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

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Abstract

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 *Fgf9*$^{-/-}$ 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*.

Keywords

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

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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 *Flk1* (*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 synergism is demonstrated in the adult retina and developing heart, in which SHH is sufficient to induce the expression of *Vegfa* and angiopoeitin 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 *Vegf* 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 FGF2 can induce angioblasts from uncommitted mesoderm and vasculogenesis from embryoid bodies (reviewed in Poole et al., 2001). However, the significance of these findings is difficult to interpret, because *Fgf2*$^{-/-}$ mice have no 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 FGF 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). Additionally, FGF2-mediated capillary morphogenesis requires VEGFR1, indicating that FGF-induced vasculogenesis might be mediated by induction of *Vegfa* expression (Kanda et al., 2004). This suggests a complex interaction through which morphogenic signals from FGF2 and VEGFA can induce vascular formation by direct, indirect and/or through synergistic mechanisms (Asahara 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 FGF10-mediated branching morphogenesis and maintains optimal SHH signaling in the sub-epithelial 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 bi-transgenic for the *rtTA-SPC* (Perl et al., 2002; Tichelaar et al., 2000) and *TRE-Fgf9-IRES-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 *rtTA-SPC* and *TRE-Fgf9-IRES-eGfp* alleles were genotyped as previously described (White et al., 2006) and maintained on an FVB background. *Fgfr1/2*$^{f/f}$ indicates mice homozygous for both *Fgfr1* (Trokovic et al., 2003) and *Fgfr2* (Yu et al., 2003) conditional floxed alleles. *Fgfr1/2*$^{\Delta/+}$ indicates mice heterozygous for a null allele of both *Fgfr1* and *Fgfr2*. *Smo*$^{f/f}$ indicates mice homozygous for the floxed smoothened allele, and were genotyped by the absence of wild-type *Smo* [adapted from Long et al. (Long et al., 2001)]. Both *Fgfr1/2*$^{f/f}$ and *Smo*$^{f/f}$ animals are phenotypically normal and live through adulthood. *Flk1-Cre* (Motoike et al., 2003) mice were mated to *Fgfr1/2*$^{f/f}$ mice to generate heterozygous *Fgfr1/2*$^{f/+}$*; Flk1-Cre* mice, which were then backcrossed to *Fgfr1/2*$^{f/f}$ mice to create *Fgfr1/2*$^{f/f}$*; Flk1-Cre* (*Fgfr1/2*^{Flk1}) conditional knockout animals. *Fgfr1/2*$^{\Delta/+}$*; Dermo1-Cre* mice were mated to *Fgfr1/2*$^{f/f}$ mice to generate *Fgfr1/2*$^{\Delta/f}$*; Dermo1-Cre* (*Fgfr1/2*^{Dermo1}) conditional knockout mice. Littermate controls were heterozygous for both *Fgfr* genes and lacked *Cre*. *Smo*$^{f/+}$*; Flk1-Cre* and *Smo*$^{f/+}$*; Actin-CreER* (Guo et al., 2002) mice were mated to *Smo*$^{f/f}$ to generate *Smo*$^{f/f}$*; Flk1-Cre* (*Smo*^{Flk1}) and *Smo*$^{f/f}$*; Actin-CreER* (*Smo*^{Actin}) animals. Littermate controls were homozygous for *Smo* but lacked *Cre*. Pregnant *Smo*^{Actin} females were injected intraperitoneally (IP) with 8 mg tamoxifen (Sigma, St Louis, MO) in sunflower seed oil (Sigma) at specified time points. Unpublished genotyping primers are (shown 5′ to 3′): *Fgfr1*^{flox} (5′ CCAGTAACTGTACCAATGAGCTGTAAGCAT and 3′ TGCCCACCATGCTCCTGCTTCCTTCAGAGC, wild-type (wt) allele is 181 bp and flox allele is 231 bp); *Fgfr2*^{flox} (5′ TTCCTGTTCGACTATAGGAGCAACAGGCGG and 3′

GAGAGCAGGGTGCAAGAGGCGACCAGTCAG, wt is 142 bp and flox is 207 bp); *Fgfr1*$^{\Delta}$ (5′ ACCTCAGGAACCTCGAATAAGCCACCATCAC and 3′ AGGTTCCCTCCTCTTGGATGACTTTAG, *Fgfr1*$^{\Delta}$ is 300 bp); *Fgfr2*$^{\Delta}$ (5′ TTCCTGTTCGACTATAGGAGCAACAGGCGG and 3′ CATAGCACAGGCCAGGTTGTTCATTTCCAT, *Fgfr2*$^{\Delta}$ is 471 bp); *Smo*^{WT} (5′ CACTGCGAGCCTTTGCGCTACAACGTGTGC and 3′ CAGTGGCCGGTCCCATCACCTCCGCGTCGC, wt is 171bp); all *Cre* (5′ GCATTACCGGTCGATGCAACGAGTGATGAG and 3′ GAGTGA ACGA ACCTGGTCGAAATCAGTGCG, 408 bp); all *lacZ* (5′ GT TGCAGTGCACGGCAGATACACTTGCTGA and 3′ GCCACTGGTGTGGGCCATAATTCAATTCGC, 389 bp); *Rosa26R-lacZ* (5′ CAAAGTCGCTCTGAGTTGTTATCAGTAAGG and 3′wt GGAGCGGGAGAAATGGATATGAAGTACTGG and 3′Δ TCCAAGAGTACTGGAAAGACCGCGAAGAGT, wt is 486bp and Δ is 332bp).

Flk1-lacZ, *Vegfa-lacZ* and the *Rosa26R-lacZ* alleles were genotyped by *lacZ* PCR, except in the case of *Rosa26R-lacZ* and *Vegfa-lacZ* in the presence of the *Smo* floxed allele, in which case a *Rosa26R-lacZ* (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% H_2O_2 (4:1) for 2 hours, rehydrated 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 PBS) 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 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 rostal-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% glutaraldehyde 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 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.

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. 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, *Fgfr1/2*$^{f/f}$; *Flk1-Cre* (*Fgfr1/2*^{Flk1}) double conditional-knockout embryos were generated (Trokovic 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 (Motoike 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. 1I,J). *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 $Fgfr1/2^{Flk1}$ 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, $Fgfr1/2^{Flk1}$ mice survive to adulthood, indicating that FGF signaling directly to endothelial cells via FGFR1 and/or FGFR2 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 ($Fgfr1/2^{Dermo1}$) (White et al., 2006). *Dermo1-Cre* efficiently targets lung mesenchyme and mesothelium but does not target the endothelial cell lineage (see Fig. S1C in the supplementary material). $Fgfr1/2^{Dermo1}$ 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-IRES-eGfp*, hereafter $Fgf9^{dox(48)}$] 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. 1O,P and data not shown). Histological analysis with the endothelial cell marker *Tie2-lacZ* 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 *Vegf* 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*$^{dox(48)}$*; 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*$^{dox(48)}$ 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 BSA showed a sparse distal capillary network (Fig. 3A) (Sorokin, 1961). The decreased capillary density in vitro is probably due to the relative hyperoxic environment of the organ cultures, which could suppress *Vegfa* expression (van Tuyl et al., 2005). By contrast, lung explants incubated with FGF9 for 48 hours formed a dense capillary network surrounding the terminal airways (Fig. 3B). As previously reported, FGF9-treated lung explant cultures also demonstrated increased mesenchyme and epithelial luminal dilation (Fig. 3E,F) (White et al., 2006). Incubation with SU5416 caused a dose-dependent decrease in lung vasculature at 48 hours (data not shown). At a concentration of 45 μM, anti-PECAM-labeled blood vessels were not detectable in SU5416-treated explants (Fig. 3A,C). However, SU5416-treated organ cultures continued to show normal in vitro branching morphogenesis (Fig. 3G). When lung explant cultures were co-incubated with FGF9 and 45 μM SU5416, minimal rescue of the SU5416-mediated reduction in the distal capillary plexus was observed (Fig. 3B,D), although FGF9 still caused the expected mesenchymal expansion and epithelial luminal dilation seen in explants treated with FGF9 only (Fig. 3F,H). At higher concentrations of SU5416 (60 μM), FGF9 showed no vascular rescue (data not shown). These data support a model in which FGF9-enhancement of *Vegfa* expression is the primary pathway for mesenchymal FGF-stimulated distal capillary formation.

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 mated homozygous floxed *Smo* mice (*Smo*$^{f/f}$) (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*$^{f/f}$*; Actin-CreER* (*Smo*^{Actin}) conditional-knockout lungs were smaller than *Smo*$^{f/f}$ 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*^{Actin} lungs was due to inactivation of SHH signaling in endothelial cells, we generated *Smo*$^{f/f}$*; Flk1-Cre* (*Smo*^{Flk1}) embryos. Development of the capillary network appeared normal in *Smo*^{Flk1} 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.

To determine whether the vascular phenotype in *Smo*^{Actin} lungs also accompanies a change in *Vegfa* expression, we generated *Smo*^{Actin}*; Vegfa-lacZ* embryos and stained for β-galactosidase activity. Surprisingly, expression of *Vegfa* in the sub-epithelial mesenchyme appeared only slightly reduced, whereas sub-mesothelial mesenchymal staining was largely absent (Fig. 4I–L). This expression pattern was also seen in explants incubated with cyclopamine (see below). Conversely, lung explants incubated with SHH protein (500 ng/ml) demonstrated an increase both in PECAM-labeled cells (Fig. 4M,N) and in *Vegfa-lacZ* staining in both sub-mesothelial and sub-epithelial mesenchyme (Fig. 4O,P). These data indicate that SHH signaling to lung mesenchyme regulates capillary network formation of the developing distal lung and is necessary for optimal expression of *Vegfa* in sub-mesothelial lung mesenchyme. These data also suggest that FGF9-mediated upregulation of HH signaling (White et al., 2006) could contribute to the vascular phenotypes described in *Fgf9* loss-of-function and gain-of-function lungs.

Differential control of VEGF expression and lung vascular development by FGF9 and SHH

Because the reduction in *Vegfa* expression seen in *Smo*^{Actin} 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,J). *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), suggesting that FGFs might also be important for vascular development in the lung. In the studies presented here, we have shown that both FGF9 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, FGF9 signals throughout both mesenchymal compartments to regulate *Vegfa* expression. Thus, FGF9 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 FGF2 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 *Fgfr1* and *Fgfr2* 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 FGF2, they showed an increase in capillary formation in lung organ cultures. Although FGF2 is a potent mitogen for endothelial cells, it is dispensable for development, because knockout animals for *Fgf2* have no known vascular development phenotype (Dono et al., 1998; Schultz et al., 1999). We show here that FGF9 is the physiologically relevant FGF required for lung vascular development. van Tuyl et al. have also examined vascular development in $Shh^{-/-}$ 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^{Actin} 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 FGF9 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 juxtapostion of the vascular plexus to the airway epithelium. Our data from *Fgf9* loss- and gain-of-function lungs supports this model (Fig. 7). In $Fgf9^{-/-}$ 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 $Fgf9^{-/-}$ lungs. Conversely, overexpression of *Fgf9* robustly increased mesenchymal *Vegfa*, resulting in a plexus that extends into the sub-mesothelial mesenchyme compartment. These data support a model in which FGF9 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 cyclopamine 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^{Actin} 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Akeson AL, Greenberg JM, Cameron JE, Thompson FY, Brooks SK, Wiginton D, Whitsett JA. Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. Dev Biol 2003;264:443–455. [PubMed: 14651929]

Asahara T, Bauters C, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 1995;92:365–371.

Auguste P, Gursel DB, Lemiere S, Reimers D, Cuevas P, Carceller F, Di Santo JP, Bikfalvi A. Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms. Cancer Res 2001;61:1717–1726. [PubMed: 11245488]

Auguste P, Javerzat S, Bikfalvi A. Regulation of vascular development by fibroblast growth factors. Cell Tissue Res 2003;314:157–166. [PubMed: 12851809]

Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL. Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 1997a;124:53–63. [PubMed: 9006067]

Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 1997b;124:4867–4878. [PubMed: 9428423]

Brown LA, Rodaway AR, Schilling TF, Jowett T, Ingham PW, Patient RK, Sharrocks AD. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. Mech Dev 2000;90:237–252. [PubMed: 10640707]

Burger PE, Coetzee S, McKeehan WL, Kan M, Cook P, Fan Y, Suda T, Hebbel RP, Novitzky N, Muller WA, et al. Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. Blood 2002;100:3527–3535. [PubMed: 12411316]

Byrd N, Becker S, Maye P, Narasimhaiah R, St-Jacques B, Zhang X, McMahon J, McMahon A, Grabel L. Hedgehog is required for murine yolk sac angiogenesis. Development 2002;129:361–372. [PubMed: 11807029]

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435–439. [PubMed: 8602241]

Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 2002;16:2743–2748. [PubMed: 12414725]

Coalson JJ. Pathology of bronchopulmonary dysplasia. Semin Perinatol 2006;30:179–184. [PubMed: 16860157]

Colvin JS, Feldman B, Nadeau JH, Goldfarb M, Ornitz DM. Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene. Dev Dyn 1999;216:72–88. [PubMed: 10474167]

Colvin JS, White A, Pratt SJ, Ornitz DM. Lung hypoplasia and neonatal death in *Fgf9*-null mice identify this gene as an essential regulator of lung mesenchyme. Development 2001;128:2095–2106. [PubMed: 11493531]

Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition [Review]. Trends Pharmacol Sci 2001;22:201–207. [PubMed: 11282421]

Del Moral PM, Sala FG, Tefft D, Shi W, Keshet E, Bellusci S, Warburton D. VEGF-A signaling through Flk-1 is a critical facilitator of early embryonic lung epithelial to endothelial crosstalk and branching morphogenesis. Dev Biol 2006;290:177–188. [PubMed: 16375885]

deMello DE. Pulmonary pathology. Semin Neonatol 2004;9:311–329. [PubMed: 15251148]

deMello DE, Sawyer D, Galvin N, Reid LM. Early fetal development of lung vasculature. Am J Respir Cell Mol Biol 1997;16:568–581. [PubMed: 9160839]

Doles J, Cook C, Shi X, Valosky J, Lipinski R, Bushman W. Functional compensation in Hedgehog signaling during mouse prostate development. Dev Biol 2006;295:13–25. [PubMed: 16707121]

Dono R, Texido G, Dussel R, Ehmke H, Zeller R. Impaired cerebral cortex development and blood pressure regulation in Fgf-2-deficient Mice. EMBO J 1998;17:4213–4225. [PubMed: 9687490]

Dyer MA, Farrington SM, Mohn D, Munday JR, Baron MH. Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. Development 2001;128:1717–1730. [PubMed: 11311154]

Ema M, Takahashi S, Rossant J. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood 2006;107:111–117. [PubMed: 16166582]

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439–442. [PubMed: 8602242]

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–676. [PubMed: 12778165]

Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995;376:66–70. [PubMed: 7596436]

Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ, Kim YH, Schreck R, Wang X, Risau W, et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res 1999;59:99–106. [PubMed: 9892193]

Galambos C, Ng YS, Ali A, Noguchi A, Lovejoy S, D'Amore PA, DeMello DE. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. Am J Respir Cell Mol Biol 2002;27:194–203. [PubMed: 12151311]

Gebb SA, Shannon JM. Tissue interactions mediate early events in pulmonary vasculogenesis. Dev Dyn 2000;217:159–169. [PubMed: 10706140]

Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. VEGF is required for growth and survival in neonatal mice. Development 1999;126:1149–1159. [PubMed: 10021335]

Greenberg JM, Thompson FY, Brooks SK, Shannon JM, McCormick-Shannon K, Cameron JE, Mallory BP, Akeson AL. Mesenchymal expression of vascular endothelial growth factors D and A defines vascular patterning in developing lung. Dev Dyn 2002;224:144–153. [PubMed: 12112468]

Guo C, Yang W, Lobe CG. A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. Genesis 2002;32:8–18. [PubMed: 11835669]

Healy AM, Morgenthau L, Zhu X, Farber HW, Cardoso WV. VEGF is deposited in the subepithelial matrix at the leading edge of branching airways and stimulates neovascularization in the murine embryonic lung. Dev Dyn 2000;219:341–352. [PubMed: 11066091]

Javerzat S, Auguste P, Bikfalvi A. The role of fibroblast growth factors in vascular development. Trends Mol Med 2002;8:483–489. [PubMed: 12383771]

Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. J Biol Chem 2003;278:8244–8249. [PubMed: 12514186]

Kanda S, Miyata Y, Kanetake H. Fibroblast growth factor-2-mediated capillary morphogenesis of endothelial cells requires signals via Flt-1/vascular endothelial growth factor receptor-1: possible involvement of c-Akt. J Biol Chem 2004;279:4007–4016. [PubMed: 14610089]

Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y, Alitalo K. Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci USA 2002;99:8868–8873. [PubMed: 12070340]

Lavine KJ, White AC, Park C, Smith CS, Choi K, Long F, Hui CC, Ornitz DM. Fibroblast growth factor signals regulate a wave of Hedgehog activation that is essential for coronary vascular development. Genes Dev 2006;20:1651–1666. [PubMed: 16778080]

Long F, Zhang XM, Karp S, Yang Y, McMahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 2001;128:5099–5108. [PubMed: 11748145]

Magnusson P, Rolny C, Jakobsson L, Wikner C, Wu Y, Hicklin DJ, Claesson-Welsh L. Deregulation of Flk-1/vascular endothelial growth factor receptor-2 in fibroblast growth factor receptor-1-deficient vascular stem cell development. J Cell Sci 2004;117:1513–1523. [PubMed: 15020678]

Mailleux AA, Kelly R, Veltmaat JM, De Langhe SP, Zaffran S, Thiery JP, Bellusci S. Fgf10 expression identifies parabronchial smooth muscle cell progenitors and is required for their entry into the smooth muscle cell lineage. Development 2005;132:2157–2166. [PubMed: 15800000]

Miller LA, Wert SE, Clark JC, Xu Y, Perl AK, Whitsett JA. Role of Sonic hedgehog in patterning of tracheal-bronchial cartilage and the peripheral lung. Dev Dyn 2004;231:57–71. [PubMed: 15305287]

Miquerol L, Gertsenstein M, Harpal K, Rossant J, Nagy A. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. Dev Biol 1999;212:307–322. [PubMed: 10433823]

Motoike T, Markham DW, Rossant J, Sato TN. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. Genesis 2003;35:153–159. [PubMed: 12640619]

Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. Dev Dyn 2001;220:112–121. [PubMed: 11169844]

Parera MC, van Dooren M, van Kempen M, de Krijger R, Grosveld F, Tibboel D, Rottier R. Distal angiogenesis: a new concept for lung vascular morphogenesis. Am J Physiol Lung Cell Mol Physiol 2005;288:L141–L149. [PubMed: 15377499]

Pepicelli CV, Lewis PM, McMahon AP. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol 1998;8:1083–1086. [PubMed: 9768363]

Pepper MS, Mandriota SJ, Jeltsch M, Kumar V, Alitalo K. Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro

and alters endothelial cell extracellular proteolytic activity. J Cell Physiol 1998;177:439–452. [PubMed: 9808152]

Perl AK, Tichelaar JW, Whitsett JA. Conditional gene expression in the respiratory epithelium of the mouse. Transgenic Res 2002;11:21–29. [PubMed: 11874100]

Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med 2001;7:706–711. [PubMed: 11385508]

Poole TJ, Finkelstein EB, Cox CM. The role of FGF and VEGF in angioblast induction and migration during vascular development. Dev Dyn 2001;220:1–17. [PubMed: 11146503]

Schachtner SK, Wang Y, Baldwin HS. Qualitative and quantitative analysis of embryonic pulmonary vessel formation. Am J Respir Cell Mol Biol 2000;22:157–165. [PubMed: 10657936]

Schultz JE, Witt SA, Nieman ML, Reiser PJ, Engle SJ, Zhou M, Pawlowski SA, Lorenz JN, Kimball TR, Doetschman T. Fibroblast growth factor-2 mediates pressure-induced hypertrophic response. J Clin Invest 1999;104:709–719. [PubMed: 10491406]

Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol 1998;141:1659–1673. [PubMed: 9647657]

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376:62–66. [PubMed: 7596435]

Sorokin S. A study of development in organ cultures of mammalian lungs. Dev Biol 1961;3:60–83.

Stenmark KR, Gebb SA. Lung vascular development: breathing new life into an old problem. Am J Respir Cell Mol Biol 2003;28:133–137. [PubMed: 12540479]

Stenmark KR, Abman SH. Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. Annu Rev Physiol 2005;67:623–661. [PubMed: 15709973]

Tichelaar JW, Lu W, Whitsett JK. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. J Biol Chem 2000;275:11858–11864. [PubMed: 10766812]

Trokovic R, Trokovic N, Hernesniemi S, Pirvola U, Vogt Weisenhorn DM, Rossant J, McMahon AP, Wurst W, Partanen J. FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. EMBO J 2003;22:1811–1823. [PubMed: 12682014]

van Tuyl M, Liu J, Wang J, Kuliszewski M, Tibboel D, Post M. Role of oxygen and vascular development in epithelial branching morphogenesis of the developing mouse lung. Am J Physiol Lung Cell Mol Physiol 2005;288:L167–L178. [PubMed: 15377493]

van Tuyl M, Groenman F, Wang J, Kuliszewski M, Liu J, Tibboel D, Post M. Angiogenic factors stimulate tubular branching morphogenesis of sonic hedgehog-deficient lungs. Dev Biol 2007;303:514–526. [PubMed: 17187775]

Vokes SA, Yatskievych TA, Heimark RL, McMahon J, McMahon AP, Antin PB, Krieg PA. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. Development 2004;131:4371–4380. [PubMed: 15294868]

Weaver M, Dunn NR, Hogan BL. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. Development 2000;127:2695–2704. [PubMed: 10821767]

Weaver M, Batts L, Hogan BL. Tissue interactions pattern the mesenchyme of the embryonic mouse lung. Dev Biol 2003;258:169–184. [PubMed: 12781691]

White AC, Xu J, Yin Y, Smith C, Schmid G, Ornitz DM. FGF9 and SHH signaling coordinate lung growth and development through regulation of distinct mesenchymal domains. Development 2006;133:1507–1517. [PubMed: 16540513]

Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, Towler DA, Ornitz DM. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development 2003;130:3063–3074. [PubMed: 12756187]

Zhao L, Wang K, Ferrara N, Vu TH. Vascular endothelial growth factor co-ordinates proper development of lung epithelium and vasculature. Mech Dev 2005;122:877–886. [PubMed: 15927453]

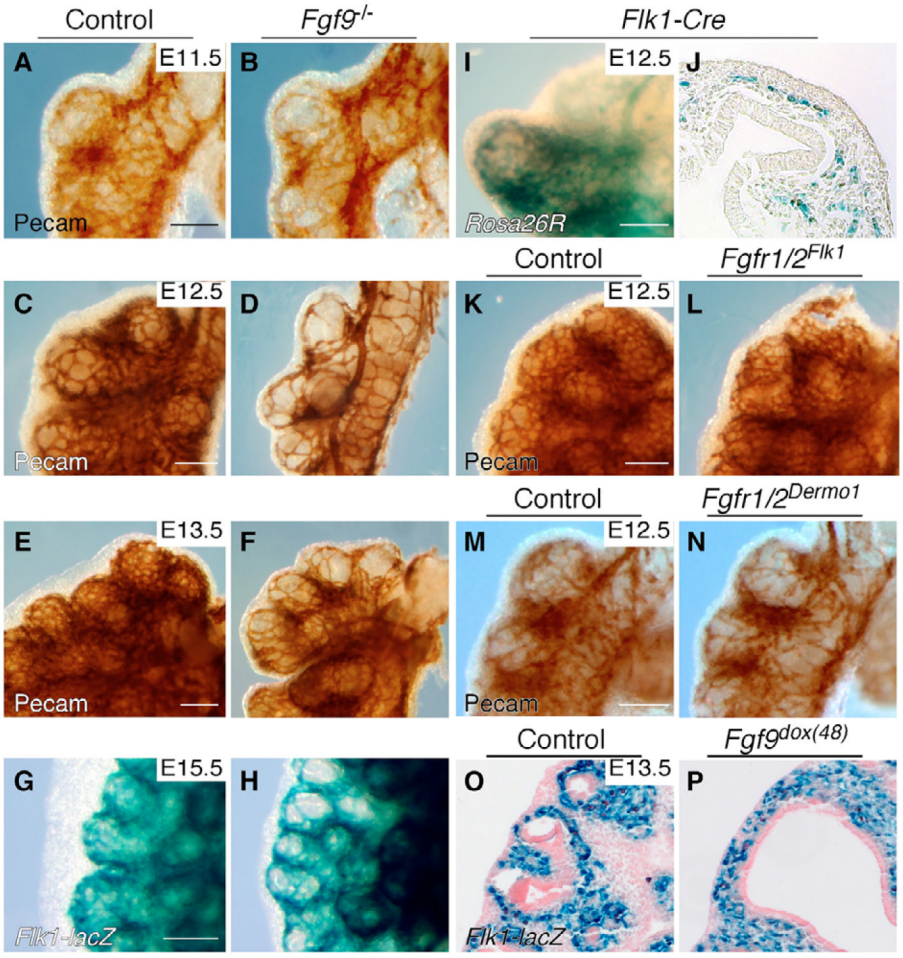

Fig. 1. FGF9 signaling via mesenchymal FGFR1/2 is necessary and sufficient for distal lung capillary development

(**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**) *Fgfr1* and *Fgfr2* double conditional knockout using *Flk1-Cre* (*Fgfr1/2*^{Flk}; L) showing no difference in distal lung vascular development compared to an *Fgfr1/2*$^{f/f}$ control (K). (**M**,**N**) *Fgfr1* and *Fgfr2* double conditional knockout using mesenchymal-specific *Dermo1-Cre* (*Fgfr1/2*^{Dermo1}), showing reduced distal lung capillary density (N) compared with an *Fgfr1/2*$^{f/f}$ control (M). (**O**,**P**) Induced *Fgf9* expression for 48 hours with doxycycline [*Fgf9*$^{dox(48)}$] 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.

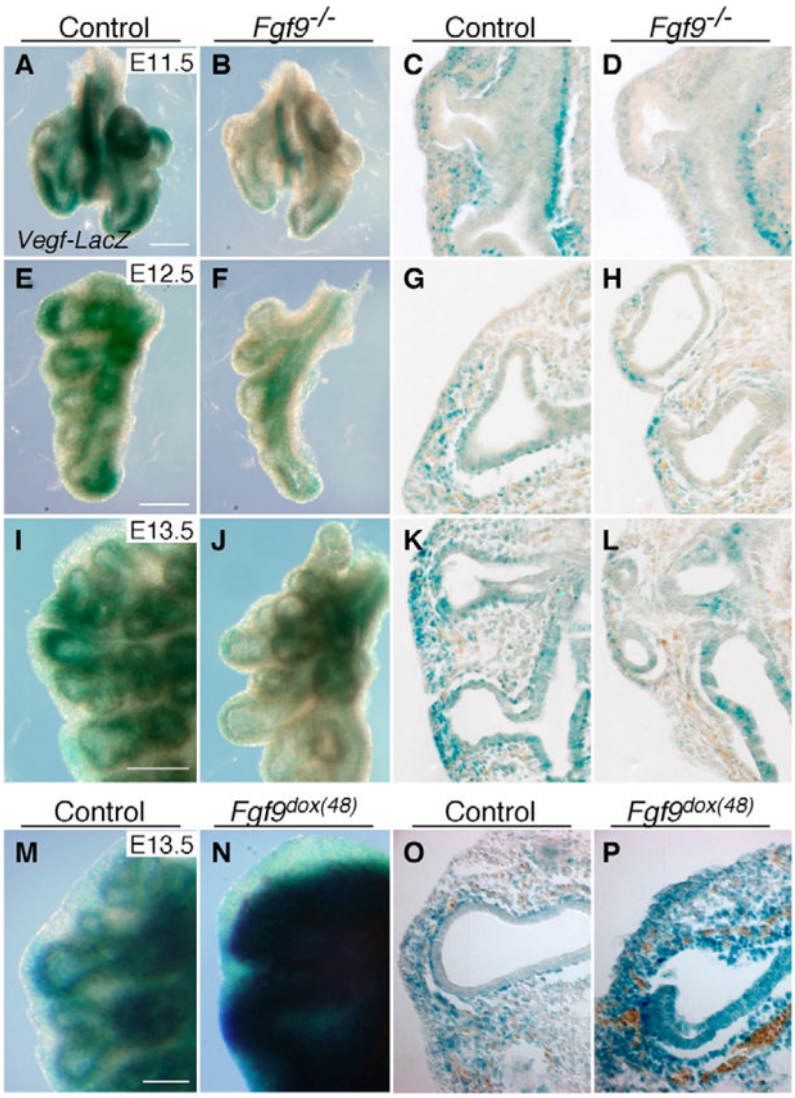

Fig. 2. *Fgf9* is necessary and sufficient for mesenchymal *Vegfa* expression in the lung

(**A**–**L**) *Fgf9*$^{-/-}$*; Vegfa-lacZ* lungs show reduced mesenchymal *Vegfa-lacZ* staining at E11.5 (B,D), E12.5 (F,H) and E13.5 (J,L) compared with control (A,C,E,G,I,K). At E11.5, no *Vegfa* is observed in the epithelium (C,D). By E13.5, levels of *Vegfa-lacZ* in the mesenchyme in *Fgf9*$^{-/-}$ lungs continue to be reduced compared with controls, but epithelial expression is comparable to controls (K,L). (**M**–**P**) *Fgf9* overexpression results in increased mesenchymal *Vegfa* in *Fgf9*$^{dox(48)}$*; Vegfa-lacZ* lungs at E13.5 (N,P). Left panels, whole-mount β-galactosidase staining; right panels, cryosections of left panels. Histology: 20× objective. Scale bars: 100 μm.

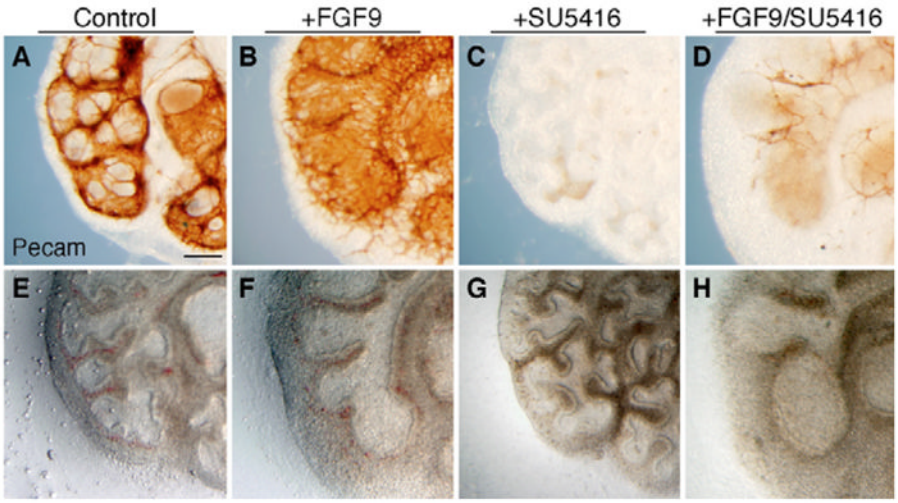

Fig. 3. FGF9 requires VEGF signaling for the formation and maintenance of the lung capillary network

(**A–D**) Incubation of E12.5 lung explants for 48 hours with 2.5 ng/μl FGF9 stimulates robust capillary formation (B) compared with untreated lung (A). The VEGFR inhibitor SU5416 (45 μM) eliminates the formation and survival of vessels (C). FGF9 is unable to stimulate growth of new distal vessels in the presence of SU5416 (D) but can partially rescue blood vessel survival in very proximal regions. (**E–H**) FGF9 stimulates luminal dilation of the epithelial tubules (F). Branching morphogenesis appears comparable to controls in SU5416-treated explants (G). In contrast to capillary development, FGF9-induced luminal dilation is not affected by SU5416 (H). Scale bar: 50 μm.

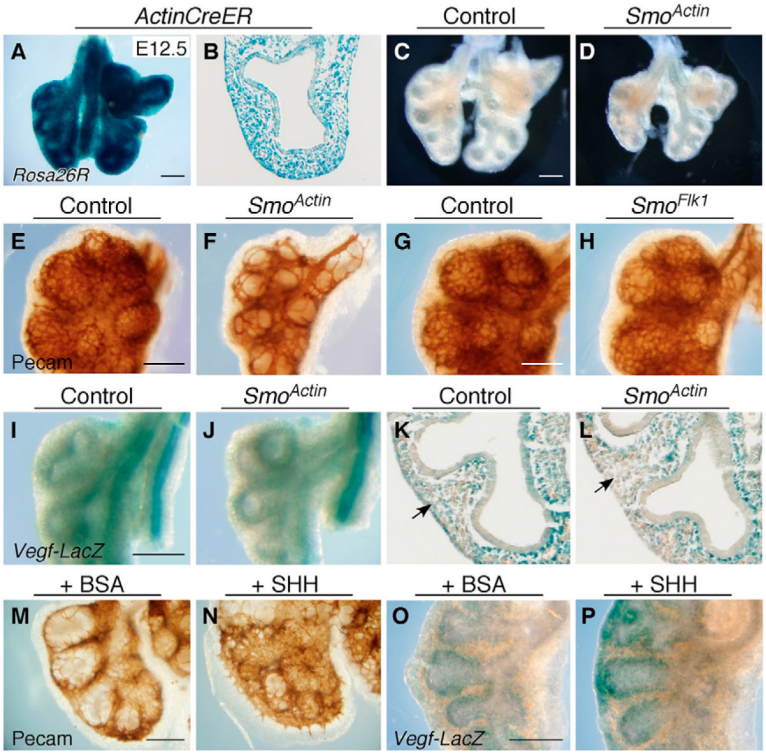

Fig. 4. SHH signaling to non-endothelial mesenchyme is necessary for distal lung capillary formation

(**A**,**B**) The *Rosa26R* allele was used to detect *Actin-CreER* activation throughout the lung mesenchyme and epithelium at E12.5 following tamoxifen injection at E9.5; (A) whole-mount and (B) frozen sections. (**C**,**D**) Conditional knockout of *Smo*, using *Actin-CreER* (Smo^{Actin}), results in lung hypoplasia (D) compared with a $Smo^{f/f}$ control (C) at E12.5. (**E**,**F**) When assessed by anti-PECAM whole-mount immunohistochemistry, Smo^{Actin} lungs showed a reduction in distal capillary network density (F) compared with $Smo^{f/f}$ controls (E) at E12.5. (**G**,**H**) Distal lung capillary density appeared similar in $Smo^{f/f}$; *Flk1-Cre* (Smo^{Flk1}; H) and $Smo^{f/f}$ control (G) mice, indicating that HH signaling to endothelial cells is not required for development. (**I–L**) Smo^{Actin} lungs (J,L) 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.

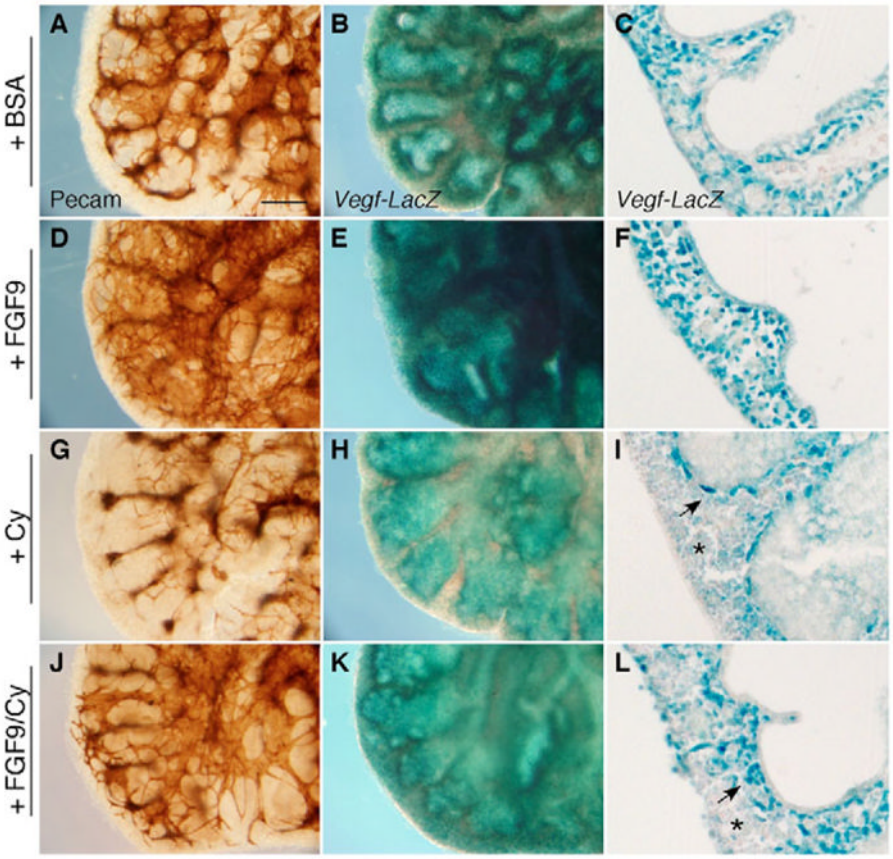

Fig. 5. FGF9 and SHH signaling are both required for capillary formation

E12.5 lung explants from *Vegfa-lacZ* mice were incubated for 48 hours with media containing BSA (**A–C**), FGF9 (**D–F**), cyclopamine (Cy; **G–I**) or FGF9 and cyclopamine (**J–L**). (A,D,G,J) Whole-mount anti-PECAM immunohistochemistry showing increased capillary formation in the presence of 2.5 ng/μl FGF9 (D) and decreased vascular development in the presence of 10 μM cyclopamine (G). FGF9 was able to only partially rescue capillary formation in the presence of cyclopamine (J). (B,E,H,K) Whole-mount preparations and (C,F,I,L) frozen sections stained for *lacZ* activity. Compared with control explants, *Vegfa* expression is increased in the presence of FGF9 (E,F) and decreased in the presence of cyclopamine (H,I). FGF9 was able to partially rescue *Vegfa* expression in the presence of cyclopamine (K,L). Notice that, in the presence of cyclopamine, *Vegfa* expression is retained only in the inner cell layer of the sub-epithelial mesenchyme (arrow in I), but is significantly reduced in the sub-mesothelial mesenchyme (asterisk in I). FGF9 increases *Vegfa* expression throughout both mesenchymal layers (F), but in the presence of cyclopamine, *Vegfa* expression is expanded only in the sub-epithelial layer (arrow in L) and remains low in the sub-mesothelial region (asterisk in L). (A–L) Lower left lobe. Histology: 20× objective. Scale bar: 100 μm.

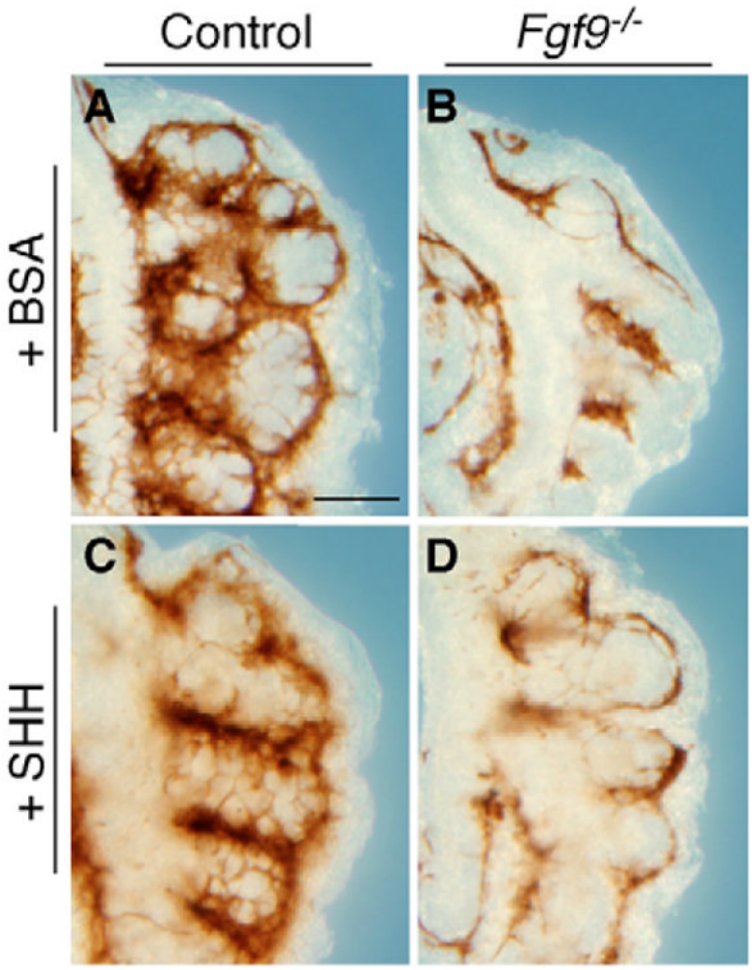

Fig. 6. SHH is not sufficient to rescue capillary plexus formation in *Fgf9*$^{-/-}$ lungs

E11.5 explant cultures of wild-type and *Fgf9*$^{-/-}$ lungs were incubated with BSA (**A**,**B**) or SHH (**C**,**D**) for 24 hours. *Fgf9*$^{-/-}$ lungs (B) formed a less dense capillary plexus compared with wild-type controls (A). In contrast to a wild-type lung (A,C), incubation of *Fgf9*$^{-/-}$ lungs with SHH did not increase capillary plexus density (D). Scale bar: 50 μm.

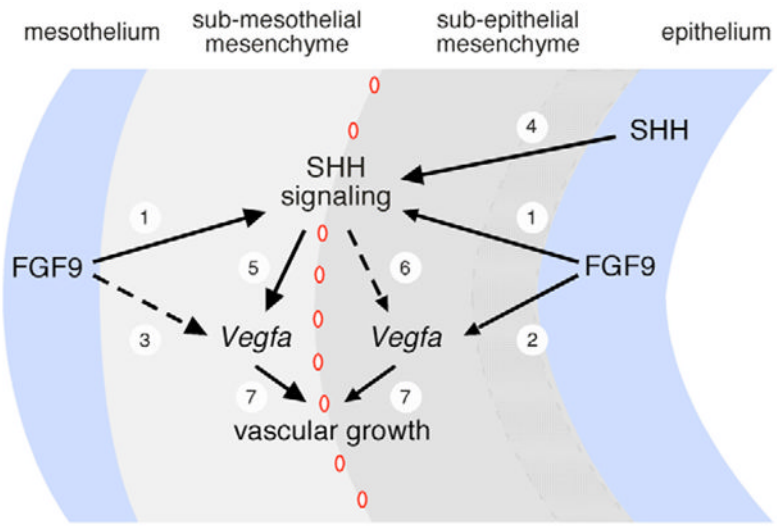

Fig. 7. Model for the regulation of *Vegfa* expression during distal lung vascular development by FGF9 and SHH signaling

(1–3) FGF9 signaling via FGFR1 and FGFR2 stimulates SHH signaling (White et al., 2006) and *Vegfa* expression both in sub-mesothelial and sub-epithelial mesenchymal layers. In the absence of SHH signaling, FGF9 has a stronger affect on *Vegfa* expression in the sub-epithelial compartment (2) compared with the sub-mesothelial compartment (3). (4) Epithelial SHH signals to lung mesenchyme (Bellusci et al., 1997a; Weaver et al., 2003). SHH signaling is required for *Vegfa* expression and subsequent vascular development both in sub-mesothelial mesenchyme (5) and sub-epithelial mesenchyme (6; except for the inner-most cell layer, stippled shading). HH signaling to *Flk1-Cre*$^{+}$ endothelial cells is not required for capillary development. (7) VEGF is a primary factor regulating vascular growth in the developing lung. Distal capillaries are denoted by red ovals located between sub-mesothelial and sub-epithelial mesenchyme layers.