FGF9 and SHH regulate mesenchymal Vegfa expression and development of the pulmonary capillary network

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The juxtaposition of a dense capillary network to lung epithelial cells is essential for air-blood gas exchange. Defective lung vascular development can result in bronchopulmonary dysplasia and alveolar capillary dysplasia. Although vascular endothelial growth factor A (Vegfa) is required for formation of the lung capillary network, little is known regarding the factors that regulate the density and location of the distal capillary plexus and the expression pattern of Vegfa. Here, we show that fibroblast growth factor 9 (FGF9) and sonic hedgehog (SHH) signaling to lung mesenchyme, but not to endothelial cells, are each necessary and together sufficient for distal capillary development. Furthermore, both gain- and loss-of-function of FGF9 regulates Vegfa expression in lung mesenchyme, and VEGF signaling is required for FGF9-mediated blood vessel formation. FGF9, however, can only partially rescue the reduction in capillary density found in the absence of SHH signaling, and SHH is unable to rescue the vascular phenotype found in Fgfa–/– lungs. Thus, both signaling systems regulate distinct aspects of vascular development in distal lung mesenchyme. These data suggest a molecular mechanism through which FGF9 and SHH signaling coordinately control the growth and patterning of the lung capillary plexus, and regulate the temporal and spatial expression of Vegfa.

KEY WORDS: Fibroblast growth factor 9 (FGF9), Vascular endothelial growth factor (VEGF), Sonic hedgehog (SHH), Lung development, Angiogenesis, Mesenchyme, Mouse

INTRODUCTION

The development of the lung as a gas-exchange organ requires precise organizational instructions to optimize blood vessel density and position adjacent to the alveolar epithelium. Although a high level of vascular refinement, remodeling and maturation occurs during postnatal development of the lung, proper capillary density and position is specified earlier in development and is required for viability at birth. Alveolar capillary dysplasia (ACD) is a lethal disorder in humans characterized by a failure of alveolar capillary formation, often accompanied by misalignment of the pulmonary veins (deMello, 2004). Bronchopulmonary dysplasia (BPD) is a chronic lung disease affecting premature infants (<1000 g) that involves impaired alveolarization and dysmorphic vascular development associated with prematurity and is thought to be exacerbated by hypoxic or mechanical injury (Coalson, 2006; deMello, 2004).

In the mouse, pulmonary vascular development matches lung branching morphogenesis from embryonic day 10.5 (E10.5) onwards and maintains a dense capillary plexus surrounding the distal epithelium (Gebb and Shannon, 2000; Parera et al., 2005; Schachtner et al., 2000). During the pseudoglandular stage (E11.5-E16.5), a complex developmental signaling network is established across the mesothelial, mesenchymal and epithelial tissue boundaries. In addition to directing mesenchymal and epithelial cell proliferation, migration and providing positional information, these signals produce cues for the development of the vasculature. Understanding how this signaling network regulates lung vascular development is necessary to understand the mechanisms leading to human lung ACDs and other vascular diseases.

Vascular endothelial growth factor A (VEGFA) is essential for endothelial cell proliferation, migration and survival (reviewed in Ferrara et al., 2003). Individual knockouts for Vegfa and for its two known tyrosine kinase receptors, Flt1 (Vegfr1) and Flik1 (Vegfr2, Kdr), result in lethality prior to the development of the lung capillary plexus (Carmeliet et al., 1996; Ferrara et al., 1996; Fong et al., 1995; Shalaby et al., 1995). In vitro lung organ culture experiments, however, have shown that VEGFA protein is sufficient to stimulate neoangiogenesis (Healy et al., 2000), to increase mesenchymal Flk1-positive cells and to promote epithelial branching morphogenesis (Del Moral et al., 2006). Conversely, sequestration of VEGFA by a soluble VEGFR1-Fc chimeric protein reduces lung vasculature and impairs epithelial development (Gerber et al., 1999; Zhao et al., 2005).

Several genetic studies have indicated that Vegfa is essential for the formation of the pulmonary vasculature and for epithelial branching morphogenesis. Vegfa is first expressed in lung mesenchyme and epithelium from E12.5-E14.5, and then becomes increasingly restricted to epithelium after E14.5 (Gebb and Shannon, 2000; Greenberg et al., 2002; Ng et al., 2001). Mice engineered to express only the non-heparin-binding VEGFA-120 isoform have significant defects in pulmonary vessel development, indicating the necessity for correct VEGFA isoform dose and spatial expression patterns (Galambos et al., 2002; Ng et al., 2001). Consistent with this requirement, directed overexpression of Vegfa from epithelium results in significant alterations in pulmonary vascular development (Akeson et al., 2003). Despite the importance of VEGFA for lung vascular development, little is known about factors that regulate Vegfa expression during early lung development.

Both hedgehog (HH) signaling and fibroblast growth factor (FGF) signaling are important for vascular formation during development, although it is unclear whether this is mediated by signaling directly to endothelial cells versus indirectly via the regulation of other vasculogenic or angiogenic factors. HH signaling can induce the aggregation of endothelial cells into tubules in vitro (Kanda et al., 2003) and, in vivo, ablation of Indian hedgehog (Ihh) or of the gene...
encoding the HH signal transduction molecule smoothened (SMO), results in severe vascular defects during murine yolk sac development (Byrd et al., 2002). Ihh is also necessary for vasculogenesis in the anterior epiblast during mouse gastrulation (Dyer et al., 2001), and zebrafish sonic-you (mutation in shh) embryos demonstrate the absence of trunk vessel formation (Brown et al., 2000). Furthermore, in chick explants, co-incubation with cyclopamine, a potent steroid alkaloid antagonist of HH signaling, inhibits endothelial tubulogenesis (Chen et al., 2002; Vokes et al., 2004). This inhibition occurs independently of VEGFA. Addition of VEGFA, however, leads to VEGFA significantly synergizing with SHH to stimulate robust vascular network formation (Vokes et al., 2004). Additional evidence for synergy is demonstrated in the adult retina and developing heart, in which SHH is sufficient to induce the expression of Vegfa and angiopoietin 2 (Ang2), thus indirectly inducing vascularization (Lavine et al., 2006; Pola et al., 2001). Furthermore, conditional knockout of Shh in lung epithelium ultimately results in fewer pulmonary blood vessels and decreased Veg expression at E18.5 (Miller et al., 2004).

FGF signaling represents a key morphogenic pathway during development that stimulates endothelial cell proliferation, migration and tube formation in a variety of contexts (reviewed in Javerzat et al., 2002). Several studies have demonstrated that FGFR2 can induce angioblasts from uncommitted mesoderm and vasculogenesis from embryonic bodies (reviewed in Poole et al., 2001). However, the significance of these findings is difficult to interpret, because Fgf2-expressing mice do not have any apparent vasculogenesis or angiogenesis phenotype, although they do demonstrate a postnatal reduction in vascular tone and low blood pressure (Dono et al., 1998). Studies that have focused on FGFR receptor 1 (FGFR1), which is expressed on endothelial cells, show that this receptor is essential for vessel formation in vitro (Burger et al., 2002; Cross and Claesson-Welsh, 2001). FGF2-mediated capillary morphogenesis in vitro (Burger et al., 2002; Cross and Claesson-Welsh, 2004) suggests a complex interaction through which morphogenetic signals from FGF2 and VEGFA can induce vascular formation by direct, indirect and/or through synergistic mechanisms (Ashara et al., 1995; Magnusson et al., 2004; Pepper et al., 1998).

Fgf9 is expressed in both mesothelium and epithelium during lung development and promotes sub-mesothelial mesenchyme proliferation, positively regulates FGFR1-mediated branching morphogenesis and maintains optimal SHH signaling in the subepithelial mesenchyme (Colvin et al., 1999; Colvin et al., 2001; White et al., 2006). Additionally, an expansion of distal endothelial cells was observed in lungs overexpressing Fgf9 (White et al., 2006). We thus hypothesized that Fgf9 might be required for lung distal capillary development, and that this could be mediated via direct signaling to endothelial cells or indirectly via regulation of VEGFA and SHH signaling. Here, we show that mesenchymal Fgf9 and SHH signaling are required for early lung distal vascular development, in which they act cooperatively to regulate both capillary plexus formation and Vegfa expression.

MATERIALS AND METHODS

Mouse strains

Fgf9+/− mice were maintained on a C57/B6 background and genotyped as described previously (Colvin et al., 2001). Fgf9+/−;dox(48) denotes mice bistransgenic for the rTA-SPC (Perl et al., 2002; Tichelaar et al., 2000) and TRE-Fgf9-RES-eGfp alleles (White et al., 2006), which together induce expression of Fgf9 in developing lung epithelium following doxycycline (Bio-Serv, Frenchtown, NJ) administration for 48 hours. The rTA-SPC and TRE-Fgf9-RES-eGfp alleles were genotyped as previously described (White et al., 2006) and maintained on an FVB background. Fgf9+/− indicates mice homozygous for both Fgf9+/− (Tichelaar et al., 2003) and Fgf9+/− mice were conditionally floxed alleles. Fgf9+/− indicates mice heterozygous for a null allele of both Fgf9+/− and Fgf2−/− animals are phenotypically normal and live through adulthood. Flik1-Cre (Motoike et al., 2003) mice were mated to Fgf9+/− mice to generate heterozygous Fgf9+/−; Flik1-Cre mice, which were then backcrossed to Fgf9+/− mice to create Fgf9+/−; Flik1-Cre (Fgf9+lox/Flox) conditional knockout animals. Fgf9+/−; Dermo1-Cre mice were mated to Fgf9−/− mice to generate Fgf9−/−; Dermo1-Cre (Fgf9+lox/Dermo1) conditional knockout mice. Littermate controls were heterozygous for both Fgf9+/− genes and lacked Cre. Smo+/−; Flik1-Cre and Smo+/−; Actin-CreER (Guo et al., 2002) mice were mated to Smo+/− to generate Smo+/−; Flik1-Cre (Smo+/−; Smo−/+ and Smo−/−; Actin-CreER (Smo−/+; Smo−/− animals. Littermate controls were homozygous for Smo but lacked Cre.

Pregnant Smo+/− females were injected intraperitoneally (iP) with 8 mg tamoxifen (Sigma, St Louis, MO) in sunflower seed oil (Sigma) at specified times. Unpublished genotyping primers are (shown 5′ to 3′); Fgf9+lox (5′ CCGTAGAATGTTCACTACGTACGATACAG, wt is 142 bp and flox is 207 bp); Fgf9+lox (5′ ACCCTAGGACACCTCGATA- AGCCACATCC and 3′ AGGTTCCTCCTCTCTGTGACTTTTAG, Fgf9−/− is 300 bp); Fgf9−/− (5′ TCTTCTTGCACTATAGGAACACA- GCCGGG and 3′ GAGAGGAGGTTCTGACAGGCGACAC- GCCGGG and 3′ CATAGCAGACGGTGTGTTGACACCT, wt is 471 bp); Smo+lox (5′ CAGGTGGACCTGCTCCGCAGGTAGATGAG and 3′ GATTGGAAGAAGGCTGGTTGATGTTGAGG, 408 bp); all lacZ−/− (5′ GTTGCAGTGCAGGGCAGATACACTTGCTGA and 3′ GCCACTG- GTTGAGCCATATCCTAAATCTGC, 389 bp); Rosa26R-luc−/− (5′ CAA- GTGCTCTCTGTTGTTGATGTTGAGG and 3′ wt is 486bp and Δ is 332bp).

Flik1-lacZ, Vegfa-lacZ and the Rosa26R-lacZ alleles were genotyped by lacZ PCRs, except in the case of Rosa26R-luc−/− and Vegfa-lacZ, in the presence of the Smo floxed allele, in which case a Rosa26R-lucZ (above) and Vegfa-lacZ-specific PCR (Miquerol et al., 1999) was used.

Whole-mount immunohistochemistry

Lung tissues were dissected in PBS, fixed overnight in 4% PFA and dehydrated to 100% methanol for storage at −20°C until use. All incubations and washes were performed at 4°C while shaking. Tissues were first incubated in methanol:30% H2O2 (1:1) for 2 hours, dehydrated to PBT (PBS/0.1% Tween-20) and then incubated with a blocking solution (2% skim milk, 1% serum, 0.1% Triton X-100) in PBT for 2 hours. The primary and secondary antibodies were incubated overnight in blocking solution. Primary and secondary antibody washes (2% skim milk, 0.1% Triton X-100) were performed once an hour for 5 hours. Visualization of secondary antibody conjugated to HRP was performed without signal amplification using the DAB kit (Vector Laboratories, Burlingame, CA), according to manufacturer’s instructions. Tissues were stained for 5-15 minutes, washed twice with PBS, dehydrated to 100% methanol for clearing and storage, and finally rehydrated for photography. Monoclonal rat anti-mouse PECAM-1 (CD31; BD Pharmingen, San Jose, CA) was incubated at 1:500. Secondary HRP-conjugated anti-rat (Chemicon, Temecula, CA) was incubated at a 1:200 dilution. All panel comparisons are from littermate tissues, which were kept in the same tube throughout the protocol, ensuring similar processing and staining and incubation time. All staining patterns are representative of at least three embryos.

Capillary density quantification

The four rostral-most distal buds at E12.5 were used for capillary density assessment. The total number of intersections between PECAM-labeled capillaries and a line at the approximate midway point along the rostral-
development, a total of three comparison sets from two separate litters were
quantified (n=3 control and 3 Fgf9−−). Whole-mount lacZ staining
To visualize Flk1-lacZ+− and Vegfa-lacZ+− signals, lungs were dissected in
ice-cold PBS, then fixed with 0.5% glacial acetic acid in PBT (PBS, 0.1%
Tween-20) for 30 minutes at 4°C. Tissues were washed in PBT twice for 10
minutes each prior to photography. All in situ data are representative of at least three
embryos.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed as described (colvin et al., 2001). Following
color reaction and methanol dehydration, larger tissues were rehydrated and saturated with 50%
glycerol in PBS prior to photography. All in situ data are representative of at least three
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caudal axis were compared from all four buds in littermate Fgf9−− and
control lungs. A total of three comparison sets from two separate litters were
quantified (n=3 control and 3 Fgf9−−). Whole-mount lacZ staining
To visualize Flk1-lacZ+− and Vegfa-lacZ+− signals, lungs were dissected in
ice-cold PBS, then fixed with 0.5% glacial acetic acid in PBT (PBS, 0.1%
tween-20) for 30 minutes at 4°C. Tissues were washed in PBT twice for 10
minutes prior to incubation with β-galactosidase staining solution (2 mM
MgCl2, 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1
mg/ml X-Gal in PBT) at room temperature in the dark. Following adequate
color reaction, tissues were again washed twice in PBT for 10 minutes each
and then dehydrated to methanol. For photography, lungs were rehydrated
and incubated with 50% glycerol in PBT to increase light penetration. All
panel comparisons are from littermate tissues kept in the same tube and
incubated with 50% glycerol in PBT to increase light penetration. All
in situ data are representative of at least three
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RESULTS

FGF9 signaling to mesenchyme is necessary and sufficient for capillary plexus formation in the
developing distal lung
Endothelial cells, or their precursors, can be identified in lung mesenchyme surrounding the growing epithelial tubes as early as E10.5 in the mouse (parera et al., 2005; Schachtner et al., 2000). Concurrent with mesenchymal and epithelial development during branching morphogenesis (E11.5-E16.5), the developing lung maintains growth of a single-layered capillary network (Gebb and Shannon, 2000; Schachtner et al., 2000). Because FGF9 signals to lung mesenchyme, we hypothesized that it might regulate pulmonary vascular development. We therefore examined Fgf9−−
lungs by whole-mount immunohistochemistry with an anti-PECAM antibody showing large
gaps between vessels in the distal capillary plexus of Flk1−−− lungs at E11.5 (B), E12.5 (D)
and E13.5 (F) when compared with control lungs (A,C,E), (G,H) Whole-mount lacZ
staining with the endothelial cell marker Flk1-lacZ showing a reduction in capillary
density around the distal epithelium in Fgf9−−−; Flk1-lacZ+− lungs at E15.5 (H)
compared with control lungs (G).

Whole-mount lacZ staining
To visualize Flk1-lacZ+− and Vegfa-lacZ+− signals, lungs were dissected in
ice-cold PBS, then fixed with 0.5% glacial acetic acid in PBT (PBS, 0.1%
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and then dehydrated to methanol. For photography, lungs were rehydrated
and incubated with 50% glycerol in PBT to increase light penetration. All
panel comparisons are from littermate tissues kept in the same tube throughout the protocol. All staining patterns are representative of at least three
embryos.

For Vegfa-lacZ histology, stained lungs were fixed in 4% PFA overnight,
soaked in 30% sucrose overnight, embedded and frozen in OTC, and
cryosectioned at 12-14 μm. Sections were dried for ~3 hours at room
temperature, dehydrated to xylene and mounted. Flk1-lacZ and Dermo1-
Cre; Rosa26R-stained sections were embedded in paraffin, sectioned at 5
μm and counterstained with eosin (Flk1-lacZ) or nuclear fast red (Dermo1-
Cre; Rosa26R).

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed as described (colvin et al., 2001). Following
color reaction and methanol dehydration, larger tissues were rehydrated and saturated with 50%
glycerol in PBS prior to photography. All in situ data are representative of at least three
embryos.

Lung organ cultures
Lung explant cultures were performed as described (White et al., 2006). FGF9 protein (Peprotech, Rocky Hill, NJ) was used at a concentration of 2.5
ng/μl, SHH-N (R&D, Minneapolis, MN) at 500ng/ml and cyclopamine (Toronto Research Chemicals, North York, Ontario, Canada) at 10 μM.

Fig. 1. FGF9 signaling via mesenchymal
FGFR1/2 is necessary and sufficient for
distal lung capillary development.
(A-F) Whole-mount immunohistochemistry
with anti-PECAM antibody showing large
gaps between vessels in the distal capillary
plexus of Fgf9−−− lungs at E11.5 (B), E12.5 (D)
and E13.5 (F) when compared with control
lungs (A,C,E). (G,H) Whole-mount lacZ
staining with the endothelial cell marker
Flk1-lacZ showing a reduction in capillary
density around the distal epithelium in
Fgf9−−−; Flk1-lacZ+− lungs at E15.5 (H)
compared with control lungs (G).

(I,J) Rosa26R-lacZ stain showing endothelial
cell-specific Flk1-Cre activity in a pattern
consistent with distal lung endothelial cells in
whole-mount (I) and frozen (J) sections.

(K,L) Fgf1 and Fgf2 double conditional
knockout using Flk1-Cre (Fgf1/2−−, L) showing
no difference in distal lung vascular
development compared to an Fgf1/2−−
control (K). (M,N) Fgf1 and Fgf2 double
conditional knockout using mesenchymal-
specific Dermo1-Cre (Fgf1/2Dermo1), showing
reduced distal lung capillary density (N)
compared with an Fgf1/2−− control (M).

(O,P) Induced Fgf9 expression for 48 hours
with doxycycline [Fgf9(Dox)], is sufficient to
induce Flk1-lacZ expression throughout lung
mesenchyme (P), compared with expression
only in the sub-epithelial mesenchyme in
control lung (O). Histological sections in O
and P were photographed through a 20×
objective. Scale bars: 50 μm.

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RESULTS

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lungs by whole-mount immunohistochemistry with an anti-PECAM antibody. At E11.5-E13.5, Fgf9−−− lungs showed a significant reduction in distal capillary network density compared with controls (Fig. 1A-F), which exhibited a dense capillary plexus overlaying the airway epithelium. Quantification of the distal vasculature at E12.5 demonstrated a significant (P<0.002) reduction (45±7%) in capillary coverage in Fgf9−−− lungs. To examine the structure of the capillary network at later stages of development, Fgf9−−−; Flk1-lacZ+− lungs were generated and
stained for β-galactosidase enzymatic activity. Flk1-lacZ efficiently labels endothelial cells throughout the embryo (Schachtner et al., 2000; Shalaby et al., 1995). At E15.5, Fgf9+/–; Flk1-lacZ+/– lungs maintained the trend observed at E11.5-E13.5, exhibiting a reduction in capillary network density surrounding the distal airway epithelium (Fig. 1G,H). Because capillary plexus density is similar throughout branching morphogenesis, the decrease in plexus density shown in Fgf9+/– lungs cannot be caused by a delay in branching. These data identify FGF9 as necessary for proper development of the distal capillary plexus during the branching morphogenesis stage of lung development.

To distinguish between FGF9 signaling directly to endothelial cells versus indirectly through lung mesenchyme, we inactivated FGF receptors either in endothelial cells or lung mesenchyme and examined distal lung capillary plexus density. To selectively inactivate FGFR signaling in endothelial lineages, Fgfr1/2Flk1–/–; Flk1-Cre (Fgfr1/2Flk1) double conditional-knockout embryos were generated (Trokovick et al., 2003; Yu et al., 2003). Flk1-Cre has been shown to effectively target floxed alleles in endothelial, hematopoietic and muscle lineages during development (Motoiko et al., 2003). Rosa26R: Flk1-Cre lungs showed a pattern of lacZ activity at E12.5, indicating effective Cre targeting to developing lung endothelial cells (Fig. 1J). Rosa26R; Flk1-Cre lungs also showed labeling of mesodermal cells present in the caudal region of the lung (data not shown); this labeling might represent progenitors with broad mesenchymal potential, as indicated by other studies (Ema et al., 2006). Examination of E12.5 Fgfr1/2Flk1 embryos revealed a lung vascular plexus that was indistinguishable from controls when labeled with anti-PECAM antibodies (Fig. 1K,L) or Rosa26R (see Fig. S1 in the ). Furthermore, Fgfr1/2Flk1 mice survive to adulthood, indicating that FGF signaling directly to endothelial cells via FGF1 and/or FGF2 is not necessary for embryonic survival (our unpublished observation). To determine whether FGF signaling to lung mesenchyme is necessary for capillary plexus formation, we examined embryos in which both Fgfr1 and Fgfr2 were inactivated throughout the developing lung mesenchyme using the Dermo1-Cre targeting allele (Fgfr1/2Dermo1) (White et al., 2006). Dermo1-Cre sufficiently targets lung mesenchyme and mesothelium but does not target the endothelial cell lineage (see Fig. S1C in the supplementary material). Fgfr1/2Dermo1 lungs exhibited decreased capillary plexus density with wider gaps between adjacent vessels (Fig. 1M,N); this reduction in capillary plexus density appeared to be similar to, although less severe than, that seen in Fgf9+/– lungs. The differences in severity are probably due to the timing of Fgfr inactivation using a conditional targeting approach versus the effects of a homozygous-null mutation in the gene encoding the ligand for these receptors.

To determine whether FGF9 could induce vascular development in lung mesenchyme, an inducible transgenic system [SPC-rtTA; TRE-Fgf9-IRESpEF] that also incorporated the Flk1-lacZ reporter allele was used to express FGF9 in lung epithelium at various time windows during lung development. Induction of Fgf9 expression for 48 hours from E12.5-E14.5 or
E15.5-E17.5 showed increased numbers of Flk1-positive cells within the lung mesenchymal compartment (Fig. 10.P and data not shown). Histological analysis with the endothelial cell marker Tie2 showed a similar pattern to Flk1-lacZ at E14.5 (data not shown). Taken together with the loss-of-function data, we conclude that FGF9 signaling via mesenchymal FGFR1 and FGFR2 is both necessary and sufficient for distal capillary plexus formation throughout the pseudoglandular stages of lung development.

FGF9 signaling to lung mesenchyme regulates Vegfa expression

Although FGF receptor activity is not required in endothelial cells, FGF signaling can promote vascular development via the induction of Vegfa expression in some systems (Auguste et al., 2001; Kubo et al., 2002; Seghezzi et al., 1998). To investigate the potential of Fgf9 to regulate Vegfa expression during lung development, we examined Vegfa-lacZ (Miquerol et al., 1999) expression in both Fgf9 gain-of-function and loss-of-function backgrounds. Consistent with previous studies, Vegfa was primarily expressed in lung mesenchyme at E11.5-E12.5, with highest levels occurring between the mesothelium and the distal epithelial tips (Gebb and Shannon, 2000; Greenberg et al., 2002) (Fig. 2A,C,E,G). Only very low levels of Vegfa-lacZ were detected in the epithelium at this stage, consistent with previous data showing that the 188 kDa isoform of Vegfa is not significantly expressed in epithelium until ~E14.5 (Greenberg et al., 2002; Ng et al., 2001). At E11.5 and E12.5, mesenchymal Vegfa-lacZ was reduced in Fgf9−/−; Vegfa-lacZ lungs compared with controls (Fig. 2A-H). Interestingly, at E11.5, no β-galactosidase expression was found in distal epithelium in controls and knockouts, and at E12.5 only very light epithelial staining was observed. At E12.5 and E13.5, Vegfa expression remained low in the mesenchyme of Fgf9−/−; Vegfa-lacZ lungs (Fig. 2H,L). However, epithelial β-galactosidase staining increased moderately at E13.5 in both Fgf9−/−; Vegfa-lacZ and control lungs, suggesting that epithelial Vegfa expression is not directly regulated by FGF9.

In contrast to Fgf9−/− lungs, Fgf9+/+; Vegfa-lacZ lungs exhibited a significant increase in β-galactosidase staining throughout the mesenchyme at E13.5, with very high expression in the sub-epithelial mesenchyme (Fig. 2M-P). Additionally, increased Vegfa-lacZ expression was found throughout the epithelium, whereas, in controls, expression remained low in epithelium (Fig. 2O,P). These data indicate that FGF9 signaling to mesenchyme is sufficient to positively regulate Vegfa expression in mesenchyme and indirectly in epithelium. Induction of Vegfa suggests a molecular pathway leading to the formation of the multi-layered vascular domain found in Fgf9−/−; H9252 lungs (Fig. 2B) (White et al., 2006).

The expression patterns in both Fgf9 loss- and gain-of-function models demonstrate that mesenchymal FGF signaling is both necessary and sufficient for Vegfa expression in lung mesenchyme, and suggest that an FGF9 to Vegfa pathway promotes distal capillary formation. To test whether the FGF9-mediated capillary formation pathway requires VEGF signaling, we used lung organ cultures co-incubated with FGF9 protein and a small molecule inhibitor (SU5416) of VEGFR signaling (Fong et al., 1999). Control lung explants isolated at E12.5 and incubated for 48 hours with SU5416 and BSA showed a sparse distal capillary network (Fig. 3A) compared with BSA-incubated controls (M-O). When assessed by anti-PECAM whole-mount immunohistochemistry, lungs showed a reduction in distal capillary network density (F) compared with Smo+/− controls (E) at E12.5. (G,H) Distal lung capillary density appeared similar in Smo+/−; Flk1-Cre (Smo+/−; H) and Smo+/− control (G) mice, indicating that HH signaling to endothelial cells is not required for development. (I-L) Smo−/− lungs (I,J) show a decrease in Vegfa-lacZ activity in mesenchyme distal to the sub-epithelial layer (arrow) in whole-lung preparations (I,J) and frozen sections (K,L) compared with controls. (M-P) Lung organ cultures incubated with 500 ng/ml of SHH-N protein show an increase in PECAM-positive cells (N) and Vegfa-lacZ staining (P) compared with BSA-incubated controls (M,O) after 24 hours. (B,K,L) Lower left lobe; (E-J) upper left lobe. Histology: 20× objective. Scale bars: 100 μm.
Smo-mediated enhancement of SHH signaling might be required for the observed FGF9 regulation of Vegfa expression or, alternatively, that SHH could act in a parallel pathway to FGF9 to regulate lung vascular development.

To assess the contribution of SHH signaling to lung capillary development, we generated Smo mice (Smo$^{fl}$) (Long et al., 2001) to Actin-CreER mice (Guo et al., 2002) to allow inactivation of the HH pathway at specific time points during lung development. In the lung, SHH activates the HH signaling pathway in sub-epithelial mesenchyme (Bellusci et al., 1997a; Weaver et al., 2003) and possibly in other cells within the mesenchymal compartment. Because Actin-CreER is inducible in most cells following the administration of tamoxifen (Guo et al., 2002), this conditional targeting strategy was expected to inactivate the HH pathway throughout the entire lung. To test the efficacy of this system in developing lung tissue, pregnant mice carrying Rosa26R; Actin-CreER embryos were given an 8 mg intraperitoneal injection of tamoxifen at E9.5 and were then examined at E12.5. Lungs from Rosa26R; Actin-CreER embryos demonstrated β-galactosidase activity throughout lung epithelium and mesenchyme (Fig. 4A,B). Using a similar paradigm to inactivate Smo, we found that Smo$^{fl}$; Actin-CreER (Smo$^{act}$) conditional-knockout lungs were smaller than Smo$^{fl}$ controls at E12.5 (Fig. 4C,D) and E13.5 (data not shown). At both time points, the lungs exhibited a severely decreased distal capillary network characterized by large gaps between the capillaries surrounding the distal epithelium (Fig. 4E,F and data not shown).

Recent reports indicate that SHH might signal directly to endothelial cells to stimulate tubulogenesis or vasculogenesis (Kanda et al., 2003; Vokes et al., 2004). To determine whether the decrease in capillary network density found in Smo$^{act}$ lungs was due to inactivation of SHH signaling in endothelial cells, we generated Smo$^{fl}$; Flik1-Cre (Smo$^{fl}$-Flik) embryos. Development of the capillary network appeared normal in Smo$^{fl}$Flik lungs at E12.5 (Fig. 4G,H), and animals with this genotype survived through adulthood with no apparent problems (data not shown). These data suggest that HH signaling to endothelial cells is not necessary for development.

SHH signaling to non-endothelial mesenchyme is necessary for lung capillary formation

Previously, we demonstrated that FGF9 signaling to lung mesenchyme promotes SHH signaling (White et al., 2006). Because SHH signaling has been shown to promote Vegfa expression and blood vessel formation in some systems (Lavine et al., 2006; Pola et al., 2001), we hypothesized that FGF9-mediated enhancement of SHH signaling might be required for the observed FGF9 regulation of Vegfa expression or, alternatively, that SHH could act in a parallel pathway to FGF9 to regulate lung vascular development.

To assess the contribution of SHH signaling to lung capillary development, we generated Smo mice (Smo$^{fl}$) (Long et al., 2001) to Actin-CreER mice (Guo et al., 2002) to allow inactivation of the HH pathway at specific time points during lung development. In the lung, SHH activates the HH signaling pathway in sub-epithelial mesenchyme (Bellusci et al., 1997a; Weaver et al., 2003) and possibly in other cells within the mesenchymal compartment. Because Actin-CreER is inducible in most cells following the administration of tamoxifen (Guo et al., 2002), this conditional targeting strategy was expected to inactivate the HH pathway throughout the entire lung. To test the efficacy of this system in developing lung tissue, pregnant mice carrying Rosa26R; Actin-CreER embryos were given an 8 mg intraperitoneal injection of tamoxifen at E9.5 and were then examined at E12.5. Lungs from Rosa26R; Actin-CreER embryos demonstrated β-galactosidase activity throughout lung epithelium and mesenchyme (Fig. 4A,B). Using a similar paradigm to inactivate Smo, we found that Smo$^{fl}$; Actin-CreER (Smo$^{act}$) conditional-knockout lungs were smaller than Smo$^{fl}$ controls at E12.5 (Fig. 4C,D) and E13.5 (data not shown). At both time points, the lungs exhibited a severely decreased distal capillary network characterized by large gaps between the capillaries surrounding the distal epithelium (Fig. 4E,F and data not shown).

Recent reports indicate that SHH might signal directly to endothelial cells to stimulate tubulogenesis or vasculogenesis (Kanda et al., 2003; Vokes et al., 2004). To determine whether the decrease in capillary network density found in Smo$^{act}$ lungs was due to inactivation of SHH signaling in endothelial cells, we generated Smo$^{fl}$; Flik1-Cre (Smo$^{fl}$-Flik) embryos. Development of the capillary network appeared normal in Smo$^{fl}$Flik lungs at E12.5 (Fig. 4G,H), and animals with this genotype survived through adulthood with no apparent problems (data not shown). These data suggest that HH signaling to endothelial cells is not necessary for development.
Differential control of VEGF expression and lung vascular development by FGF9 and SHH

Because the reduction in Vegfa expression seen in SmoActin conditional-knockout lungs is less severe than that seen in Fgf9–/– lungs, it is unlikely that SHH signaling is the sole downstream regulator of FGF9-mediated vascular development. To investigate the relationship between FGF9 and SHH signaling in regulating Vegfa expression and vascular development, we determined whether high levels of FGF9 signaling could rescue capillary plexus formation and Vegfa expression in lungs devoid of HH signaling. To address this issue, we used an in vitro organ culture system and cyclopamine to block HH signaling (Mailleux et al., 2005; White et al., 2006). Cyclopamine-treated explants exhibited severely impaired distal vascular development (Fig. 5A,G). In these explants, large vessels running alongside the proximal epithelium remained, but the capillary network adjacent to the distal epithelium was significantly reduced or absent. Mesenchymal Vegfa-lacZ expression was correspondingly reduced in cyclopamine-treated explants, except within a single layer of sub-epithelial mesenchyme, consistent with our in vivo data (Fig. 5H,I, Fig. 4L). Compared with cyclopamine treatment alone, explants treated with both FGF9 and cyclopamine showed increased Vegfa expression and increased distal vascular development (Fig. 5G). Vegfa-lacZ expression was increased in the sub-epithelial compartment, from a single layer of sub-epithelial mesenchyme in explants treated with cyclopamine only (Fig. 5I) to several layers of cells in combined FGF9 and cyclopamine-treated explants (Fig. 5L). Vegfa-lacZ expression, however, could not be rescued in the sub-mesothelial compartment (Fig. 5F,L). Similar results were found in E11.5 explants incubated with cyclopamine and FGF9-loaded heparin beads at E11.5 for 48 hours (data not shown). When lung explants were treated with only FGF9, robust blood vessel formation was induced throughout the mesenchyme, consistent with in vivo data showing capillary extension towards the mesothelium (Fig. 1P, Fig. 3B, Fig. 5D). As predicted, Vegfa-lacZ expression was enhanced throughout the mesenchyme in FGF9-treated organ cultures (Fig. 5B,C,E,F), similar to what was observed in vivo (Fig. 2M-P). These data indicate that a spatially specific pattern of mesenchymal Vegfa expression is differentially regulated by FGF9 and SHH signaling in sub-mesothelial and sub-epithelial mesenchymal compartments. Furthermore, distal capillary plexus formation appears dependent on Vegfa expression in sub-mesothelial mesenchyme, and Vegfa expression in this region requires both FGF9 and SHH signaling. Vegfa expression in sub-epithelial mesenchyme appears to be less-dependent on SHH but responsive to FGF9.

Finally, we addressed whether SHH is sufficient to stimulate lung vascular formation in an Fgf9–/– background. Fgf9–/– lung organ cultures were treated with BSA or SHH and examined for vascular development by anti-PECAM immunohistochemistry. In contrast to wild-type tissues, which exhibited an increase in PECAM-labeled capillary sprouts in response to SHH (Fig. 6A,C), SHH-treated Fgf9–/– lung explants demonstrated no difference in the amount of PECAM-labeled cells compared to BSA-treated Fgf9–/– controls (Fig. 6B,D).

DISCUSSION

Air-blood gas exchange requires a dense capillary network to encapsulate the distal respiratory tree (Stenmark and Abman, 2005). Impaired development of this vascular network – caused by mutation, prematurity or injury – can cause life-threatening deficiencies in pulmonary function that are often manifest as bronchopulmonary dysplasia and alveolar capillary dysplasia (Coalson, 2006; deMello, 2004). In previous studies, we identified two mesenchymal domains in the developing lung – the sub-mesothelial domain and the sub-epithelial domain – both of which differentially respond to FGF9 and SHH signals (White et al., 2006).

We showed that FGF9 serves to regulate the overall size of the lung by coordinating mesenchymal growth with signals that regulate epithelial development (Colvin et al., 2001; White et al., 2006). FGF9 does this by signaling to sub-mesothelial mesenchyme to stimulate proliferation and Fgf10 expression, and to the sub-epithelial mesenchyme, in which it acts to maintain optimal SHH signaling. SHH supports mesenchymal cell proliferation and survival, and modulates the expression pattern of FGF10 (Mailleux et al., 2005; Pepicelli et al., 1998; White et al., 2006), which signals back to airway epithelium to regulate branching (Bellusci et al., 1997b; Weaver et al., 2000).

During lung development, expansion of lung mesenchyme is essential for growth of the pulmonary tree. The mesenchymal compartment also contains developing vasculature and bronchial smooth muscle (deMello et al., 1997; Mailleux et al., 2005; Stenmark and Gebb, 2003). As the lung matures, most mesenchyme is consumed, leaving only a dense capillary network tightly juxtaposed to airway epithelium. Examination of the origins of the capillary network identified the formation of a primitive capillary plexus between the sub-mesothelial and sub-epithelial mesenchymal domains (Fig. 7) (Gebb and Shannon, 2000; Schachtner et al., 2000; White et al., 2006). Because FGF9 and SHH regulate proliferation and survival of these two mesenchymal domains, we reasoned that these signaling molecules might also regulate the formation of the intervening capillary plexus. In various systems, FGF and HH signals have been shown to regulate each other, and to regulate vascular development, by signaling either directly to endothelial cells or indirectly to surrounding mesenchyme to regulate the expression of angiogenic factors such as Vegf. In the developing lung, we found that overexpression of FGF9 resulted in the expansion of the primitive capillary plexus (White et al., 2006),
suggested that FGFs might also be important for vascular development in the lung. In the studies presented here, we have shown that both FGFR9 signaling and SHH signaling are required for formation of the pulmonary capillary plexus, but do so indirectly by regulating the level and pattern of Vegfa expression in lung mesenchyme. We also showed that SHH is required for Vegfa expression in the more distal sub-mesothelial compartment, but not in proximal sub-epithelial mesenchyme that is adjacent to sites of SHH expression. By contrast, FGFR9 signals throughout both mesenchymal compartments to regulate Vegfa expression. Thus, FGFR9 appears to be more important for the level of Vegfa expression, whereas SHH appears to be more important for patterning Vegfa expression.

**FGF and SHH do not signal to Flk1-positive endothelial cells**

Many studies have shown that FGFR2 is sufficient to signal to endothelial cells to promote vascular development (Auguste et al., 2003). In the studies presented here, and in previous studies examining coronary artery development (Lavine et al., 2006), we have shown that conditional deletion of both Fgrf1 and Fgrf2 in endothelial cells, using either Flk-1-Cre or Tie1-Cre targeting alleles, did not cause vascular abnormalities during development. Similarly, we show here that inactivation of the HH pathway, by conditionally targeting a floxed allele of Smo in endothelial cells with Flk1-Cre, did not perturb vascular development. These studies suggest that FGF and HH signaling are not directly necessary for endothelial cell development. By contrast, inactivation of FGF receptors or the HH signaling pathway in lung mesenchyme resulted in reduced capillary plexus density, strongly suggesting that these signaling pathways regulate lung vascular development indirectly by regulating the expression of another factor (Fig. 7). Consistent with this model, examination of Vegfa expression revealed modulation by both FGF and HH signaling in lung mesenchyme.

A recent report by van Tuyl et al. explored the relationship between lung vascular development and branching morphogenesis (van Tuyl et al., 2007). In the presence of FGFR2, they showed an increase in capillary formation in lung organ cultures. Although FGFR2 is a potent mitogen for endothelial cells, it is dispensable for development, because knockout animals for Fgfr2 have no known vascular development phenotype (Dono et al., 1998; Schultz et al., 1999). We show here that FGFR9 is the physiologically relevant FGF required for lung vascular development. van Tuyl et al. have also examined vascular development in Sdh-lh lungs; however, they were not able to detect a defect in vascular development prior to E14.5 or detect a change in Vegfa expression (van Tuyl et al., 2007). By contrast, in Smo-kam conditional-knockout lungs, we see a significant decrease in capillary density at earlier stages and decreased expression of mesenchymal Vegfa-lacZ. A possible explanation for this discrepancy might be compensation for loss of SHH by another HH ligand, as has been observed during prostate development (Doles et al., 2006). Because we use a conditional knockout of the obligate signal transducing molecule for all HH ligands, we avoid issues of ligand redundancy and compensation.

**Vegfa is regulated by FGFR9 and SHH and is essential for capillary plexus formation in the lung.**

During normal lung development, Vegfa shows a dynamic pattern of expression. Vegfa is first observed at E11.5 in mesenchyme and, by E13.5, Vegfa expression becomes activated in airway epithelium. We speculate that this pattern of expression drives initial formation of the vascular plexus in the mid-mesenchymal regions and, later, modulates the juxtaposition of the vascular plexus to the airway epithelium. Our data from Fgfr9 loss- and gain-of-function supports this model (Fig. 7). In Fgfr9-/- lungs, at E11.5 and E12.5, the level of Vegfa in lung mesenchyme decreased, which resulted in a capillary plexus of decreased complexity. After E13.5, although the density of the capillary plexus and the level of mesenchymal Vegfa remained low, epithelial Vegfa appeared normal. Epithelial Vegfa expression correlates with proper positioning of vessels next to epithelium in Fgfr9-/- lungs. Conversely, overexpression of Fgfr9 robustly increased mesenchymal Vegfa, resulting in a plexus that extends into the sub-mesothelial mesenchyme compartment. These data support a model in which FGFR9 signaling to mesenchyme regulates the level of mesenchymal Vegfa, which in turn specifies the density of the early lung vascular plexus.

By contrast, our data suggest that endogenous levels of SHH primarily regulate the pattern of Vegfa expression rather than the level. Loss of SHH signaling either by conditional knockout of Smo or treatment of organ cultures with cycloamine led to a lung capillary plexus of decreased density. In both cases, mesenchymal Vegfa expression appears at the same intensity in the sub-epithelial mesenchyme as controls, but is absent in the sub-mesothelial mesenchyme. These data suggest that sub-mesothelial Vegfa is necessary for vascular plexus formation in the developing lung and sub-epithelial Vegfa is not sufficient alone. In organ cultures treated with high levels of SHH protein, Vegfa levels in both mesenchyme regions is increased. This indicates that SHH might be permissive for Vegfa expression in the sub-mesothelial mesenchyme and that overabundance of SHH can lead to increased Vegfa levels indirectly. Alternatively, SHH might be required for the initiation of Vegfa expression (as indicated by loss-of-function experiments) and to
regulate Vegfa expression levels (as indicated by gain-of-function experiments) in sub-mesothelial mesenchyme. Previous studies using cyclopamine support the permissiveness model, mediated by cell survival. Mesenchymal cell death occurs at higher levels in organ cultures lacking SHH signaling (Weaver et al., 2003; White et al., 2006), and thus the decrease in sub-mesothelial Vegfa found in Smo<sup>fl/fl</sup> conditional knockouts and cyclopamine-treated lung explants could be secondary to decreased cell survival, which would manifest as an apparent decrease in Vegfa expression. Supporting a model for survival-mediated permissiveness, cyclopamine-mediated cell death can be partially rescued by FGF9 addition (White et al., 2006), as can capillary development and Vegfa expression.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/2/3743/DC1

**References**


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