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Developmental Biology 258 (2003) 154–168
DEVELOPMENTAL BIOLOGY
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Temporal effects of Sprouty on lung morphogenesis

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Received for publication 8 November 2002, revised 17 January 2003, accepted 27 January 2003

Abstract

Paracrine signaling mediated by FGF-10 and the FGF-R2IIIb receptor is required for formation of the lung. To determine the temporal requirements for FGF signaling during pulmonary morphogenesis, Sprouty-4 (Spry-4), an intracellular FGF receptor antagonist, was expressed in epithelial cells of the fetal lung under control of a doxycycline-inducible system. Severe defects in lobulation and severe lung hypoplasia were observed when Spry-4 was expressed throughout fetal lung development (E6.5–E18.5) or from E6.5 until E13.5. Effects of Spry-4 on branching were substantially reversed by removal of doxycycline from the dam at E12.5, but not at E13.5. In contrast, when initiated late in development (E12.5 to birth), Spry-4 caused less severe pulmonary hypoplasia. Expression of Spry-4 from E16.5 to E18.5 reduced lung growth and resulted in perinatal death due to respiratory failure. Expression of Spry-4 during the saccular and alveolar stages, from E18.5 to postnatal day 21, caused mild emphysema. These findings demonstrate that the embryonic-pseudoglandular stage is a critical time period during which Spry-sensitive pathways are required for branching morphogenesis, lobulation, and formation of the peripheral lung parenchyma.

Keywords: Pulmonary; Organogenesis; Fibroblast growth factor; Progenitor cells

Introduction

Formation of the murine lung is initiated on E9.0–E9.5 by evagination of tissue from the lateral, esophageal–tracheal sulcus from the foregut endoderm. Respiratory tubules elongate and branch as the epithelial cells proliferate within the splanchnopleural mesenchyme. Stereotypic branching of the respiratory tubules is achieved by precise temporal and spatial signals that control cell shape, proliferation, differentiation, and/or migration to form the conducting airways (trachea, bronchi, and bronchioles) and peripheral lung parenchyma, including the alveoli. Formation of the pulmonary vasculature, including pulmonary arteries, capillaries, and veins, accompanies the respiratory tubules, ultimately providing a rich vascular supply to the alveolar regions to accommodate gas exchange after birth.

While the mechanisms underlying the orderly process of branching morphogenesis of the lung are as yet poorly defined, increasing evidence supports the critical role of signaling by fibroblast growth factor receptors, their ligands, and antagonists in the process. Inhibition of FGF receptor signaling in the developing respiratory epithelium with a dominant-negative FGF receptor or a truncated FGF-R2 isoform of the FGF receptor caused pulmonary hypoplasia in transgenic mice *in vivo* (Celli et al., 1998; Peters et al., 1994). Similarly, targeted deletion of FGF-10 caused complete pulmonary agenesis (Min et al., 1998). Selective proliferation and migration of subsets of respiratory epithelial cells are required to accomplish the precise pattern of branching characteristic of the mammalian lung.

Drosophila Spry (Dspry) is an antagonist of FGFR signaling during tracheal morphogenesis (Hacohen et al., 1998; Placzek and Skaer, 1999). During formation of the Drosophila trachea, DSpry prevents neighboring cells from elaborate branching, functioning in a non-cell-autonomous

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doi:10.1016/S0012-1606(03)00106-4

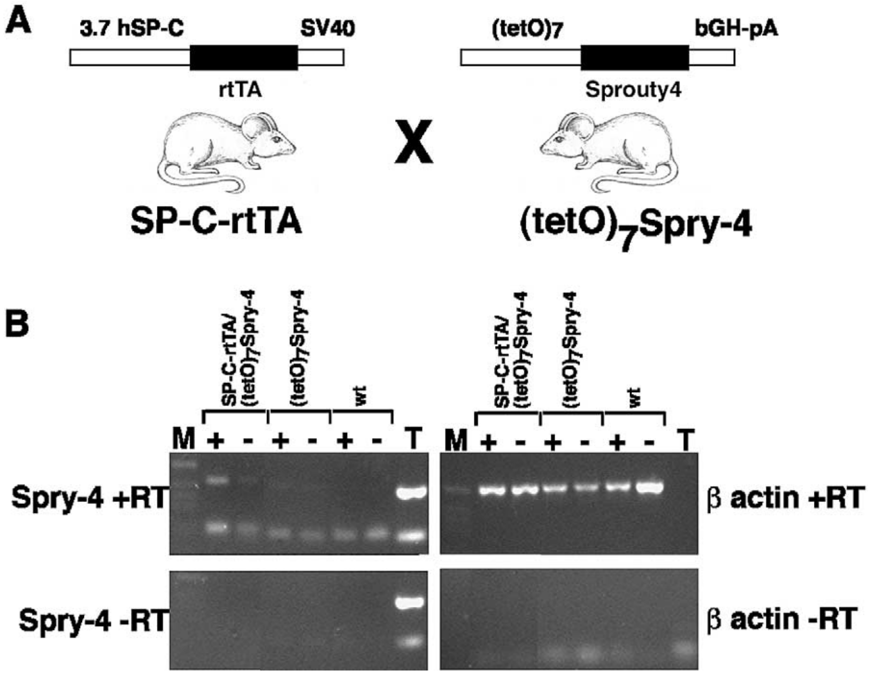


Fig. 1. Constructs used for conditional expression of Spry-4. (A) The murine Spry-4 cDNA was inserted into $(tetO)_7$CMV-bGH-polyA vector, and the resulting transgenic mice were bred to SP-C-rtTA transgenic mice to produce double transgenic mice. After doxycycline, double transgenic progeny express Spry-4 mRNA. (B) Transgene-specific murine Spry-4 and β-actin mRNAs were assessed by RT-PCR in adult mouse lung after 48 h of doxycycline (+) and compared with untreated animals (−). M, marker; SP-C-rtTA/$(tetO)_7$Spry-4, double transgenic mice; $(tetO)_7$Spry-4, single transgenic; wt, wild type mice; T, Tail DNA (+) on doxycycline; (−) off doxycycline. (Left panels) Spry-4; +RT, cDNA reaction with reverse transcriptase, −RT, cDNA reaction without reverse transcriptase; (right panels) for β-actin. Double transgenic mice expressed the transgene at high levels following doxycycline. Low levels of Spry-4 mRNA detected in the absence of doxycycline had no observable biological effect.

manner (Hacohen et al., 1998). More recent data support the concept that DSpry is an intracellular inhibitor of FGF signaling, and that its membrane localization is essential for function (Casci et al., 1999; Impagnatiello et al., 2001). During Drosophila eye development and oogenesis, DSpry also inhibited FGFR signaling in a cell-autonomous manner controlling the number of neurons and glia cells (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). Since DSpry interferes with signaling from various receptor-tyrosine kinases (RTK), such as Torso and Sevenless, DSpry may function as a general inhibitor of RTK signal transduction (Casci et al., 1999; Reich et al., 1999). DSpry associates with various intracellular components of the FGFR signaling cascade, namely Gap1 and Drk, the Drosophila homologue of Grb2 (Casci et al., 1999). During eye development, DSpry inhibits p42/44 mitogen-activating protein (MAP) kinase activation at the level of Ras (Casci et al., 1999), whereas during wing development, it may interfere downstream of RAF (Reich et al., 1999).

Three human, four murine, and two avian genes encoding homologues of Drosophila Spry were identified (Chambers and Mason, 2000; de Maximy et al., 1999; Hacohen et al., 1998; Minowada et al., 1999; Tefft et al., 1999). Mspry-1 and Mspry-2 inhibited FGF- and VEGF-induced endothelial cell proliferation and differentiation, by repressing pathways leading to p42/44 MAP kinase activation (Impagnatiello et al., 2001). During zebrafish development, Spry-4 expression is colocalized and dependent on FGF-8 and FGF-3 signaling. Sprouty antagonizes by interfering with FGF signaling downstream of FGFR1, downregulating MAP kinase activity. Loss of Spry expression resulted in overexpression of FGFs (Fürthauser et al., 2002). Blocking the FGF pathway in early *Xenopus* embryos inhibits mesoderm induction and results in truncation of the anterior–posterior axis (Nutt et al., 2001).

Expression of murine Spry-2 and -4 resulted in the repression of FGF-mediated limb development in chicken (Minowada et al., 1999), whereas ablation of Spry-2 expression in the cultured embryonic mouse lungs led to an increase in branching of respiratory tubules, a process induced by FGFs (Tefft et al., 1999). Constitutive expression of murine Spry-2 in the developing mouse lung using the SP-C promoter reduced branching and inhibited epithelial proliferation (Mailleux et al., 2001). As observed with DSpry, expression of murine and avian Spry was stimulated by FGF activity, providing feedback regulation of the signal transduction pathway (Chambers and Mason, 2000; Hacohen et al., 1998; Mailleux et al., 2001; Minowada et al., 1999).

While FGF-10 and FGF-R2IIIb are critical for formation of the lung, it is presently unclear whether FGF signaling is continuously required for proliferation of pulmonary epithe-

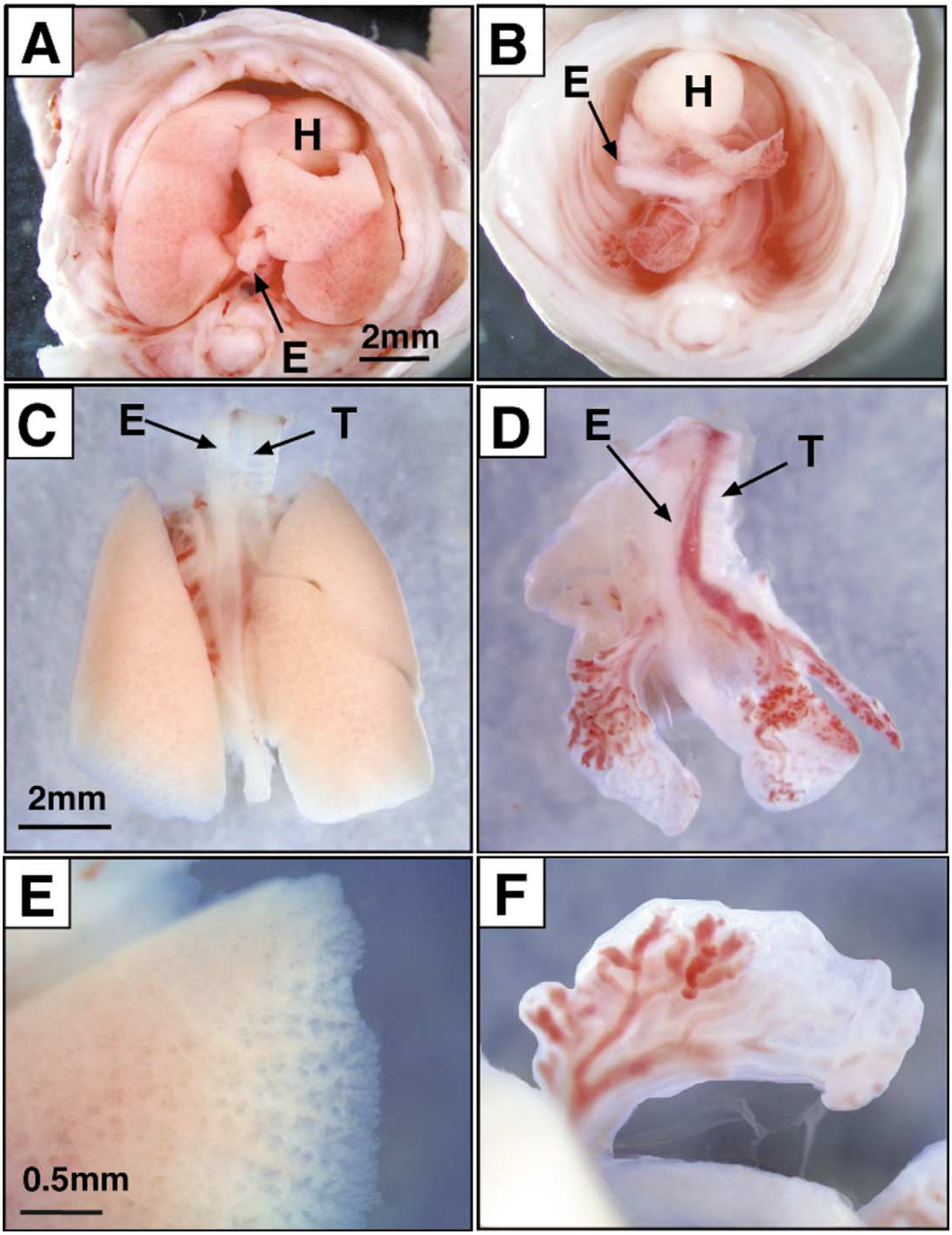
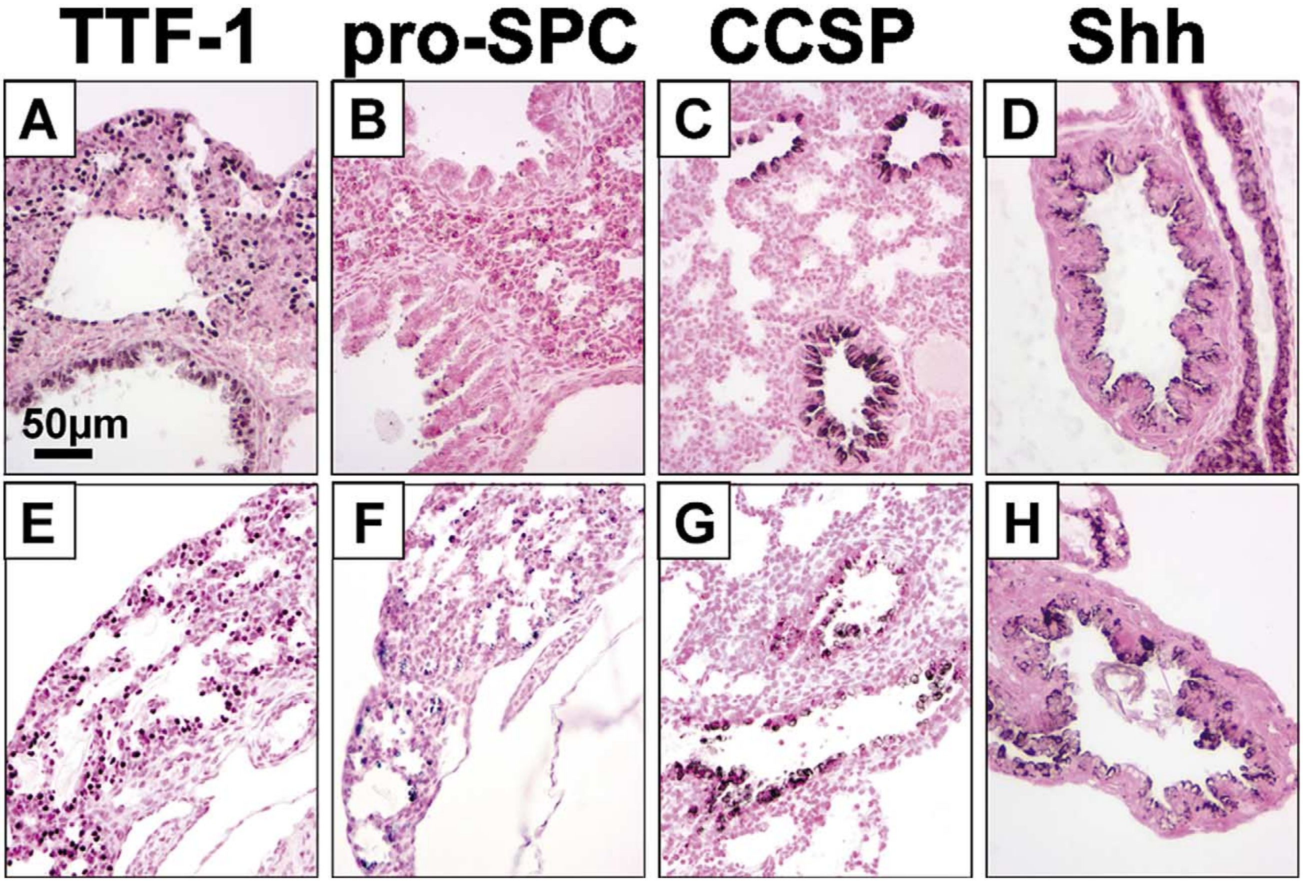


Fig. 2. Lung morphology at E16.5 after continuous expression of Spry-4. Morphology of wildtype (A, C, E) and double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 (B, D, F) embryos are shown at E16.5. The dams were treated with doxycycline from E6.5 to E16.5 (A, B) Whole thorax; (C, D) lung and heart; (E, F) higher magnification of left lung lobe. E, esophagus; H, heart; T, trachea. In the double transgenic mouse, the chest cavity is of normal size and shape but nearly empty, reflecting severe lung hypoplasia (B), reduction in primary and secondary branching resulted in three lobes (D), and loss of lung periphery (D, F). Airways are blood-filled, outlining the bronchial tree (D, F). Size bars equal 2 mm in (A–D), 0.5 mm in (E, F).

lial cells forming the lung or whether FGFs influence commitment or survival of critical subsets of lung progenitor cells at precise times during its morphogenesis. In the present work, we demonstrate that Spry's antagonistic action on FGF-mediated lung formation is temporally restricted. Spry-4 inhibited branching morphogenesis prior to E13 without altering cell differentiation. Expression of Spry-4 during the late perinatal and postnatal period caused emphysema. The results indicate that Spry-4-sensitive pathways are required for commitment or expansion of a critical subset of lung progenitor at precise stages of lung development.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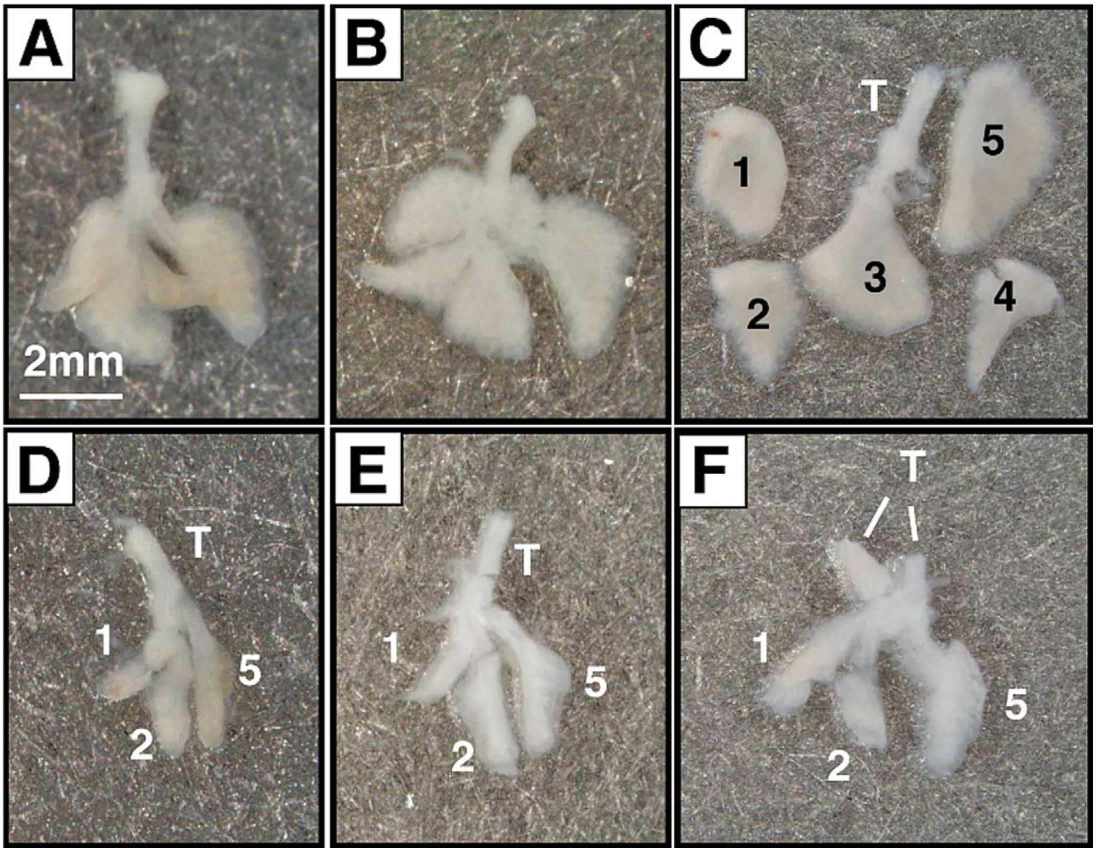
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Materials and methods

Transgenic constructs

To antagonize FGFR signaling during lung morphogenesis, transgenic mice bearing a construct consisting of the rtTA binding element $(tetO)_7$ linked to an inactive CMV minimal promoter driving expression of murine Spry-4, $(tetO)_7$Spry-4, were generated and mated to transgenic SP-C-rtTA mice. SP-C-rtTA mice are transgenic mice expressing the rtTA activator under the control of the human surfactant protein C (SP-C) promoter and have been described previously (Tichelaar et al., 1999; Perl et al., 2002a). Transgenic $(tetO)_7$Spry-4 mice were generated by oocyte injection of a construct in which the mouse Spry-4 cDNA was inserted between the $(tetO)_7$CMV promoter and the 3′ untranslated region of the bovine growth hormone gene. Founders were identified by PCR analysis and confirmed by Southern blot analysis. Single transgenic $(tetO)_7$Spry-4 or SP-C-rtTA mice were without abnormalities and were maintained in microisolator cages. Double transgenic mice were generated by crossing SP-C-rtTA activator and $(tetO)_7$Spry-4 operator mice. Founder lines were screened for utility based on the phenotype of hypoplastic lungs after tetracycline induced transgene activation. Lung hypoplasia was observed in two distinct Spry-4-expressing founder lines. Line 9.1 was chosen for further investigations.

Genotyping

Transgenic mice were identified by using PCR primers specific for each transgene (5′ SP-C promoter: 5′-GAC ACA TAT AAG ACC CTG GTCA-3′ and the 3′ primer in rtTA coding sequence (5′-AAA ATC TTG CCA GCT TTC CCC-3′), (5′ tetO promoter: 5′-CAC CGG GAC CGA TCC AGC-3′) and the 3′ Spry-4 primer (5′-GAA GTG CTG CTA CTG CTG CTT ACAG-3′). Amplification of PCR products was performed as following: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C (SP-C-rtTA), or 60°C (tetO-Spry-4) for 30 s, and extension at 72°C for 30 s followed by a 5-min extension at 72°C.

Animal use and doxycycline administration

Animals were housed in pathogen-free conditions in accordance with institutional guidelines. Animals were mated and gestation dated by detection of the vaginal plug and correlated with length and weight of each pup at the time of sacrifice. Dams bearing double transgenic pups were maintained on doxycycline in the food (25 mg/g; Harlan Teklad, Madison, WI) or drinking water with doxycycline (Sigma Chemical Co., St. Louis, MO) at 1 mg/ml for various timespans. The mice were killed by either placing them in a CO_2 chamber or by injecting them with 0.2–0.3 cc anesthetic containing ketamine, xylazine, and acepromazine. All experiments were done with at least three pregnant females, resulting in at least five double transgenic mice per time point and per assay and compared with single and double transgenic littermates.

RT-PCR

Adult mice were placed on doxycycline for 48 h prior to killing. Lung tissues were homogenized in Trizol reagent (Life Technologies, Rockville, MD), and RNA was extracted according to the manufacturer's instructions. RNA was treated with DNase prior to cDNA synthesis. RNA (5 μg) was reverse transcribed and analyzed by PCR for Spry-4 mRNA. These primers were also used for genotyping. For normalization, PCR for β-actin mRNA was performed. To control for DNA contamination, PCRs for Spry-4 and β-actin were also conducted on samples in the absence of reverse transcriptase in the cDNA reaction.

Lung histology and in situ hybridization

Mouse embryos were harvested according to their gestational age. Animals were decapitated, and the thorax was fixed overnight in 4% paraformaldehyde at 4°C. Lungs of postnatal day 21 double transgenic mice and controls lungs were inflation fixed overnight at 4°C by using 4% paraformaldehyde at 25 cm of pressure. All samples for histology were washed with PBS, dehydrated through a graded series of ethanol, and processed for paraffin embedding. Sections (5 μm) were loaded onto silianized slides. All stainings were performed on at least three different animals for each marker.

Fig. 3. Expression of TTF-1, CCSP, proSP-C, and Shh. Immunohistochemical analyses of the expression of TTF-1 (A, E), proSP-C (B, F), and CCSP (C, G) and Shh (D, H) on E17.5–E18.5-old wild type (A–D), and SP-C-rtTA/$(tetO)_7$Spry-4 (E–H) lung sections. The dam was treated with doxycycline from E6.5 until killing. Note the presence of respiratory epithelium (TTF-1-positive), type II cells and precursors (proSP-C-positive), and nonciliated bronchiolar cells (CCSP-positive) in the hypoplastic lungs. While peripheral epithelial cells were markedly decreased in number, morphology and immunostaining of the remaining cells were appropriate for the saccular stage of development. Spatial distribution and levels of expression of Shh were maintained in the conducting airways. Bar, 50 μm.

Fig. 4. Spry-4 caused defects in branching morphogenesis at E12.5. Lungs of wild type (A–C) and double transgenic SP-C-rtTA/$(tetO)_7$Spry-4 (D–F) littermates at E12.5 (A, D), E13.5 (B, E), and E14.5 (C, F) are shown. The dams were treated with doxycycline from E6.5 to day of sacrifice. Five lobes were observed in the wild type mice, while three lobes were observed in mutant animals. Lungs are shown at the same magnification. 1, cranial; 2, medial; 3, caudial; 4, accessory; 5, left lobe; T, trachea. Bar, 2 mm.

In situ hybridization was performed with ^{35}S-nucleotide labeled riboprobes to assess the sites of expression of FGF-10, FGF-18, Spry-2, and Spry-4 mRNAs using sense and antisense probes from full-length mouse cDNA clones. Sense and antisense riboprobes were generated from pGEM-T (Promega, Madison, WI) containing the full-length FGF-10, FGF-18, Spry-2, or Spry-4 cDNAs that were transcribed *in vitro* with a Riboprobe transcription kit (Promega, Madison, WI). Conditions and solutions for hybridization are essentially as previously described (Wert et al., 1993). Slides were dipped in Kodak NTB2 emulsion, exposed for 1–4 weeks, and developed with Kodak D19 developer following manufacturer's protocols.

Immunohistochemistry

Tissue sections were stained with hematoxylin–eosin, orcein, and trichrome. Immunohistochemistry for thyroid transcription factor 1 (TTF-1), proSP-C, CCSP, SHH, and platelet endothelial cell adhesion molecule-1 (PECAM-1) were performed as previously described (Tichelaar et al., 2000; Whitsett et al., 2002). For BrdU incorporation, animals were injected intraperitoneally with bromodeoxyuridine (BrdU; 1 mg/g body weight) and killed 2 h thereafter. BrdU-labeled cells were detected by immunohistochemical staining using mouse monoclonal antibody in accordance with the manufacturer's instructions (Zymed Laboratories, Inc., San Francisco, CA).

Lung explant culture

Lungs were isolated from mouse embryos at embryonic day E12.5 from SP-C-rtTA and (tetO)$_7$Spry-4 crosses, yielding 25% double transgenic progeny. Lungs were cultured on a 13-mm diameter and 0.8-μm pore size Track-Etch membrane floating in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM). Lungs were cultured in the presence and absence of doxycycline (10 μg/ml; Sigma, St. Louis, MO). Human recombinant FGF-7 (Prepro Tech. Inc., Rocky Hill, NJ) was diluted in PBS and added to each well at final concentrations of 15 ng/ml. Lungs were cultured at 37°C in a 5% humidified 95% CO_2 air incubator. Photographs were taken at the same magnification with the use of a Nikon SMZ-U stereoscope and a Nikon Cool PIX5000 digital camera. All photographs were processed in Adobe Photoshop and Aldus Freehand.

Results

Spry-4, normally expressed in the lung mesenchyme, was the most active FGF antagonist of various family members tested (De Maximy et al., 1999; M.-A.I. and G.C., unpublished observations). Spry-4 was therefore expressed in the respiratory epithelium of transgenic mice. Doxycycline inducible (tetO)$_7$Spry-4 transgenic mice were generated and crossed to transgenic mice in which the human surfactant protein-C promoter controls synthesis of the reverse tetracycline transactivator (SP-C-rtTA) (Perl et al., 2002a; Tichelaar et al., 2000) (Fig. 1A). Spry-4 mRNA was readily detected in lungs of double transgenic SP-C-rtTA/(tetO)$_7$Spry4 mice when treated with doxycycline. Spry-4 mRNA was undetectable in the absence of the rtTA transgene (Fig. 1B), and was barely detectable in double transgenic mice in the absence of doxycycline. Treatment of the dam with doxycycline induced expression of transgenes in lungs of double transgenic mice within 16 h, and transgene expression ceases approximately 24–48 h after removal of the drug (Perl et al., 2002a; Tichelaar et al., 2000).

Continuous expression of Spry-4 caused severe lung hypoplasia

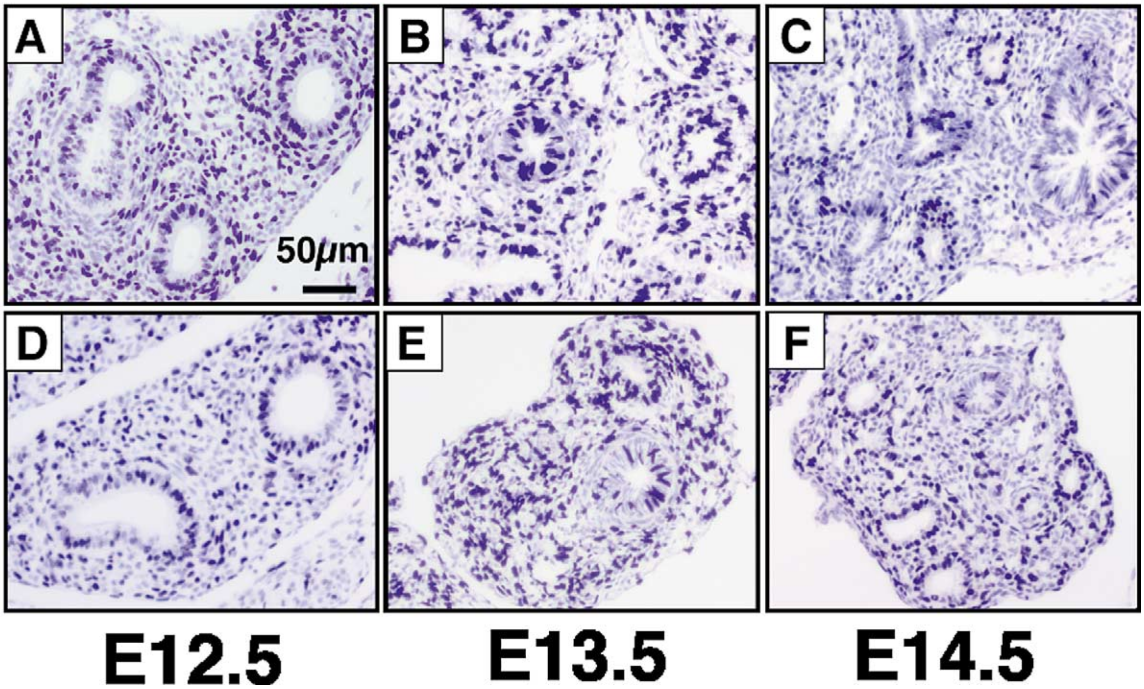
The (tetO)$_7$Spry-4 transgene was transmitted in ratios predicted by Mendelian inheritance. When the dam was maintained on doxycycline from E6.5–E16.5, a marked reduction in pulmonary parenchyma was noted in all double, but single transgenic mice or wild type littermates (Fig. 2). Hypoplastic lungs were found in otherwise normal chest cavities (Fig. 2B). Spry-4 markedly inhibited branching morphogenesis, generally resulting in lungs with three, instead of five lobes (Fig. 2D). While trachea and lobar bronchi were present, the mass of peripheral lung parenchyma was markedly reduced (Fig. 2F). Thus, expression of Spry-4 throughout lung development caused severe pulmonary hypoplasia (Fig. 2B, D, and F). Decreased lobulation caused by Spry-4 was observed as early as E12.5 (see Fig. 4D).

Spry-4 does not alter epithelial cell differentiation

Electronmicroscopic analyses of the respiratory epithelium on E16.5 demonstrated stage appropriate differentiation of type II cells and Clara cells in both conducting and

Fig. 5. Mitotic index by BrdU staining. Lung histology and BrdU incorporation were assessed in wild type (A–C) and double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 (D–F) littermates maintained on doxycycline from E6.5 until killing at E12.5 (A, D), at E13.5 (B, E), and at E14.5 (C, F). Mitotic index was determined by BrdU immunohistochemistry and described as percentage of total cells. At all time points, the percentage of mitotic cells and the epithelial mesenchymal ratio was unchanged. Bar, 50 μm.

Fig. 6. Effects of Spry-4 during lung bud culture. Lungs of E12.5 controls and double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 embryos were cultured for 60 h in the presence and absence of doxycycline. Branching was not inhibited in controls in the presence of doxycycline (A, D, G, J). Branching was not inhibited in double transgenic lung explants in the absence of doxycycline (B, E, H, K). Culture of double transgenic lung explants in the presence of doxycycline inhibited branching (C, F, I, L); note the continued elongation of the nonbranching tubules in the double transgenic lungs in the presence of doxycycline. Size bar, 1 mm.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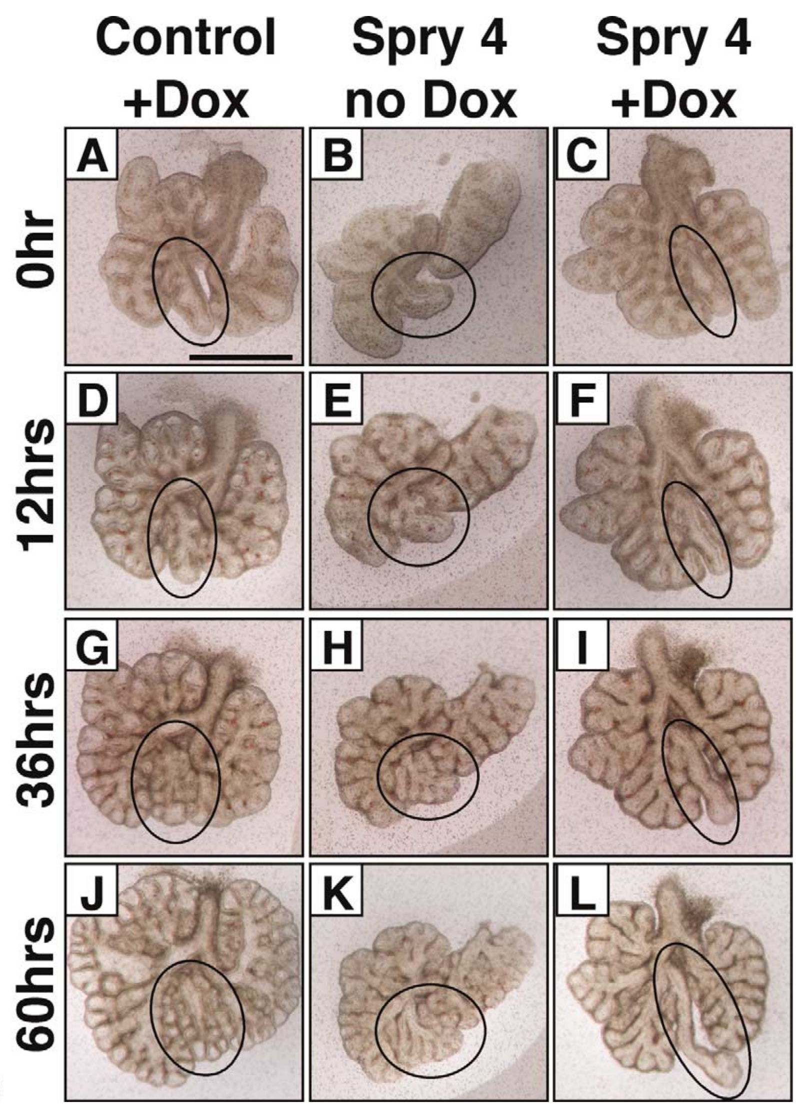
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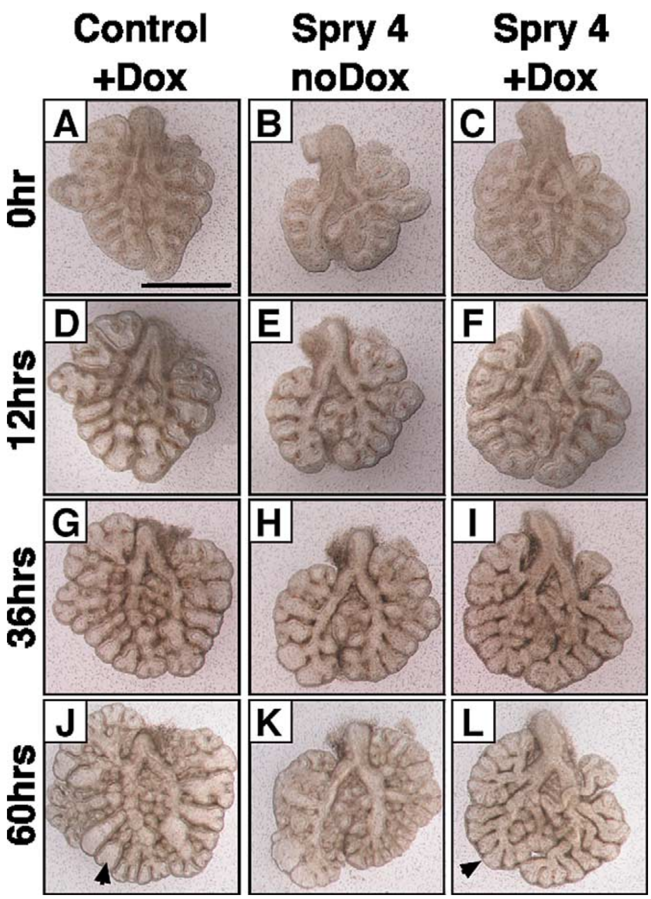

Fig. 7. Spry-4 inhibits effects of FGF-7 in vitro. Lungs of E12.5 controls and double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 embryos were cultured for 60 h in the presence of 15 ng/ml FGF-7. Cystic lesions were observed in the presence of doxycycline in control lung explants (A, D, G, J) and in explants cultured from double transgenic mice in the absence of doxycycline (B, E, H, K). FGF7-induced cyst formation was inhibited in lungs from double transgenic mice in the presence of doxycycline (C, F, I, L). Size bar, 1 mm.

peripheral airways (data not shown). Type II cells, with nascent lamellar bodies and extensive microvillar surfaces, were observed. Ciliated cells were readily apparent in the conducting airways (data not shown). Immunohistochemical staining for differentiation markers selective for proximal and peripheral respiratory epithelial cells was assessed in hypoplastic lungs from the double transgenic embryos during the saccular stage of development (E17.5–E18.5). The spatial pattern of expression of TTF-1 (Fig. 3A and E) and proSP-C (Fig. 3B and F), both markers of distal respiratory epithelial cells, was maintained. However, reduced numbers of TTF-1 and proSP-C staining cells were observed, consistent with a general decrease in the mass of peripheral respiratory epithelial cells. Respiratory epithelial cells in the peripheral airways stained for proSP-C, indicating maturation and differentiation of the alveolar type II cells. Conducting airways from Spry-4-expressing mice were lined by ciliated and nonciliated respiratory epithelial cells, the latter staining for CCSP, a Clara cell differentiation marker (Fig. 3C and G). Staining for CCSP was maintained in the conducting airways, indicating that cellular differentiation continued despite the branching defect caused by Spry-4. Immunohistochemistry for sonic hedgehog (Shh) also did not reveal any differences in its spatial distribution or levels of expression (Fig. 3D and H). Thus, the proximal–distal patterns of gene expression along the developing airways were not disturbed by Spry-4.

Early lobulation defects and lung hypoplasia in Spry-4-expressing mice

Lung morphogenesis was assessed in double transgenic mice treated from E6.5 to E12.5, E6.5 to E13.5, and E6.5 to E14.5 (Fig. 4). Mutant lungs had three instead of five lobes (Fig. 4D–F). Cranial right lobe, medial right lobe, and left lobe were present but smaller; the caudal right and accessory lobes were absent. Histological analysis of the mutant lungs on days E12.5, E13.5, and E14.5 demonstrated relatively normal histology of trachea, and lobar bronchi in spite of severe reductions in peripheral lung tissue. Decreased numbers of peripheral bronchioles and loss of peripheral lung buds were observed, although the organization of epithelial and mesenchymal structures was generally maintained (Fig. 5).

Effects of Spry-4 on cell proliferation

Mitotic indices were assessed by BrdU incorporation in both mesenchyme and epithelial compartments of wild type and Spry-4-expressing mice maintained on doxycycline from E0 at E12.5, E13.5, and E14.5 (Fig. 5). BrdU-positive and-negative cells in the epithelium and mesenchyme were counted from complete cross-sections of lungs from five Spry-4-expressing and wild type pups at each time point. The labeling index, the percentage of positive cells from total number of cells counted, was not changed at any age. At E13.5 and E14.5, the epithelial–mesenchymal ratio of labeled and unlabeled cells was not altered (Fig. 5). At E12.5, the percentage of all epithelial cells was moderately reduced; however, the difference was not statistically significant (17.4 $\pm$ 1.87% to 12.3 $\pm$ 2.47%; P = 0.132) by ANOVA.

Spry-4 inhibits branching and FGF-7-induced cyst formation in vitro

Lungs from E12.5 embryos derived from timed matings of SP-C-rtTA and (tetO)$_7$Spry-4 mice were cultured in the presence and absence of doxycycline (Fig. 6). Branching of the lungs from single transgenic or wildtype mice was not altered by doxycycline. Lung branching was inhibited in explants from double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 mice in the presence, but not in the absence of doxycycline. However, elongation of lung tubes was not inhibited in

Spry4-expressing lung explants (Fig. 6C, F, I, and L), indicating ongoing proliferation of the residual tubules. Lungs from double transgenic E12.5 embryos were cultured in the presence of 15 ng/ml FGF-7 (Fig. 7). After 36 and 60 h of culture, dilated cysts were found in FGF-7-treated lung explants from double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 mice and in control lungs but not in doxycycline-treated lungs of double transgenic mice. Thus, Spry-4 blocked branching morphogenesis and inhibited effects of FGF-7 effects *in vitro.*

Expression patterns of FGF7, FGF-10, FGF-18, Spry-2, and Spry-4 mRNA

FGF-10, FGF-18, Spry-2, and Spry-4 mRNAs were assessed in E13.5 wild type and doxycycline-treated double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 lungs by in situ hybridization analyses (Fig. 8). FGF-10 mRNA was detected in the mesenchyme at the tips of epithelial buds in the control mice (Fig. 8A). Epithelial expression of Spry-4 increased FGF-10 mRNA in the mesenchyme at the tips of the lung tubules and along the tubes (Fig. 8B). Distribution of FGF-18 mRNA was unaltered by Spry-4 expression, but the intensity of the signal was slightly increased (Fig. 8C and D). Expression of FGF7, BMP4, and FGFR2IIIb mRNAs was unchanged (data not shown). Spry-2 mRNA (Fig. 8E and F) was detected in the epithelial cells at the tips of the lung tubes in wild type and affected lungs at comparable levels. Endogenous Spry-4 mRNA was detected diffusely throughout the mesenchyme at low levels in wild type and mutant lungs (Fig. 8G and H). Transgenic Spry-4 mRNA was expressed at high levels in most of the respiratory epithelial cells in doxycycline-treated double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 lungs (Fig. 8H). In situ hybridization with sense probes revealed no specific hybridization (data not shown).

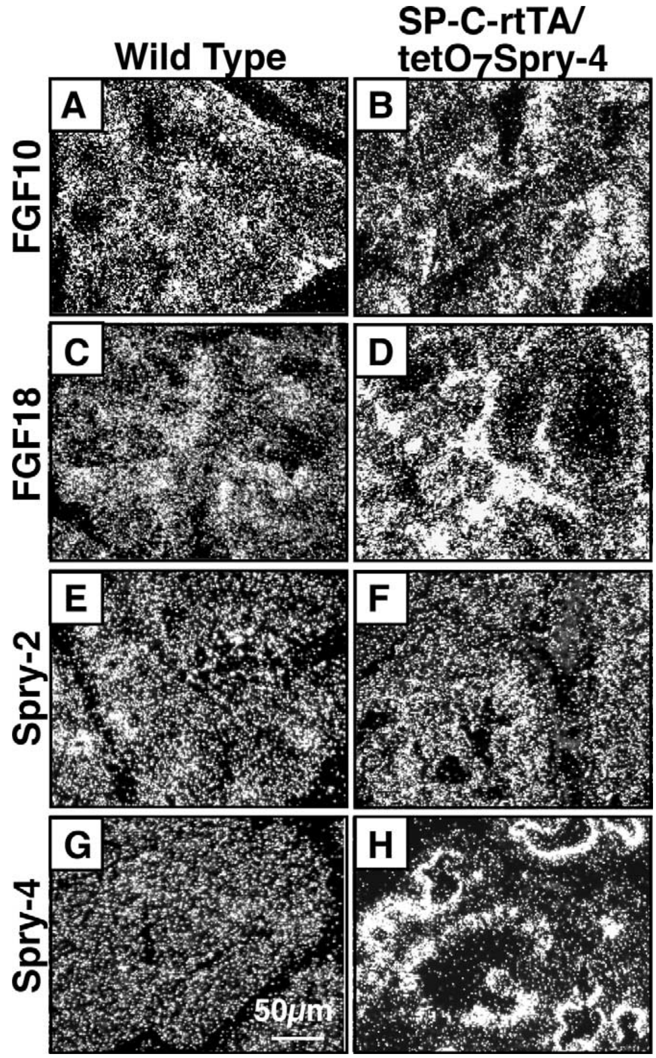

Fig. 8. FGF-10, FGF-18, Spry-2, and Spry-4 mRNAs at E13.5. Lungs from control (A, C, E, G) and transgenic SP-C-rtTA/(tetO)$_7$Spry-4 (B, D, F, H) embryos at E13.5 (doxycycline from E6.5–E13.5) were analyzed for FGF-10 (A, B), FGF-18 (C, D), Spry-2 (E, F), and Spry-4 (G, H) mRNA by in situ hybridization. No specific staining was found in sense-probe controls (bar, 50 μm).

Reversibility of lung lobulation at E12.5

While continuous expression of Spry-4 caused severe lung hypoplasia, it is unclear whether effects of Spry-4 were related to inhibition of branching and proliferation throughout lung morphogenesis or to the failure of expansion of progenitor cells that normally form the peripheral lung parenchyma. In order to address whether Spry-4 affects lung morphogenesis throughout development or its actions were restricted in time, dams were treated with doxycycline before E13.5 or after E12.5, and lungs were compared at the end of embryonic development at E18.5 (Fig. 9). Lungs of untreated double transgenic mice were of normal size and comparable to single transgenic and wildtype littermates (Fig. 9A). After continuous doxycycline treatment from E0.5 to E18.5, lungs of double transgenic mice were markedly hypoplastic and consisted of only three lobes (Fig. 9B). Expression of Spry-4 from E6.5 to E12.5 produced hypoplastic lungs with three instead of five lobes at E12.5 and E13.5 (Fig. 4D and E). To determine the reversibility of the effects of Spry-4, dams were treated with doxycycline from E6.5, removed from doxycycline on E12.5 or E13.5, and lung morphology was assessed at E18.5. When doxycycline was provided to the dam from E6.5 to E12.5 and then removed, lobulation was corrected, five lobes being present at E18.5. Caudal and accessory lobes were fused, but separate main stem bronchi were observed. Peripheral lung tissue was extensively restored, and epithelial cells expressed appropriate proximal (CCSP) and distal (SP-C) cell markers after removal from doxycycline (Fig. 9C, and data not shown). In contrast, termination of doxycycline 1 day later (E13.5) resulted in hypoplastic lungs with three lobes at E18.5. Spry-4 expression until E13.5 resulted in loss of the ability to reinitiate branching of both the right caudal and the accessory lobes (Fig. 9D). Thus, prior to E12.5, Spry-4 reversibly inhibited lung lobulation. Inhibitory effects of Spry-4 on branching morphogenesis were not reversible after E13.5. Expression of Spry-4 from E12.5 to E18.5 did not influence lobulation (Fig. 9E).

Effects of Spry-4 on lung growth and survival in the perinatal period

To investigate the temporal effects of Sprouty4 on lung growth, pregnant dams were given doxycycline at various time points before or after E12.5. Fetal lung weights were assessed at E18.5 and expressed as percent of total body weight. After expression of Spry-4 from E12.5 or E13.5 to E18.5, lung/body weight ratios ($1.1 \pm 0.04\%$) were significantly more reduced than in mice treated from E6.5 to E12.5 ($1.5 \pm 0.14\%$; $P = 0.03$) or from E14.5 and E16.5 to E18.5 ($1.4 \pm 0.11\%$; $P = 0.02$) by ANOVA. Thus, E12.5–E13.5 is a critical time period during which both branching and growth of the lung are most sensitive to Spry-4.

Treatment of double transgenic mice with doxycycline from E14.5 or E16.5 to E18.5 significantly reduced lung weight from $2.6 + 0.05\%$ to $1.4 + 0.11\%$ ($P < 0.0001$) by ANOVA. Thus, Spry-4 inhibits lung growth between E16.5 and E18.5. Double transgenic mice treated with doxycycline between E14.5 and E18.5 or E16.5 to E18.5 had no defects in lobulation (data not shown) but never survived perinatal due to respiratory failure. Electron microscopy and expression studies for SP-C and SP-B showed no obvious developmental defects in these lungs. Thus, the perinatal cause of death in these mice remains unclear.

Expression of Spry-4 from E18.5 and throughout postnatal alveolarization did not interfere with perinatal survival and caused noninflammatory emphysema in mice detected at PN 21 days of age (Fig. 10). Expression of Spry-4 for 3 months in adult mice had no effect on changes in lung architecture (data not shown). Thus, Spry-4 expression in the perinatal period did not cause respiratory failure but modestly impaired alveolarization.

Vascular development

Vascular components of the lung were identified by immunohistochemistry for platelet endothelial cell adhesion molecule (PECAM) during the saccular stage of lung development (E17.5–E18.5) (Fig. 11). Large pulmonary vessels were apparent in the lungs of all mice. Peripheral pulmonary vessels were markedly reduced in the hypoplastic lungs seen after continuous treatment of the dam with doxycycline (Fig. 11B). The distribution and intensity of PECAM staining in the hypoplastic tissue was not altered by expression of Spry-4 from E6.5 to E13.5 or from E12.5 to E18.5 (Fig. 11C and D).

Discussion

Effects of Spry-4, an intracellular inhibitor of FGF-signaling, on lung growth and morphogenesis were dependent on the stage of development. Inhibitory effects of Spry-4 on branching were observed before E13.5, and were reversible until, but not after, this age. Maintenance of cell differentiation and proliferation in residual tissues support the concept that the Spry-4 inhibits commitment or expansion of critical populations of progenitor cells in the embryonic period of development. Effects of Spry-4 between E16.5 and E18.5 in gestation revealed inhibitory effects on lung growth and postnatal survival. Expression of Spry-4 in the perinatal period caused focal emphysema. Taken together, these findings support the concept that FGF signaling is required for early branching morphogenesis and for survival and proliferation of progenitor cells that contribute to the peripheral lung later in development.

Both Spry-2 and Spry-4 are expressed in epithelial cells and surrounding mesenchyme of the fetal lung, respectively. Neither Spry-2 nor Spry-4 encodes a leader sequence, and both are thought to act in a cell-autonomous manner to inhibit tyrosine kinase signaling following FGF activation. While Spry is thought to act primarily as an inhibitor of the FGF pathways, in some tissues (e.g., the retina), EGF signaling was also inhibited by DSpry (Kramer et al., 1999; Egan et al., 2002). The marked inhibitory effects of Spry-4 on lung morphogenesis are similar to those previously observed after expression of a dominant-negative FGF receptor or Spry-2 in epithelial cells of the developing lung (Peters et al., 1994; Mailleux et al., 2001). Together with previous studies that demonstrated the arrest of lung formation in FGF-10 and FGF-R2IIIb transgenic mice, the present observations are consistent with the inhibitory effects of Spry-4 on FGF signaling. In contrast, inhibition of EGFR signaling as seen in waved-2 mice (Luetteke et al., 1994) or mice deficient of a functional EGF receptor (Miettinen et al., 1997; Sibilia and Wagner, 1995) did not perturb lung morphogenesis, postnatal survival, or lung architecture. Thus, the present findings are consistent with inhibitory effects of Spry-4 primarily on FGF-, rather than EGF-dependent pathways.

Loss of lobar bronchi and abnormalities in lobulation, seen after Spry-4 expression, are consistent with the temporal and spatial expression of the transgene. The SP-C promoter is active early in lung morphogenesis, being readily detected by E10 (Wert et al., 1993). As demonstrated in Perl et al. (2002b), doxycycline-induced hSP-C driven transgene expression is highly restricted to the progenitor cells of the peripheral lung epithelium before E8.5. After E10, the hSP-C promoter is active in most of the epithelial cells in the periphery of the fetal mouse lung and in epithelial cells of the conducting airways. The trachea was unaltered in the SP-C-rtTA/$(tetO)_7$Spry-4 mice, in spite of marked inhibition of peripheral lung growth. In contrast, distal trachea and bronchi were absent in FGF-10 gene-

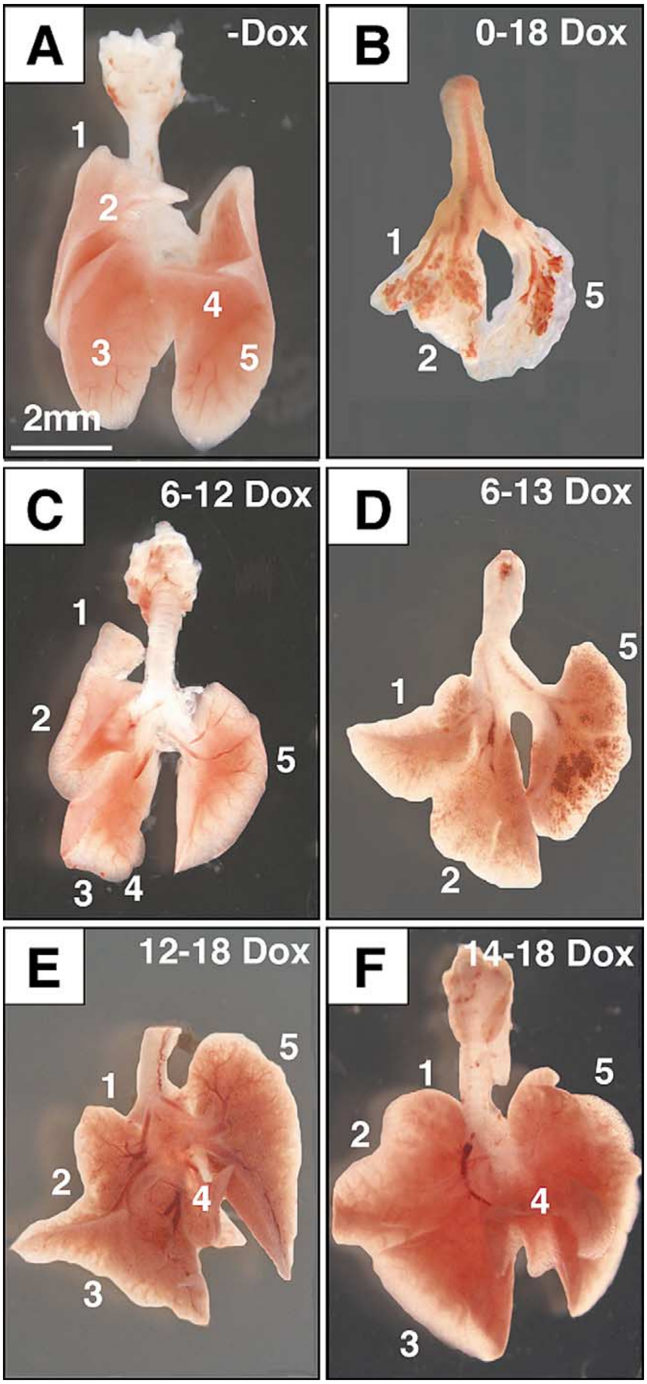

Fig. 9. Reversibility of the effects of Spry-4 on lung lobulation. Lungs from E18.5 double transgenic SP-C-rtTA/$(tetO)_7$Spry-4 mice are shown. All lobes are indicated by numbers: 1, cranial; 2, medial; 3, caudal; 4, accessory; 5, left lobe. Dams were untreated (A) or treated with doxycycline from E0 to E18.5 (B), E6.5 to E12.5 (C), E6.5 to E13.5 (D), E12.5 to E18.5 (E), and E14.5 to E18.5 (F). (Bar, 2 mm).

targeted mice. Although SP-C transgenes are expressed as early as E10 and persist at high levels thereafter, Spry had no observable effect on formation of the trachea. This observation may be related to the spatial and temporal restriction of rtTA expression under the control of the SP-C or to precise stochiometric requirements for Spry-4 to exert its inhibitory effects. Alternatively, growth of the more proximal elements of the trachea may not be sensitive to Spry-4.

Spry-4 inhibited growth of the lung periphery but did not alter cytodifferentiation of respiratory epithelial cells. The proximal–distal patterns of epithelial cell differentiation were maintained in the residual lung tissue in the Spry-4 transgenic mice. Epithelial cell markers, including CCSP, were expressed in nonciliated epithelial cells at appropriate times and sites. In spite of expression of Spry-4 in peripheral respiratory epithelial cells, these cells expressed appropriate differentiation-dependent markers and developed ultrastructural features consistent with normal lung differentiation. Vascular and other mesenchymal components of the lung were lost in proportion to the diminished epithelial compartment. These findings are consistent with the maintenance of a careful balance between mesenchymal and epithelial components of the lung during morphogenesis, a process likely to be determined by the appropriate production of signaling molecules between the mesenchyme and epithelium.

The specificity, timing, and substantial reversibility of lung hypoplasia seen after withdrawal of doxycycline, and with it the cessation of Spry-4 expression, support the concept that Spry-4-sensitive pathways are not required throughout lung morphogenesis for proliferation of lung epithelial cell progenitors. Spry-4 expression did not change the percentage of mitotic cells. Expression of Spry-4 in lung explants inhibited the branching of existing lung tubes but did not inhibit their elongation. Thus, the loss of peripheral lung epithelium in development is not readily explained by an ongoing inhibition of cell proliferation. Rather, effects of Spry may be mediated by a temporally restricted requirement for FGF signaling that mediates commitment and/or expansion of subsets of peripheral lung progenitor cells during the embryonic phase of lung morphogenesis at or before E12.5. This finding is consistent with the findings that FGF10 is expressed at highly restricted sites at the tips of respiratory tubes (Weaver et al., 2000). Loss of this pool of progenitors presumably is related to the loss of signaling from FGF-10 via the FGFR2IIIb receptor, which reduced branching, whereas the mitotic index in the remaining cell pool was maintained. Between E14.5 and E16.5, lung growth appears to be less sensitive to Spry-4 expression. The concept that FGF signaling is required for survival and proliferation of a subset of progenitor cells is supported by findings in which progenitor cells of the peripheral lung were identified at the tips of lung tubules and at sites of lateral branching at E11.5. Expansion of these progenitor cells did not occur until E12.5, and these cells contribute to the majority of epithelial cells from which the peripheral lung is formed (Perl et al., 2002b). Effects of Spry-4 were less severe before E12.5 and after E14.5. Thus, E12.5–E14.5 appears to be a critical time period during which progenitor cells require FGF signals for lobulation and lung growth.

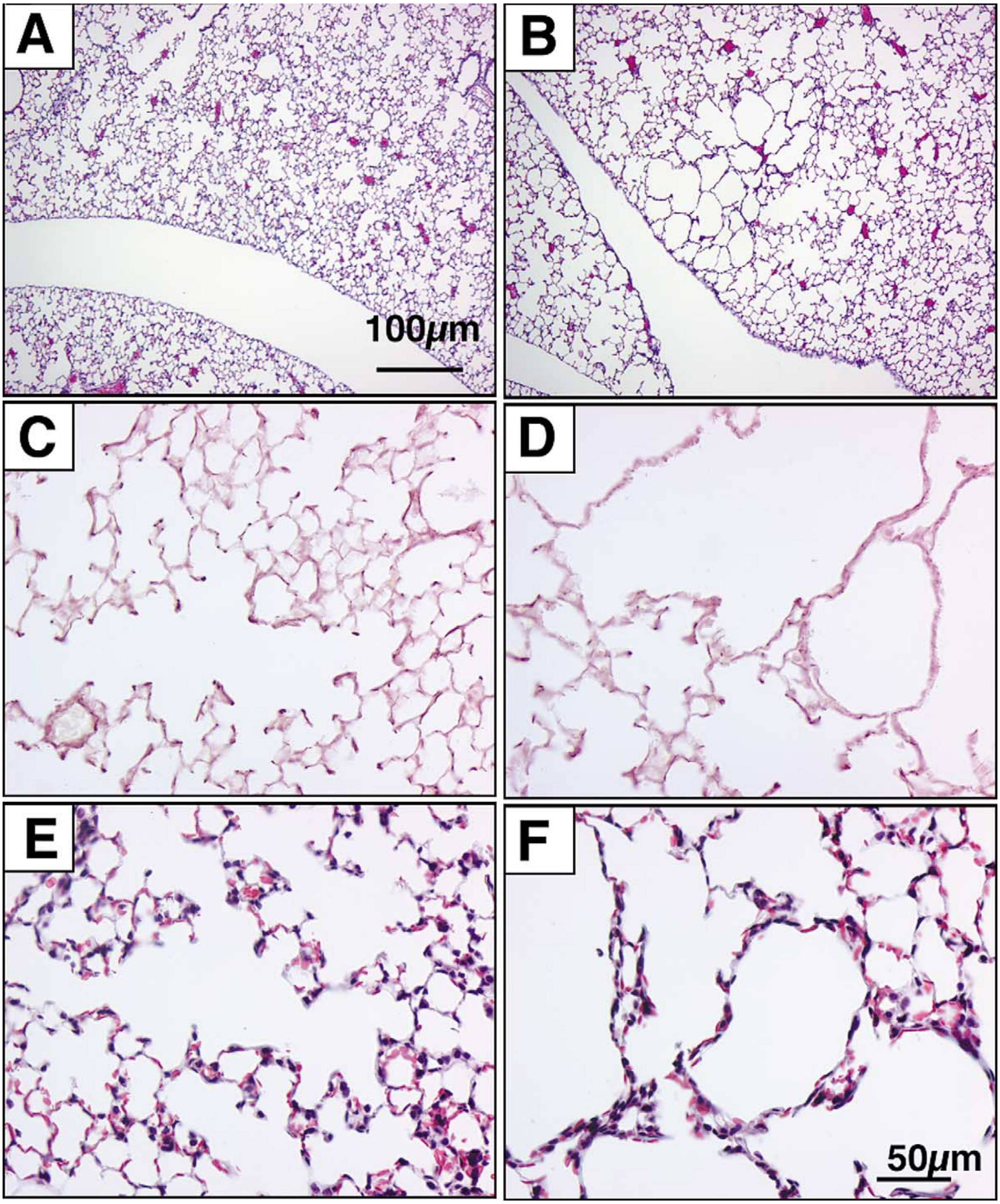

Fig. 10. Emphysema after expression of Spry-4 from E18.5 to PN21. Histology of lungs from double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 9 (B, D, F) mice and control littermates (A, C, E) are shown after doxycycline treatment from E18.5 to PN21. Lungs were stained with hematoxilyn and eosin (A, B), orcein (C, D), and trichrome (E, F). Focal emphysema was detected without evidence of inflammation or fibrosis. Elastin staining (C, D) was unchanged. (A, B: bar, 100 μm; C–F: bar, 50 μm).

The effects of Spry-4 on growth and lobulation were largely reversible until E13.5. Furthermore, Spry-4 had lesser effects on lung structure when the mice were treated with doxycycline after E13.5. Since peripheral lung growth in the Spry-4-expressing mice was not restored unless doxycycline was removed before E13.5, severe lung hypoplasia caused by Spry may reflect a permanent loss of peripheral lung progenitor cells whose commitment, maintenance, and early expansion require FGF signaling during a critical time period. The present findings are consistent with recent studies in which FGF signaling was inhibited by using a FGFR-Hfc (Hokuto et al., 2003). In these studies, effects of FGFR-Hfc on lung growth and lobulation were less reversible when compared with mice expressing Spry-4. Spry-4 an-

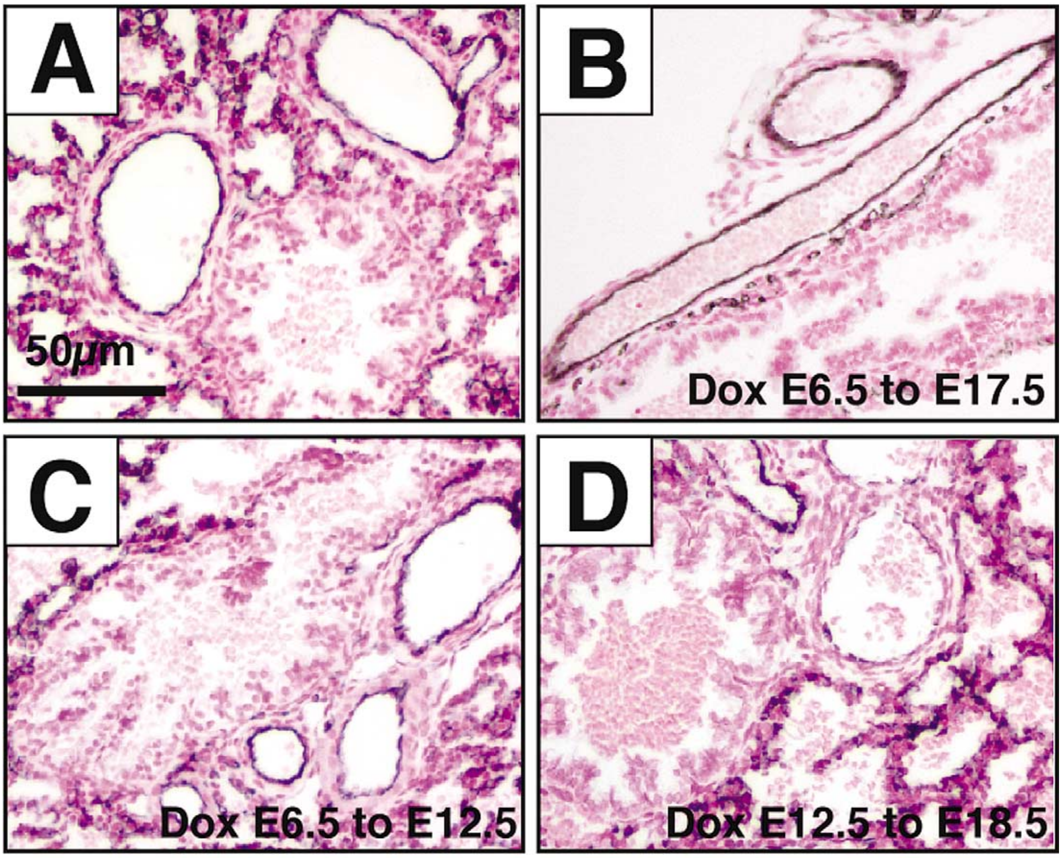

Fig. 11. PECAM staining of the pulmonary vasculature. PECAM was assessed in lungs from E17.5-old wild type (A) and double transgenic SP-C-rtTA/(tetO)$_7$Spry-4 at E17.5 (B) and at E18.5 (C, D) mice. The dam was treated with doxycycline from E6.5 to E17.5 (A, B), E6.5 to E12.5 (C), or E12.5 to E18.5 (D). While large pulmonary arteries were present in lungs from mice expressing Spry-4, capillaries were deficient in the lung parenchyma consistent with the loss of alveolar structures (B). Histology and immunohistochemical staining for PECAM revealed no obvious malformations at E18.5 when Spry-4 was expressed from E6.5 to E12.5 or E12.5 to E18.5 (C, D) (Bar, 50 μm). Note: Red blood cells in (B) and (D) are due to a preparation artifact and not due to hemorrhages.

tagonizes FGFR signaling in the epithelium, whereas FGFR-Hfc is likely to antagonize FGFR signaling in both epithelium and mesenchyme. From the differences in these two models, we hypothesize that FGF signaling may also occur from the epithelium to the mesenchyme, perhaps resulting in the survival and proliferation of epithelial progenitor cells.

The finding that expression of Spry-4 enhanced FGF-10 and FGF-18 expression in the mesenchyme may reflect a compensatory effect for the lack of FGF signaling from the mesenchyme to the epithelium.

Inhibition of FGFR signaling by Spry-4 expression from E17.5 to PN28 caused focal and mild emphysema at PN28, demonstrating that progenitor cells involved in alveolarization are sensitive to Spy in the early postnatal period. The role of FGF signaling in postnatal alveolarization is supported by findings in FGFR3/FGFR4 double knockout mice (Weinstein et al., 1998). Inhibition of FGFR signaling by expression of a soluble FGFR (FGFR-Hfc) from E14.5 to E18.5 caused postnatal emphysema. In contrast, expression of FGFR-Hfc during postnatal alveolarization had no effect on lung morphology (Hokuto et al., 2003). Taken together, these data support the concept that progenitor cells, which form the secondary alveolar septae, are dependent on FGF/FGFR signaling during the canalicular and saccular stage but not during alveoloarization per se. Spry-4 expression during the canalicular and saccular stage results in perinatal lethality.

The timing of the signaling events mediating lung hypoplasia in our experiments is dependent on the time of onset and reversibility of the doxycycline-dependent transgene. In previous studies, SP-C-rtTA-dependent expression of luciferase in the adult lung was detected 6 h after treatment of double transgenic mice or in the fetal lung within 12 h after administration of doxycycline to the dam. Levels of transgene expression decreased rapidly within 24 h after removal of doxycycline in adult mice (Perl et al., 2002a; Tichelaar et al., 2000). Likewise, the concentration of doxycycline required to induce levels of Spry-4 sufficient to inhibit FGF signaling also complicates an estimation of the precise timing in the present experiments. Nevertheless, our studies suggest that Spry-4-dependent pathways are most critical for survival and expansion of peripheral progenitor cells before the end of the pseudoglandular period of lung development (E13–E14).

The studies presented support a model of temporal-restricted FGF signaling, which is required for commitment and survival of a restricted subset of progenitor cells that forms the majority of the peripheral lung in the fetus. A model in which continuous activity of FGF signaling is required for proliferation and differentiation throughout pulmonary morphogenesis is not supported by our findings. The observed reversibility of the effects of Spry-4 on branching suggests that peripheral lung progenitor cells are capable of establishing cell lineages in the late pseudoglandular period, after the formation of lobar bronchi, but before proliferation of the peripheral lung that occurs from E13.5 and thereafter. Later in fetal development (E13.5–E14.5), signaling via Spry-4-sensitive pathways exerts a lesser effect on lung growth and morphogenesis. Postnatal lung morphogenesis was not greatly affected by Spry-4. While recovery from lung hypoplasia following expression of Spry-4 was temporally restricted, the ability of the lung to resume branching morphogenesis was remarkable. Such observations may be of critical importance in understanding clinical syndromes causing lung hypoplasia and emphysema. In human infants, loss of amniotic fluid and chest compression by other organs as in diaphragmatic hernia or oligohydramnios caused by renal abnormalities often cause lung hypoplasia, resulting in respiratory failure at birth. Effects of oligohydramnios are highly temporally restricted, being most marked in midgestation (weeks 16–20) during the canalicular phase of human lung development. Lethal lung hypoplasia is generally not seen after loss of amniotic fluid later in gestation (after 24 weeks).

Acknowledgments

We thank Mildred Stahlman from Division of Pediatrics and Pathology, Vanderbilt University Medical Center (Nashville, TN) for electron microscopy analysis; Paula Blair and Leigh Anne Miller for technical assistance on the immunohistochemistry. This work is supported by National Institutes of Health HL56387 and Austrian FWF Schroedinger J1959-GEN.

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