. 1A, Shh is expressed throughout the epithelium, with the highest levels in the tips of the distal buds. By contrast, Ptc is detected at high levels in the mesenchyme around the end buds, and also possibly at low levels in the distal epithelium (Fig 1B-F). This pattern of expression persists at least until 16.5 dpc when epithelial and mesenchymal cells can be recognized easily. Studies by others had shown Ptc expression in the chicken lung restricted to the mesenchyme but had not reported the localization of high levels around the end buds (Marigo et al., 1996). Overlapping or adjacent domains of expression for Shh and Ptc have already been described in other systems. For example, in the chicken limb bud, Ptc and Shh are both expressed in the posterior mesoderm (Marigo et al., 1996). In the mouse neural tube, with the onset of Shh transcription in the floor plate, Ptc and Shh expression briefly overlap before resolving into complementary patterns. By 9 dpc, Ptc RNA begins to disappear from the floor plate where Shh is strongly expressed, but remains high in adjacent cells (Goodrich et al., 1996). The finding that Ptc expression in the lung is high around Shh-expressing end buds suggests that during normal development SHH protein secreted by the buds is signaling to the adjacent mesenchyme via a PTC-dependent pathway, since in Drosophila Ptc upregulation is a consistent feature of cells responding to a HH signal. The inter-relationship of Shh and Ptc expression is further supported by northern blot analysis that shows a co-ordinated decline in the level of both transcripts as development proceeds (Fig. 2).

The effect of overexpressing Shh in transgenic mouse lungs

To investigate the role of SHH in lung development, we generated transgenic embryos in which mouse Shh is overexpressed throughout the distal epithelium under the control of the SP-C-enhancer/promoter (Fig. 3). Histological analysis reveals an increase in the ratio of interstitial mesenchyme to distal epithelial tubules in the transgenic lungs that begins at 16.5 dpc (Fig. 5E-F) and is most pronounced just before and at birth (Figs 5M-P, 6), when transgenic lungs clearly contain extensive mesenchyme. This excess mesenchyme probably interferes with sacculation and the formation of functional alveoli needed for postnatal survival. This process is normally associated with the thinning of the mesenchyme and the close apposition of capillaries to the terminal epithelial sacs.

It is not yet clear why an abnormal phenotype is not observed in transgenic lungs until 16.5 days p.c in spite of a clear overexpression of the transgene at 15.5 dpc (Fig. 3A-D). One hypothesis is that Shh expression driven by the transgene is not high enough to generate an abnormal phenotype before 16.5 dpc. In support of this hypothesis, SP-C expression is dramatically increased around day 15 pc when acinar tubules differentiate at the lung periphery (Wert et al., 1993).

The finding that the interstitial mesenchyme is more abundant in the transgenic lungs compared with controls raises the possibility that Shh overexpression stimulates mesenchymal cell proliferation and/or inhibits cell death. BrdU labeling shows no difference in cell proliferation between normal and transgenic lungs at 15.5 dpc, consistent with the absence of an abnormal phenotype discussed above. However, by day 16.5 pc, about a two-fold increase in the rate of proliferation of both mesenchymal and epithelial cells is observed. At present we do not know whether SHH acts directly as a paracrine growth factor on the mesenchyme, or whether it acts indirectly through upregulating an autocrine growth factor, and/or inhibiting an autocrine growth inhibitor. However, it seems unlikely from our in situ hybridization studies that SHH acts through dramatically changing the expression of Fgf7, Wnt2, or Bmp4, genes encoding potential autocrine growth factors (see below and data not shown). BrdU labeling also shows an increase in
Role of Shh in lung development

the proliferation of the epithelium of transgenic lungs at 16.5 dpc. The high level of *Ptc* expression observed in the mesenchyme around the end buds of the normal lung (Fig. 1) suggests that mesenchymal cells represent the primary target for the paracrine action of SHH in vivo, and that the distal epithelial cell response is secondary to an effect on the mesenchyme. However, there is some evidence that *Ptc* is expressed at low levels in the distal epithelium (Fig. 1), so that we cannot at this time rule out an autocrine stimulatory effect of SHH. Unfortunately, it was not possible from the in situ hybridization studies to say whether *Ptc* is upregulated in the epithelial cells of SP-C-Shh lungs, a finding that would have supported the idea of an autocrine role.

A mitogenic activity for SHH/HH has previously been reported in other systems. For example, in vitro, SHH has a mitogenic effect on mouse presomitic mesoderm (Fan et al., 1995) and in Drosophila ectopic hh expression induces overproliferation of somatic cells in the ovary (Forbes et al., 1996) and excess growth and duplication of structures derived from imaginal discs (e.g. Basler and Struhl, 1994). Recently, it has been shown that mutations in the human *Ptc* gene are present in patients with hereditary basal cell nevus syndrome, a

Fig. 9. Shh overexpression upregulates *Ptc* mRNA levels. In situ hybridization of sections with an ^35^S-labeled antisense mouse *Ptc* riboprobe. (A,C) Bright- and (B,D) dark-field micromicrographs showing *Ptc* expression in 16.5 dpc normal (A,B) and transgenic lung (C,D). Comparable levels of expression are observed. (E,G) Bright- and (F,H) dark-field micrographs showing *Ptc* expression in 17.5 dpc normal (E,F) and transgenic lung (G,H). A dramatic upregulation of *Ptc* in the transgenic lung is observed. High magnification of the normal (IJ) and transgenic (KL) lungs in the periphery (IK) or in the center (JL) of the lung. The high magnification shows more grains in the transgenic versus the normal lung. The level of expression is not comparable between lungs at different ages. a, future alveoli. Scale bar, (A-H) 280 μm; (I-L) 46 μm.

Fig. 10. SHH does not upregulate *Bmp-4* in vivo. *Bmp4* and *Shh* expression in control and transgenic lung as revealed by in situ hybridization of sections with ^35^S-labeled antisense mouse *Bmp4* and *Shh* riboprobes. (A) Bright-field and (B) dark-field photomicrographs of a 16.5 dpc normal lung showing *Bmp4* expression in the distal epithelium (de) and in the mesenchyme (m). (C) Bright-field and (D) dark-field photomicrographs of a 16.5 dpc transgenic lung showing similar levels of *Bmp4* expression compared to the control. (E) Bright-field and (F) dark-field photomicrographs of a 16.5 dpc normal lung showing *Shh* expression in the distal epithelium. (G) Bright-field and (H) dark-field photomicrographs of a 16.5 dpc transgenic lung showing overexpression of *Shh* in the distal epithelium. The levels of expression between *Shh* and *Bmp4* are not comparable. Scale bar, 240 μm.
condition which is associated with basal cell carcinoma in the skin, tumors of the nervous system, and developmental patterning defects. It is therefore likely that, in the future, SHH signaling will be shown to be associated with changes in proliferation in many different target cells in vivo (Johnson et al., 1996).

Inhibition of cell death in SP-C-Shh transgenic lungs was experimentally difficult to investigate because of the low number of cells undergoing this process in the normal lung. We previously reported that by 16.5 dpc only 0.04% of the cells could be labeled by TUNEL or Nick translation in the normal lung (3 cells labeled out of 8000) (Bellusci et al., 1996). Therefore, we cannot at present exclude the possibility that Shh overexpression inhibits cell death in the developing lung. However, no histological evidence of major apoptosis was seen in the transgenic lung (see Figs 5L,P, 6).

While Shh overexpression affects cell proliferation in transgenic lungs does it also alter cell differentiation? No difference could be seen in the pattern and level of expression of CFTR, SP-A, SP-B, SP-C and CC-10 between transgenic and normal lung at 17.5 dpc (Fig. 8) suggesting that epithelial cells differentiate into mature proximal and distal epithelial cell types. Abundant, differentiated Type II alveolar cells containing lamellar bodies, and extracellular surfactant protein, could also be identified in the 19 day pc transgenic lung (Fig. 6D). This is in contrast to the situation in SP-C-TGFβ1 transgenic lungs where an increase in the amount of mesenchyme is associated with an inhibition in epithelial differentiation (Zhou et al., 1996). Cells consistent with Type I cells were also seen at the electron microscopic level (Fig. 6). However, in the absence of specific molecular markers for Type I cells, it is difficult to conclude that the respiratory epithelium of the SP-C-Shh transgenic lung differentiates completely normally.

Conservation of the hedgehog signaling pathway in lung development

A striking aspect of embryonic development is the conservation of patterning pathways between invertebrates and vertebrates. In Drosophila, HH induces the expression of target genes (Wg, Dpp and Ptc itself) by opposing the repressing activity of Ptc. Other genes have been identified in the HH signaling pathway, including smo (Alcedo et al., 1996), fused (Fz) and cubitus interruptus (Ci), as well as cyclic AMP-dependent protein kinase A (PKA), which also represses downstream target genes of HH (Ingham, 1993; Hooper, 1994; Lepage et al., 1995). Vertebrate homologues of some of these genes have been identified, e.g. the three genes Gli, Gli2 and Gli3 that encode zinc-finger proteins related to Ci (Hui et al., 1994). We have shown here that in the lung, Shh overexpression leads to upregulation of Ptc expression in the mesenchyme at least, which responds by increased proliferation. It remains to be seen whether homologs of other components of the HH signaling pathway are also involved in this response.

Shh and Bmp4 pathways are independent

Previously, we proposed a model in which SHH secreted by the distal tip epithelium regulated the expression of Bmp4 and/or Wnt2 in the adjacent mesenchyme (Bellusci et al., 1996). The fact that the expression of these two genes does not appear to change following overexpression of Shh, and the fact that the phenotypes of SP-C-Shh and SP-C-Bmp4 transgenic lungs are very different, argue against this model, and suggest that the SHH and BMP4 pathways are independent. The possibility still remains that Bmp4 is involved in lung morphogenesis by locally inhibiting epithelial proliferation at the tips of the end buds, thereby allowing differential proliferation of the epithelial compartment and subsequent branching.

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Role of Shh in lung development


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