Wnt7b regulates mesenchymal proliferation and vascular development in the lung

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SUMMARY

Although the Wnt signaling pathway regulates inductive interactions between epithelial and mesenchymal cells, little is known of the role that this pathway plays during lung development. Wnt7b is expressed in the airway epithelium, suggesting a possible role for Wnt-mediated signaling in the regulation of lung development. To test this hypothesis, we have mutated Wnt7b in the germline of mice by replacement of the first exon with the lacZ-coding region. Wnt7b∗/− mice exhibit perinatal death due to respiratory failure. Defects in early mesenchymal proliferation leading to lung hypoplasia are observed in Wnt7b∗/− embryos. In addition, Wnt7b∗/− embryos and newborn mice exhibit severe defects in the smooth muscle component of the major pulmonary vessels. These defects lead to rupture of the major vessels and hemorrhage in the lungs after birth. These results demonstrate that Wnt7b signaling is required for proper lung mesenchymal growth and vascular development.

Key words: Wnt7b, Lung, Mesenchyme, Proliferation, Vascular smooth muscle

INTRODUCTION

Proper lung development is essential for postnatal survival, and pulmonary defects are a leading cause of neonatal illness and mortality in humans (Guyer et al., 1999). Development of the mouse lung closely parallels that of the human lung and as such provides a powerful model in which to study lung development. Formation of the mouse lung begins at ~E9.5 of development by budding from the foregut endoderm. This early lung endoderm, which is surrounded by mesodermally derived mesenchyme, undergoes branching morphogenesis to produce the three-dimensional arborized network of airways required for postnatal respiration in mammals (Warburton et al., 2000). During branching morphogenesis, the pulmonary mesenchyme gives rise to several different cell types, including smooth muscle of the upper airways and the pulmonary vasculature. In addition, the mesenchyme produces essential growth factors and signaling molecules required for airway epithelial development and branching including members of the fibroblast growth factor (FGF) family (reviewed by Warburton et al., 2000). In turn, the epithelium also produces signaling molecules important for mesenchymal differentiation and proliferation including bone morphogenetic protein 4 (BMP4) and sonic hedgehog (SHH) (Bellusci et al., 1997; Belluscì et al., 1996; Litingtung et al., 1998; Pepicelli et al., 1998). Therefore, the inductive interactions between mesenchyme and epithelium are crucial to the generation and patterning of the mammalian lung.

Like several other organ systems, the lung is patterned in a proximodistal manner. Proliferation in airway epithelium occurs at a higher level in distal versus proximal regions during development (Hogan and Yingling, 1998; Warburton et al., 2000). Distinct epithelial cell lineages arise from the common epithelial precursor cells to generate the necessary cell types for adult respiration including alveolar epithelial type 1 (AEC-1) and type 2 (AEC-2) cells. AEC-1 cells themselves are thought to differentiate from AEC-2 cells through an as yet uncharacterized mechanism (Evans et al., 1975). AEC-1 and AEC-2 cells are found only in the distal airway epithelium during development. AEC-1 cells are required to form the thin, diffusible stratum between the airway lumen and the pulmonary capillary network, whereas AEC-2 cells produce surfactant to maintain the proper airway surface tension for respiration. In addition, defects in surfactant protein expression cause neonatal distress and death in humans (Ballard, 1996; Nogee et al., 1994). As the airway epithelium develops proximally, cell types such as Clara epithelial cells and ciliated epithelium of the upper airways become the primary cells types populating this region of the lung. The proper proximodistal differentiation of the airway epithelium is essential for proper lung development, as shown by the neonatal lethality of mice expressing the BMP inhibitors noggin and gremlin, which disrupt proximodistal patterning (Lu et al., 2001; Weaver et al., 1999). Thus, this patterning in the lung results in the establishment of a large, functional airway surface area that is capable of efficiently exchanging gases with the environment and expelling inhaled particulate matter.

The lung mesenchyme is also patterned in a proximodistal
manner. This is exemplified by the recruitment of smooth muscle surrounding the proximal airways and proximal blood vessels and not the distal airways and blood vessels. This patterning is closely linked to that observed in the epithelium as blocking BMP signaling in a distal epithelial cell autonomous manner results in the disruption of proximodistal patterning of both the epithelium and mesenchyme, including the ectopic appearance of smooth muscle surrounding the distal airways (Weaver et al., 1999). Thus, the proper level of proliferation and differentiation along a proximodistal axis is necessary for several aspects of lung development; the elucidation of the signals involved in these processes will provide important insights into embryonic lung development.

The Wnt growth factor family is comprised of at least 18 different secreted ligands that interact with 10 known frizzled receptors. Wnt signals are key regulators of cell proliferation, polarity and differentiation (reviewed by Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Wnts signal through multiple pathways, the best studied of these being the β-catenin/LEF-TCF pathway also known as the canonical pathway (Parr and McMahon, 1994; Willert and Nusse, 1998). In this pathway, secreted Wnt proteins bind to the cell membrane receptors of the frizzled family, inhibiting GSK-3β-mediated phosphorylation of β-catenin. Hypophosphorylated β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it heterodimerizes with members of the LEF/TCF transcription factor family. A role for Wnt signaling in lung development is suggested by the observation that several Wnt genes are expressed in the developing lung mesenchyme and/or epithelium. In particular, Wnt7b is expressed at high levels in distal airway epithelium (Pepicelli et al., 1998; Weidenfeld et al., 2002). However, the functions of Wnt7b and the Wnt signaling pathway in lung development are unknown.

To determine the role that Wnt7b plays during development, we generated mice bearing a mutant Wnt7b allele in which the lacZ-coding region replaces the coding region of the first exon. Wnt7blacZ mice die perinatally due to respiratory failure. The lungs of Wnt7blacZ/+ mice do not inflate, are hypoplastic and show extensive hemorrhage at birth. Lung hypoplasia in Wnt7blacZ/+ embryos is evident as early as E12.5 of development and is due, at least in part, to decreased cell proliferation in the distal mesenchyme adjacent to the growing tips of the distal airway epithelium where Wnt7b is normally expressed. Wnt7blacZ/+ embryos also exhibit defects in late airway epithelial maturation. Histological analysis of Wnt7blacZ/+ neonatal mice and embryos reveals that vascular smooth muscle is hypertrophic and, at birth, apoptotic, which leads to vessel rupture and hemorrhage. These results demonstrate that Wnt7b signaling is necessary for proliferation and growth of the lung mesenchyme and for maturation of the airway epithelium.

**MATERIALS AND METHODS**

**Generation of Wnt7blacZ mice**

Wnt7b mouse BAC clones were obtained from a commercial source (Incyte, Palo Alto, CA). Southern blotting and PCR were used to map the mouse Wnt7b gene. PCR was used to generate 3.5 kb of 5′ flanking region which includes the Wnt7b 5′ untranslated sequence but lacks the endogenous Wnt7b initiating ATG. This fragment was fused to the coding sequence of the bacterial lacZ sequence followed by a SV40 poly(A) sequence (Zhang et al., 2001). A 6.5 kb 3′ BamHI fragment was cloned into the BamHI site of pPNT and the 7.5 kb arm/lacZ-coding sequence was cloned into the NotI/Xhol sites of pPNT to generate the pWnt7blacZ-KO vector. This targeting vector was linearized with NotI and electroporated into R1 ES cells, which were selected in 250 μg/ml G418 and 0.2 μM FIAU for 8 days at which time resistant clones were picked and analyzed by Southern blotting for identification of homologous recombinants. Two correctly targeted clones were injected into C57BL/6 E3.5 blastocysts and chimeric mice from each clone were mated for germline transmission of the allele. The phenotype was identical for animals derived from both ES clones on a 129S6 background or 129S6-C57BL/6 mixed background.

**RT-PCR and co-transfection assays**

For RT-PCR, total RNA was extracted from wild-type and Wnt7b–/– E14.5 lung tissue using Trizol. Two micrograms of RNA was subjected to reverse transcriptase-cDNA synthesis using the Superscript II enzyme (Gibco-BRL). Ten percent of each RT reaction was then subjected to PCR using the following cycling conditions:

- short distance – 94°C for 20 seconds, 65°C for 1 minute, 72°C for 1 minute, 35 cycles;
- long distance – 98°C for 20 seconds, 68°C for 6 minutes.

For short distance PCR, the Takara Ex Taq enzyme was used, while for the long distance PCR, the Takara Taq enzyme was used (Panvera, Madison, WI). The oligos used for these reactions are as follows: A, 5′-GGGGTTCACCATGTTGCCAAGCG-3′; B, 5′-GGGGATCTGACATTGTCAAGAC-3′; C, 5′-AACTGGTGGCT- GCAGCACTGG-3′; D, 5′-CCGACGTGTCACTCGCCCTG-3′; E, 5′-CCGAATTCAGACACCGTGAGTAC-3′; GAPDH, 5′-GGATCTACGTGGTTCTACCAC-3′; and GAPDH, 3′-CCGAGGACAACGTCGCTCG-3′.

To generate the full-length Wnt7b and truncated Wnt7b cDNA expression vectors, Wnt7b was amplified from mouse embryonic lung cDNA using the following oligonucleotides: Wnt7b, 5′ full-length 5′-CAGGGATTCAGACAGAGGAATTTGCAAAAGTTG-3′; Wnt7b, 5′ truncated 5′-CGAGAATTCCTCGAGAATTTGCAAAAGTTG-3′; Wnt7b, 3′-5′-AACTGGTGGCTGAAACGTCGCTCG-3′; and Wnt7b, 3′-5′-GGATCTACGTGGTTCTACCAC-3′. The amplified products were cloned into the pcDNA3 vector. An HA epitope tag was incorporated at the 3′ end of each cDNA. HEK-293 cells were transfected with these expression constructs using Fugene 6 (Roche Biochemicals). After 48 hours, cells were stained with a monoclonal antibody to the HA epitope (HA.11, Convance) and analyzed by fluorescent microscopy.

**Histological procedures**

For in situ hybridization, embryos were fixed in 4% paraformaldehyde for 24-48 hours, depending on age. Fixed embryos were dehydrated through increasing ethanol concentrations and embedded in paraffin for 24-48 hours, depending on age. Fixed embryos were dehydrated through increasing ethanol concentrations and embedded in paraffin. Paraffin sections of 5μm were used for radioactive in situ hybridization as well as immunohistochemistry. The aquaporin-5, SP-C, CC10, SHH probes have been previously described (Lu et al., 2001; Pepicelli et al., 1998). In situ hybridization was performed using a previously published protocol (Kuo et al., 1997). β-gal staining was performed as previously described (Kim et al., 1997). Further details on histological protocols can be found at the Molecular Cardiology Research Center (http://www.uphs.upenn.edu/mcrc/histology/histologyhome.html).

**Cell proliferation assays**

Immunohistochemistry using a phospho-histone H3 monoclonal antibody was used to detect mitotic cells (clone 6G3, Cell Signaling Technology) (Hans and Dimitrov, 2001; Saka and Smith, 2001). Briefly, sections from paraformaldehyde fixed E14.5 wild-type and Wnt7b–/– embryos were probed with the phospho-histone H3 monoclonal antibody at a 1:250 dilution overnight at room temperature. Slides were washed and then probed with rabbit anti-
mouse HRP antibody at a 1:100 dilution. After a final set of washes, slides were developed using a commercially available kit (Vector Laboratories). Slides were counterstained with Hematoxylin. Positive cells, as well as the total cell number, were counted in an approximately 60° radius of growing epithelial tubules in three adjacent slides from four different embryos of each indicated genotype using the NIH Image 1.62 software.

Cardiac acrylic resin injections for visualizing vascular abnormalities
To visualize the pulmonary vascular defects in Wnt7blacZ–/– embryos, Batson 17 acrylic resin (Polysciences, Warrington, PA) was injected into the ventricles of the heart of E18.5 Wnt7blacZ–/– and wild-type littermates until the embryonic vasculature was filled. After hardening, soft tissue was digested with maceration solution (Polysciences, Inc.) at 37°C for 3 days. Photographs of lung vascular network casts were taken on a Leica Model MZ125 dissecting microscope with a Leica digital camera.

RESULTS
Generation of a Wnt7blacZ mutant mouse
The coding sequences of the first exon of mouse Wnt7b were replaced with the coding sequence of the lacZ gene by homologous recombination in embryonic stem cells (Fig. 1A). The resulting Wnt7b gene lacks the endogenous ATG initiation codon as well as the predicted signal peptide sequence, which should eliminate the ability of Wnt7b to be secreted (Nielsen et al., 1997). This strategy was employed because previous experiments have shown that the signal peptide sequence is required for Wnt activity in Xenopus embryos and cell culture (Mason et al., 1992; McMahon and Moon, 1989). Germline transmission of the targeted allele was confirmed by Southern blotting of genomic DNA using the indicated probe (Fig. 1B).

RT-PCR was used with RNA from E14.5 Wnt7b+/+ and Wnt7b–/+ embryonic lungs to detect possible spliced products in the 3′ region of the Wnt7b gene as well as splicing that could occur from either the 5′UTR or the lacZ sequences to the 3′ region. As shown in Fig. 1C, we do not detect any spliced products stemming from the 5′ first exon or from the lacZ sequences either by standard PCR or by long distance PCR (Fig. 1C and data not shown). However, transcripts were detected that contained both exon 3 and 4 in the Wnt7blacZ–/– lung RNA, albeit at markedly lower levels than in wild-type animals (Fig. 1C). This raised the possibility that a truncated Wnt7b protein could be produced from the Wnt7blacZ–/– allele. HEK-293 cells were transfected with either a full-length Wnt7b expression construct or a construct that contained a Wnt7b cDNA that lacked the sequence transcribed by the first exon that are deleted in Wnt7blacZ–/– mice to determine whether the potential truncated Wnt7b cDNA would produce a stable, secreted protein. Using an antibody against the hemagglutinin

Fig. 1. Targeting strategy for generation of the Wnt7blacZ mice. (A) Schematic of Wnt7b targeting construct. (B) Southern blot of a litter of newborn mice indicating the wild-type allele (10 kb) and the mutant allele (5 kb), resulting from an EcoRI restriction enzyme digest. (C) RT-PCR analysis of mRNA from E14.5 mouse lungs from Wnt7blacZ–/– embryos. The Wnt7blacZ targeted allele and the oligonucleotide used for RT-PCR are shown. No amplifiable transcripts were obtained using oligo combinations A/C or A/E (data not shown) from Wnt7blacZ–/– embryos while wild-type (+/+ or +/−) mRNA produced a robust signal. Low levels of transcript were obtained using oligos D and E with lung cDNA from Wnt7blacZ–/– embryos. (D) HEK-293 cells were transfected with either HA-tagged full-length or truncated (trunc) Wnt7b cDNAs. The truncated Wnt7b cDNA represented the complete coding region from the last three exons, which are still present in the Wnt7blacZ–/– allele. Cells are counterstained with DAPI to visualize the nucleus. Scale bar: 40 μm.
(HA) epitope, transfected cells were stained for the cellular localization of the full length and truncated Wnt7b proteins. The full length Wnt7b protein was located throughout the cytoplasm and cell periphery (Fig. 1D). However, the truncated Wnt7b protein was localized in a perinuclear pattern reminiscent of the endoplasmic reticulum (ER) (Fig. 1D). Retention of proteins in the ER is thought to result from improper folding and subsequent degradation of mutant protein products (Lippincott-Schwartz et al., 1988). These data indicate that although the Wnt7b lacZ allele produces low levels of transcripts at the 3’ end of the gene, any resulting protein made from this transcript is not likely to be stable or secreted.

The Wnt7b lacZ allele recapitulates the endogenous pattern of mouse Wnt7b expression

The substitution of the first exon with the coding sequences of the lacZ gene in the Wnt7b lacZ allele allowed us to carry out a detailed examination of the expression pattern of Wnt7b during development. Previous reports using in situ hybridization have shown that Wnt7b is expressed in the airway epithelium of the lung, proximal tubules of the forming kidney, the spinal cord and the brain (Hollyday et al., 1995; Parr et al., 1993; Pepicelli et al., 1998; Rubenstein et al., 1999; Weidenfeld et al., 2002). Staining of E12.5-E14.5 embryos for β-galactosidase expression shows that our Wnt7b lacZ allele accurately recapitulates the endogenous expression pattern (Fig. 2A). Wnt7b lacZ expression is observed in the frontal edge of the future cerebral cortex (B, yellow arrowhead), in a narrow band across the roof of the midbrain (B, black arrowhead) and the medulla oblongata (C, arrowheads). Histological sections of E12.5 embryos reveal expression in the ependymal (D,L, arrow) and the outer marginal layers (D,L, arrowhead) of the neural tube and the corpus striatum (cs), pons (pons) and the medulla oblongata (mo) (H,I). β-galactosidase expression is observed in the interdigital mesenchyme of the forming limb bud (E, arrowhead) but is excluded from the apical ridge (F, arrowhead). High levels of β-galactosidase expression are observed in the developing lung (G, lu) and trachea, and are restricted to the airway epithelium (J, arrowheads indicate lung epithelium, arrow indicates trachea; K, arrowheads). By E14.5, expression in the airway epithelium is restricted to the distal regions (K, compare white arrowheads with black arrowhead). β-Galactosidase expression is observed in the developing skin at E14.5 (M), in the metanephric tubules of the developing kidney (N, arrowheads), and in the epithelium of the bile duct (O, arrowhead). Scale bars: 1.5 mm in G; 750 μm in H; 500 μm in I; 350 μm in J; 250 μm in K,L,O; 125 μm in N; 75 μm in M.
unknown regions of Wnt7b expression such as in the developing bile duct.

**Wnt7b**lacZ−/− mice die quickly after birth due to respiratory failure

Wnt7b**lacZ−/−** mice were mated to produce Wnt7b**lacZ+/−** offspring. Eighty-five 2-week-old mice from Wnt7b**lacZ+/−** crosses were genotyped and no live homozygous null mice were found (Table 1). We then determined the time of death of Wnt7b**lacZ−/−** mice. The expected Mendelian ratio was obtained at all embryonic time points, including immediately post-delivery (Table 1). However, examination of newborn pups revealed that approximately 25% of them gasped for breath, appeared cyanotic and died within 10 minutes of delivery (Fig. 3A,B). Examination of wild-type lungs showed that they were expanded by inhalation of air (C), which can be observed as air bubbles in the distal regions of the lung (D). The Wnt7b**lacZ−/−** lungs were collapsed and hypoplastic, lacking visible air in the lung periphery (E).

Histology of these lungs revealed the collapsed nature of the airways in Wnt7b**lacZ−/−** mice and the overall smaller size of the lungs (F,G). Lungs from E12.5 Wnt7b**lacZ−/−** and Wnt7b**lacZ+/−** embryos were removed and stained for β-galactosidase expression to reveal the extent of lung hypoplasia (H). Cross-sections from E14.5 wild-type and Wnt7b**lacZ−/−** embryos further reveals the extent of lung hypoplasia (I,J).

Table 1. Genotype of Wnt7b**lacZ−/−** mice during embryogenesis and adulthood

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E9.5-E14.5</th>
<th>E16.5-E18.5</th>
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<td>−/−</td>
<td>21</td>
<td>15</td>
<td>17 (dead)</td>
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Wnt7b**lacZ−/−** embryos display lung hypoplasia

To further characterize the lung defects in Wnt7b**lacZ−/−** embryos, we examined histological sections from Wnt7b**lacZ+/−** and Wnt7b**lacZ−/−** embryos at E12.5 and E14.5. Both whole-mount and sections from these time points revealed that lung hypoplasia is noticeable as early as E12.5 of development (Fig. 3H-J). Although airway development is largely normal at these time points, a reduced amount of distal mesenchyme, adjacent to the growing epithelial tubules, is observed (Fig. 4A-F). In wild-type lungs this distal mesenchyme is several cell layers thick (Fig. 4A,C,E). However, in Wnt7b**lacZ−/−** embryos, this mesenchyme is extremely thin, sometimes no more than one or two cell layers thick, resulting in the airway tubules forming immediately next to the mesothelium (Fig. 4B,D,F). These observations suggest that growth and or differentiation of distal lung mesenchyme is affected in Wnt7b**lacZ−/−** embryos.
To determine whether cell proliferation is affected in Wnt7b lacZ–/– embryos, histological sections were stained with a phospho-histone H3 monoclonal antibody to detect cells undergoing mitosis. Quantification of the mitotic cells in the distal mesenchyme of wild-type and Wnt7b lacZ–/– embryos shows that proliferation is reduced at E12.5 by approx. two-thirds in Wnt7b lacZ–/– embryos (Fig. 4I). By E14.5, this difference in proliferation in the distal mesenchyme between wild-type and Wnt7b lacZ–/– embryos was no longer evident (Fig. 4G-I). Furthermore, the number of mitotic airway epithelial cells was unchanged at both E12.5 and E14.5 (Fig. 4C,D,G-I). These data indicate that Wnt7b acts as an important mitogen for distal mesenchyme during early lung development.

**Differentiation of lung epithelium in Wnt7b lacZ–/– embryos**

Lung epithelium differentiates during mid to late gestation, resulting in distinct cell lineages along a proximodistal axis (Warburton et al., 2000). To determine whether lung epithelial cell differentiation had occurred properly in Wnt7b lacZ–/– embryos, markers for proximal (CC10) and distal (SP-C) epithelial differentiation were used for in situ hybridization of E18.5 Wnt7b lacZ–/– and wild-type littermates. CC10 expression is normally confined to non-ciliated Clara epithelial cells of the proximal airways, whereas SP-C is expressed exclusively in alveolar type 2 (AEC-2) cells of the distal airways. In situ hybridization reveals that Wnt7b lacZ–/– embryos express CC10 and SP-C at normal levels and in a pattern similar to that in wild-type littermates (Fig. 5A-D). These data suggest that proximodistal cell patterning is unaffected in Wnt7b lacZ–/– embryos.

From E16.5 through the first 2 weeks after birth, the mouse lung undergoes further differentiation to produce the thin, diffusible surface area used for efficient gas exchange. One of the primary differentiation events that occur during this time is the differentiation of alveolar epithelial type 1 cells (AEC-1), which comprise approximately 95% of the airway surface area and are responsible for gas exchange between blood and air in postnatal animals. Aquaporin 5 encodes a water channel and is expressed in AEC-1 cells in the lung beginning at ~E17.5 (Lee et al., 1997). As previously reported, aquaporin 5 is expressed in the distal airways of the lung at E18.5 (Fig. 5E,F) (Lee et al., 1997; Yang et al., 2002). However, in Wnt7b lacZ–/– mice, aquaporin 5 expression is severely attenuated, suggesting that later stages of lung epithelial differentiation are defective (Fig. 5F).

Transmission electron microscopy was performed to verify whether AEC-1 cell differentiation was defective, as suggested by the attenuated aquaporin 5 expression. AEC-1 cells are distinctive in appearance and are characterized by their squamous morphology. The distal airways of E18.5 Wnt7b lacZ–/– embryos contained large quantities of cuboidal AEC-2 cells (Fig. 5H, arrowheads). Surfactant is also observed in the airway lumen (Fig. 5H, notice ‘S’ and arrow). However, almost no AEC-1 cells were observed in Wnt7b lacZ–/– lungs, whereas wild-type littermates did contain AEC-1 cells (Fig. 5G, arrow). Together, these results suggest that Wnt7b lacZ–/– embryos display a late lung epithelial differentiation/maturation defect that correlates with decreased numbers of AEC-1 cells.

**Wnt7b lacZ–/– embryos exhibit thin mesenchyme and reduced distal mesenchyme proliferation at E12.5.** Wnt7b lacZ–/– embryos at E12.5 and E14.5 exhibit thinner mesenchyme than do Wnt7b lacZ+/− littermates, with the airway epithelium growing almost directly next to the mesothelium of the lung (A,B,E,F, arrows). Samples were stained for β-galactosidase expression to better visualize the airway epithelium (A,B,E,F). Staining of lung tissue with a monoclonal antibody to phospho-histone H3, which detects mitotic cells, shows decreased staining in the distal mesenchyme of Wnt7b lacZ–/– embryos at E12.5 (C,D, yellow arrowheads). By E14.5, no significant difference in cell proliferation is observed between Wnt7b lacZ–/– and Wnt7b lacZ+/− embryos in the lung (G,H). Quantification of cell proliferation shows that distal lung mesenchyme proliferation is reduced by approximately two-thirds in E12.5 Wnt7b lacZ–/– embryos (I). Scale bars: 150 μm in A,B; 125 μm in C,D; 250 μm in E,F; 200 μm in G,H. dm, distal mesenchyme; de, distal epithelium.

**Wnt7b lacZ–/– embryos display pulmonary vascular defects**

Wnt7b lacZ–/– mice show hemorrhage surrounding the large pulmonary vessels at birth, suggesting a pulmonary vascular defect in these mice. This hemorrhage is not observed in any
other region of the embryos or neonates, indicating a specific defect in the lung vasculature. Examination of the large blood vessels in the lung at E18.5 shows that many of them are dilated and contain a thickened smooth muscle cell layer (Fig. 6A-D). The number of smooth muscle or endothelial cells does not appear to be increased, suggesting a hypertrophic response and not increased cell proliferation. This is supported by the lack of an increase in phospho-histone H3-positive cells in the pulmonary vasculature of Wnt7blacZ–/– embryos (data not shown). At birth, severe hemorrhage is observed surrounding the major pulmonary vessels (Fig. 6G,H). This suggests that Wnt7blacZ–/– embryos and mice have dilated and weakened pulmonary vessels that rupture at birth, possibly due to the increased pulmonary blood flow or mechanical strain on the lungs that occurs at birth in association with diaphragm contractions.

To obtain a more thorough picture of blood vessel development in Wnt7blacZ–/– embryos, we injected methacrylate resin into the ventricles of the heart to fill the embryonic vasculature. After the soft tissue of the embryo is digested away, a cast of the cardiovascular system can be visualized (Merscher et al., 2001). As expected, wild-type E18.5 embryos have an extensive pulmonary vascular network with reiterated branching of the large vessels into smaller ones (Fig. 6E). However, in E18.5 Wnt7blacZ–/– embryos, reiterated branching of the smaller pulmonary vessels is reduced (Fig. 6F). In addition, the diameter of the branched vessels is increased, supporting the above histological analysis that indicates dilation of pulmonary blood vessels.

**Aberrant smooth muscle α-actin expression and increased cell death in pulmonary vascular smooth muscle of Wnt7blacZ–/– neonates**

The dilated blood vessels and hemorrhage observed in the lungs of Wnt7blacZ–/– embryos and neonates suggested possible defects in vascular smooth muscle cell (VSMC) differentiation. To detect whether smooth muscle had differentiated properly in Wnt7blacZ–/– neonates, smooth muscle α-actin immunohistochemistry was performed. In the lung, smooth muscle is normally found surrounding the upper bronchial and tracheal airways and the pulmonary vasculature in late development. Some of the large hemorrhagic blood vessels in Wnt7blacZ–/– neonates contained noticeably less smooth muscle, while most had increased smooth muscle α-actin staining, which was probably due to hypertrophy (Fig. 7E,F). Both types of vessels displayed extensive hemorrhage (Fig. 7E,F). By contrast, bronchial smooth muscle was well formed and observed surrounding all of the large upper airways of both wild-type and Wnt7blacZ–/– neonates (Fig. 7G,H).

TUNEL staining was performed on wild-type and Wnt7blacZ–/– lung tissue to determine whether increased cell death was observed in the pulmonary blood vessels of Wnt7blacZ–/– mice. As shown in Fig. 7J, VSMCs of Wnt7blacZ–/– neonatal mice exhibited extensive TUNEL staining when compared with wild-type littermates (Fig. 7J). This was not observed in bronchial smooth muscle cells (Fig. 7K) or in the vascular smooth muscle of wild-type littermates (Fig. 7I). This data suggests that Wnt7b is important for the differentiation and/or maintenance of pulmonary VSMCs in the lung.

To determine whether the smaller capillaries in the lung, which lack vascular smooth muscle, were affected in Wnt7blacZ–/– embryos, lung tissue from neonatal mice was immunohistochemically stained with an antibody to
platelet endothelial adhesion molecule (PECAM). PECAM staining of both wild-type and Wnt7b-lacZ–/– littermates is normal, with the capillary plexus staining strongly (Fig. 7L,M). This suggests that lung vascular endothelial development in Wnt7b-lacZ–/– embryos and neonates exhibit lung hypoplasia characterized by reduced mesenchymal cell proliferation early in development and, in addition, exhibit a delay in lung epithelial maturation. Later in development, Wnt7b-lacZ–/– embryos and neonates develop pulmonary VSMC defects, which lead to vessel rupture and hemorrhage at birth. Together, these data demonstrate for the first time a key role for Wnt signaling in lung development.

**Wnt7b and lung mesenchymal proliferation**

Wnt7b is the only Wnt gene that we have found to be expressed exclusively in the airway epithelium of the lung during early embryonic development. Other Wnt genes, such as Wnt2, Wnt2b and Wnt11 are expressed in the mesenchyme of the developing lung (Lako et al., 1998; Lin et al., 2001; Monkley et al., 1996). However, there is no reported lung phenotype in Wnt2-null mice, suggesting that any role Wnt2 may play is rescued through overlapping expression of other Wnt genes such as Wnt2b or Wnt11 (Lako et al., 1998; Lin et al., 2001; Monkley et al., 1996). Our data show that Wnt7b plays an essential role in proliferation of the lung mesenchyme. This observation is noticeable early in development in mesenchymal cells directly adjacent to the growing airway epithelium where Wnt7b is expressed. However, at E14.5 and later in development, proliferation is not noticeably affected. Interestingly, early mesenchyme differentiation does not appear to be affected in Wnt7b-lacZ–/– embryos, as demonstrated by normal expression of Wnt2 and FGF-10 (data not shown). Thus, Wnt7b is required for normal lung mesenchymal proliferation in a narrow window of development prior to E14.5.

Regulation of cell proliferation is a key function of Wnt signaling and various members of the Wnt family have been implicated in regulating proliferation in the forming limb, hematopoietic cells, and intestinal and mammary epithelium (Bradley and Brown, 1995; Edwards et al., 1992; Lickert et al., 2000; Morin, 1999; Reya et al., 2000; Van Den Berg et al., 1998; Wong et al., 1998; Yamaguchi et al., 1999). Our data indicate that Wnt7b signals derived from the lung epithelium regulate lung mesenchymal proliferation. Regulation of cell proliferation by Wnts involves both the β-catenin canonical pathway as well as through protein kinase C-dependent mechanisms (Morin, 1999; Murray et al., 1999; Wong et al., 1998). Further investigation will be necessary to determine whether Wnt7b signals through β-catenin-dependent or independent pathways during lung development.
SHH and BMP4 also appear to affect cell proliferation in both mesenchymal and epithelial cells of the lung. Overexpression of SHH in the distal airway epithelium of the lung causes increased cell proliferation in both mesenchymal and epithelial cell types (Bellusci et al., 1997). In addition, Shh-null mice have a significantly reduced level of cell proliferation in both the mesenchyme and airway epithelium (Litingtung et al., 1998; Pepicelli et al., 1998). Over-expression of BMP4 results in decreased cell proliferation in the epithelium and an increase in the mesenchyme (Bellusci et al., 1996). In Wnt7b knockout embryos, SHH and BMP4 expression do not appear to be affected, suggesting that these genes are not regulated by Wnt7b signaling (data not shown). In turn, Wnt7b expression is not affected in Shh-null embryos (Pepicelli et al., 1998). Together, these data suggest that cell proliferation in lung morphogenesis is controlled by several distinct pathways (Fig. 8A).

**Wnt7b and lung epithelial cell differentiation and maturation**

Wnt7b knockout embryos exhibit normal proximal-distal epithelial cell differentiation as shown by the expression patterns of SP-C and CC10. This indicates that Wnt7b does not regulate this proximodistal patterning process in airway epithelium in mice. Expression of surfactant by electron microscopy and immunohistochemical staining for surfactant proteins B and C indicates that production of surfactant is not grossly compromised in Wnt7b knockout embryos (Fig. 6 and data not shown). However, Wnt7b knockout embryos exhibit a decrease in expression of the late differentiation marker gene aquaporin 5. Coupled with a decrease in AEC-1 cells in the lungs of Wnt7b knockout embryos, these data suggest that late lung maturation is disrupted in Wnt7b knockout embryos. The differentiation of AEC-2 cells into AEC-1 cells late in lung development is necessary to form the thin, diffusible stratum between the airway lumen and the pulmonary capillary network (reviewed by Warburton et al., 2000). Several other mouse models that have defects in late lung maturation such as epidermal growth factor receptor-null mice, Cutl1 null mice and lung-specific GATA6 dominant-negative transgenic mice do not exhibit significant hypoplasia, suggesting that maturation and hypoplasia are not necessarily linked during mouse lung development (Ellis et al., 2001; Miettinen et al., 1997; Yang et al., 2002). In addition, many of these mice die quickly after birth because of respiratory failure, supporting the importance of these late maturation events in postnatal

![Defective smooth muscle integrity in Wnt7b knockout embryos and mice.](image-url)
of the smooth muscle component of the vessel wall. The vascular defects in Wnt7b lacZ–/– embryos and neonates are unlikely to be linked to lung hypoplasia because other mouse models that have severe lung hypoplasia such as the Vsmc haploinsufficient (Vsmc–/–) embryos and mice exhibit pulmonary VSMC hypertrophy and apoptosis, suggesting that Wnt7b affects late mesenchymal development because smooth muscle in the lung differentiates from the mesenchyme. Because Wnt7b is not expressed in vascular smooth muscle cells in the lung, several possibilities exist for the role of Wnt7b in vascular development including: (1) it may be required for maintenance of the pulmonary vascular smooth muscle phenotype by acting as a paracrine growth factor; (2) it may be necessary for the expression of other auto- or paracrine factors in mesenchymal or epithelial cell lineages required for VSMC survival; or (3) loss of Wnt7b signaling could result in defective differentiation of VSMCs, leading to a block in development and degradation of the smooth muscle component of the vessel wall. The vascular defects in Wnt7b lacZ–/– embryos and neonates are unlikely to be linked to lung hypoplasia because other mouse models that have severe lung hypoplasia such as the Fgf9 knock-out mice exhibit completely normal vascular development (Colvin et al., 2001). Because Wnt7b expression decreases during late gestation in the lung (i.e. after E16.5) and is expressed at only low levels in the adult mouse lung (Gavin et al., 1990) (data not shown), Wnt7b is likely to play a role during the early events of pulmonary vascular smooth muscle differentiation. Thus, Wnt7b may initiate a mesenchymal differentiation program that propagates from the distal to proximal regions as the lung grows, resulting in the proper differentiation of vascular smooth muscle. In this model, as the mesenchyme differentiates and progresses from the distal towards the proximal region, vascular smooth muscle differentiates and begins to surround the endothelial tubes, establishing the support structure of the large blood vessels in the lung (Fig. 8A,B). Loss of Wnt7b function does not result in loss of vascular smooth muscle specification, but does lead to the inability of vascular smooth muscle to maintain its integrity and survive (Fig. 8B). This degradation in the vessel wall could be due to a form of pulmonary hypertension which, in turn, could result in blood vessel wall failure. Thus, inactivation of Wnt7b leads to defective smooth muscle differentiation, degradation of the vessel wall, and perinatal hemorrhage. The observation that vascular smooth muscle but not bronchial smooth muscle is affected in Wnt7b lacZ–/– embryos supports a model in which different types of pulmonary smooth muscle are regulated by specific signaling and transcriptional programs. Distinct differences between the molecular programs regulating vascular and visceral smooth muscle development have been identified previously. In particular, transcriptional mechanisms that drive vascular smooth muscle development have been shown to be dependent on the activity of serum response factor, a transcriptional regulator enriched in cardiac and smooth muscle lineages (Kim et al., 1997; Li et al., 1997; Strobeck et al., 2001). The vascular phenotype of Wnt7b lacZ–/– embryos provides an important example of the differences in the responses of vascular and bronchial smooth muscle to the Wnt signaling pathway. Interestingly, a recent report implicates the Wnt pathway in the regulation of vascular smooth muscle development (Wang et al., 2002). In particular, β-catenin was elevated in VSMCs from balloon angioplasty-injured aortas, and forced expression of β-catenin increased VSMC proliferation and inhibited apoptosis. These same studies showed that expression of a dominant-negative TCF4

**Fig. 8.** A model for the role of Wnt7b in lung development. (A) Wnt7b is expressed at the distal tips of the airway epithelium in a pattern similar to that observed with BMP4 and overlapping that of SHH. In addition, Wnt7b is expressed in an increasing gradient from the proximal-to-distal airway epithelium. FGFs are expressed in the mesenchyme and are known to regulate epithelial branching and proliferation. However, because BMP-4 and SHH expression is unchanged in Wnt7b lacZ–/– embryos and Wnt7b expression is unchanged in Shh-null mice, Wnt7b regulates mesenchymal proliferation and differentiation through a unique pathway. (B) Lung vasculature is composed of both endothelium (red) and vascular smooth muscle (VSMC, blue), and develops in parallel with the airways (green). Loss of Wnt7b function results in defects in vascular smooth muscle differentiation and/or survival leading to a hypertrophic response (change from dark blue to light blue), degradation of the vessel wall and eventual rupture of the weakened vessels.
blocked the β-catenin induced inhibition of apoptosis. These data suggest that β-catenin dependent signaling increases cell proliferation and inhibits apoptosis in VSMCs and support the hypothesis that Wnt7b signals through a β-catenin-dependent pathway in pulmonary VSMCs.

The phenotype of Wnt7b lacZ/+ mice contrast with those recently reported for another Wnt7b mutant, which displays a much earlier defect in amnion and chorion fusion, causing lethality at E10.5 (Parr et al., 2001). Our targeting strategy involved the deletion of the coding sequences of the first exon of Wnt7b, while Parr et. al. targeted a region of the third exon (Parr et al., 2001). Although our allele did generate a very low level of transcripts at the 3' end of the gene, the allele generated by Parr et. al. may be expected to produce transcripts from at least the first two exons of Wnt7b. As C-terminal truncated Wnts have been used to inhibit Wnt signaling in a dominant-negative manner, a possible explanation for the differences between our results and those of Parr et. al. could be that these authors have generated a dominant-negative allele (Baker et al., 1999; Hoppler et al. 1996; Mullor et al., 2001). Alternatively, the Wnt7b lacZ allele might be a hypomorph rather than a true null allele. However, this latter explanation seems unlikely as we deleted the region of the transcript encoding the signal peptide and show that any resulting truncated protein (if generated) is not likely to be stable or secreted, which is supported by previous reports showing that the signal peptide is required for Wnt activity in Xenopus embryos and cell culture (Mason et al., 1992; McMahon and Moon, 1989).

In summary, our findings present in vivo evidence that Wnt7b plays a crucial role during lung development, regulating mesenchymal proliferation, late epithelial maturation and pulmonary vascular smooth muscle differentiation and/or survival. Our data demonstrate that signaling from the lung epithelium is necessary for the normal development of lung mesenchyme and demonstrate for the first time that Wnt signals play a key role in this process.

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