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VEGF and endothelium-derived retinoic acid regulate lung vascular and alveolar development

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Yun EJ, Lorizio W, Seedorf G, Abman SH, Vu TH. VEGF and endothelium-derived retinoic acid regulate lung vascular and alveolar development. Am J Physiol Lung Cell Mol Physiol 310: L287–L298, 2016. First published November 13, 2015; doi:10.1152/ajplung.00229.2015.—Prevention or treatment of lung diseases caused by the failure to form, or destruction of, existing alveoli, as observed in infants with bronchopulmonary dysplasia and adults with emphysema, requires understanding of the molecular mechanisms of alveolar development. In addition to its critical role in gas exchange, the pulmonary circulation also contributes to alveolar morphogenesis and maintenance by the production of paracrine factors, termed “angiocrines,” that impact the development of surrounding tissue. To identify lung angiocrines that contribute to alveolar formation, we disrupted pulmonary vascular development by conditional inactivation of the Vegf-A gene during alveologenesis. This resulted in decreased pulmonary capillary and alveolar development and altered lung elastin and retinoic acid (RA) expression. We determined that RA is produced by pulmonary endothelial cells and regulates pulmonary angiogenesis and elastin synthesis by induction of VEGF-A and fibroblast growth factor (FGF)-18, respectively. Inhibition of RA synthesis in newborn mice decreased FGF-18 and elastin expression and impaired alveolarization. Treatment with RA and vitamin A partially reversed the impaired vascular and alveolar development induced by VEGF inhibition. Thus we identified RA as a lung angiocrine that regulates alveolarization through autocrine regulation of endothelial development and paracrine regulation of elastin synthesis via induction of FGF-18 in mesenchymal cells.

VEGF; lung development; alveolar development; alveolarization; FGF-18; retinoic acid; vascular development

Disruption of lung alveolar formation or lung maintenance may lead to pulmonary diseases such as bronchopulmonary dysplasia (BPD) in premature infants and emphysema in adults, respectively (7, 30, 46, 52). Lung histology in fatal BPD includes findings of fewer and larger alveoli with abnormal alveolar capillary structure due to arrested development, which contributes to poor gas exchange, respiratory distress, and pulmonary hypertension (4, 12). Loss of alveoli by progressive destruction is the main histological feature of adults with emphysema (52). Understanding basic mechanisms of normal alveolar formation and maintenance may further identify mechanisms contributing to the pathogenesis of BPD and emphysema and lead to novel strategies to prevent and treat these diseases.

Lung morphogenesis requires complex and finely orchestrated reciprocal interactions among many cell populations comprising the airways and the alveoli, including epithelial cells, vascular cells, smooth muscle cells, fibroblasts, and other mesenchymal cells (20). Mechanisms of alveolarization are not completely understood, although many critical cellular and molecular factors have been identified (30, 40, 42, 46). Of particular significance is the pulmonary vasculature, which develops in close spatial proximity to the conducting airways and the alveolar epithelium. The specific patterning of the pulmonary arteries, veins, and capillaries in relation to the airways and respiratory epithelium is essential for the gas-exchange function of the lung and suggests interdependent development of these two lung tissue components.

Development of the pulmonary vasculature is dependent on vascular endothelial growth factor A (VEGF-A), a potent angiogenic factor essential for embryonic vascular development. Targeted null mutation in the Vegf-A gene causes early embryonic lethality (9, 15, 58). Using conditional targeted inactivation of the Vegf-A gene selectively in lung epithelium, our laboratory has previously showed that pulmonary vascular development during fetal lung organogenesis depends on VEGF-A produced by the epithelium, and that inhibition of pulmonary capillary formation impairs primary septation and saccular development (56). VEGF-A is also essential for postnatal lung alveolar development and maintenance, as inhibition of VEGF-A signaling by either pharmacological or genetic approaches causes deleterious effects on alveolar formation and structures (17, 21, 24, 25, 35, 48, 51). However, molecular mechanisms through which impaired vascular development disrupts alveolar formation remain uncertain.

Retinoic acid (RA) is a morphogen necessary for normal lung development and can also induce lung regeneration following injury (29, 31). The mechanisms whereby RA regulates lung development and regeneration are not well understood, but may partly be through regulation of pulmonary vascular development (1, 11). RA may also modulate alveolarization by regulating elastin, a key molecule in normal alveologenesis (28, 32, 33, 37, 38, 54, 57). However, the relevant source of RA in the lungs is not clear, and whether RA induces elastin directly or through intermediate molecular targets remains unknown.

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Fibroblast growth factor (FGF)-18 belongs to the FGF family, which has more than 20 members with diverse roles throughout embryonic life, including early lung development (36, 45). FGF signaling is necessary for normal alveolar formation, as mice deficient in both FGF receptors-3 and -4 exhibit impaired alveologenesis (53). FGF-18 can bind and activate both of these receptors, and exhibit impaired alveologenesis (53). FGF signaling is necessary for normal alveolar development throughout embryonic life, including early lung development (14, 59). However, since Fgf18 null mice die at birth, the role of FGF-18 during alveolar development remains unknown. Past studies suggest that FGF-18 may regulate the development and function of the smooth muscle and alveolar myofibroblast lineages, as well as elastin expression, which are critical for normal septation in the lung (10, 34, 43, 55).

We hypothesized that RA may be a VEGF-induced endothelial-derived “angiocrine” that plays a critical regulatory role in distal lung growth during the alveolar stage of postnatal development. We further hypothesized that the effects of RA on septation may be mediated through induction of FGF-18 and enhanced elastin production. To test these hypotheses, we conditionally inactivated the Vegf-A gene selectively in lung epithelium during postnatal alveolar development. We show that epithelium-derived VEGF-A is essential for alveolar capillary formation, and that impaired lung capillary development disrupts alveolar morphogenesis. We further demonstrated that this defect is associated with impaired elastin deposition and identified RA as an endothelium-derived factor that regulates alveolar development by autocrine regulation of endothelial cell development and paracrine induction of mesenchymal FGF-18 expression and elastin expression.

**Materials and Methods**

All animal procedures and protocols were reviewed and approved by the Animal Care and Use Committees at the University of California San Francisco and the University of Colorado Denver Health Sciences Center.

**Generation of mice with conditional and targeted inactivation of the Vegf-A gene in lung epithelium**

Transgenic mice in which the Vegf-A gene is conditionally inactivated in lung epithelium was generated using a combination of Cre/loxP-mediated gene ablation and tetracycline (Tet)-On systems, as previously reported (56). Vegf-A/loxP mice were mated with Spc-rTA and (tetO)7-Cre mice to obtain SpC-rTA (+/−);Vegf-A/loxP (+/+ or +/−) and (tetO)7-Cre (+/−);Vegf-A/loxP (+/+) progeny, which were further intercrossed to generate offspring including the desired triple transgenic, Spc-rTA (+/−);(tetO)7-Cre (+/−);Vegf-AloxP (+/+) genotype. Treatment with doxycycline (Dox) induces Cre expression in lung epithelial cells and causes recombination of the floxed exon 3 of the Vegf-A gene and inactivation of the gene in the lung epithelium in the triple transgenic pups. Pups were treated with Dox (Sigma, St. Louis, MO) at 2 mg/ml in the drinking water, which was changed every 3 days, from postnatal day 1 (P1) until death. Littermates that are not triple transgenic were used as controls.

**Tissue collection and histology**

Littermate control and triple transgenic mice were killed following Dox treatment. Excised lungs were fixed overnight in 4% paraformaldehyde prepared in phosphate-buffered saline. Samples were processed, paraffin-embedded, and sectioned (6 μm) before staining, according to standard procedures: hematoxylin and eosin (H&E) for alveolar morphology, and modified Hart’s method to visualize elastic fibers. (We are grateful to Dr. Barry Starcher for providing elastin staining reagents.) For mean linear intercept (MLI) measurement, three H&E-stained sections from each genotype were selected for analysis. The MLI was calculated as the linear sum of the lengths of all lines randomly drawn across the images, divided by the number of intersections between alveolar walls and the lines. A minimum of 100 intercepts from 20 lines drawn across the lung in an unbiased fashion was obtained for each lung. Data are presented as means ± SD. Statistical analysis was done using the Student’s t-test.

**Immunohistochemistry**

Sections were deparaffinized and rehydrated and endogenous peroxidase quenched with 3% H2O2 in methanol for 15 min. After blocking with 5% normal serum, sections were incubated with primary antibodies [rat anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1) monoclonal antibody, Clone MEC 13.3, BD Pharmingen, San Diego, CA; 1:100] diluted in PBS overnight at 4°C. Sections were washed in PBS, incubated with biotinylated secondary antibody, washed in PBS, and then incubated with the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA), washed in PBS and developed with diaminobenzidine substrate, and lightly counterstained with methyl green.

**RNA isolation and quantitative real-time RT-PCR analysis**

Total RNA from postnatal lung tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, or Life Technologies, Rockville, MD), according to the manufacturer’s instructions, and treated with RNase-free DNase (DNA-free; Ambion, Austin, TX). One microgram of total RNA was reverse transcribed by using superscript II (Invitrogen), and the resulting cDNA was used in either PCR or real-time PCR reactions using gene-specific primers or primers/probe sets. Primers/probes were designed using Primer Express software (Applied Biosystems, Foster City, CA), based on sequencing data from National Center for Biotechnology Information databases. The 5’ and 3’ modifications of all the probes are FAM and BHQ, respectively. The mean number of cycles to threshold (Ct) of fluorescence detection was calculated for each sample, and the results were standardized to the mean Ct of murine glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for each sample. The relative level of mRNA abundance was determined by comparative Ct method, and results were expressed as percentage of the level of a control group. The sequences for the primers/probe sets used are shown in Table 1.

**Primary Lung Fibroblast Cultures**

Primary mouse lung fibroblasts were isolated from lungs of 1-wk-old mice, as previously described (8, 27). Briefly, lungs from 1-wk-old mice were dissected, rinsed in Ca2+ and Mg2+-free Hank’s balanced salt solution (HBSS), minced, and digested in HBSS containing 0.3 mg/ml type IV collagenase and 0.5 mg/ml trypsin at 37°C for 60 min. During digestion, the tissues were pipetted through a 25-ml pipette at 20-min intervals to aid cell dissociation. Following digestion, an equal volume of cold culture medium (DMEM/Ham’s F-12 50:50 mix containing 10% FBS, glutamine, and Pen/Strep) was added, the undigested materials were allowed to settle, the supernatant was removed, and the cells were pelleted by centrifugation, then resuspended in fresh culture medium, and plated. For RA treatment of lung fibroblasts, cells were seeded at 3.3 × 10^5 cells/cm² and treated with RA at 1 nM concentration for 48 h. The sequences for the primer pairs used in RT-PCR are shown in Table 2.

**Isolation of P6 Embryonic Lung Endothelial Cells**

Lungs from P6 Tie2-EGFP (enhanced green fluorescent protein) mice were dissected, rinsed in PBS, minced, and digested in 1 mg/ml collagenase-dispase (Roche Applied Science, Indianapolis, IN) in DMEM/Ham’s F-12 supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, 10 μg/ml amphotericin B, and 0.1 mM 2-mercaptoethanol.
PBS for 60 min at 37°C. The digestion was stopped by the addition of BSA to 0.5% final concentration. The cell mixture was filtered through a 40-µm cell strainer, centrifuged, washed with PBS, and resuspended to 10⁷ cells/ml with PBS containing 25 mM HEPES and subjected to FACS sorting for green fluorescent protein positive cells.

Immortomouse-Derived Lung Endothelial Cell Cultures

Lung endothelial cells derived from the immortomouse were a generous gift from Dr. Mette Johansen (23). The immortomouse carries a transgene expressing an interferon-inducible temperature labile SV40 Large T antigen under control of an myosin heavy chain class I promoter. At the permissive temperature (32°C) the SV40 labile Large T antigen is stable but it is rapidly degraded at the nonpermissive temperature (37°C). This mouse line allows immortalization of cells cultured at the permissive temperature. Cells were subcultured routinely in DME/F12 with 20% FCS, 50 μg/ml heparin (Sigma, St. Louis, MO), 20 U/ml of IFN-γ (R&D Systems, Minneapolis, MN), and Pen/Strep at the permissive temperature (32°C). For VEGF treatment, the cells were cultured in media without IFN-γ at the nonpermissive temperature (37°C).

Disulfiram Treatment

To determine the effects of RA on early postnatal lung development, we treated newborn CD1 mouse pups with disulfiram during P2–P14. Disulfiram (Sigma, St. Louis, MO) was dissolved in DMSO and diluted in corn oil and administered by daily intraperitoneal (IP) injections at a dose of 2.5 mg·kg⁻¹·day⁻¹. Control animals received IP injections of corn oil only.

Fetal Pulmonary Artery Endothelial Cell Isolation, Cultures, and Treatment

To further examine VEGF-RA interactions during lung development, pulmonary artery endothelial cells (PAECs) were harvested from the proximal pulmonary arteries of late gestation fetal sheep at day 140 (day 147 term), as previously described (18). Immunohistochemistry with standard endothelial markers confirmed the cell phenotype. PAECs (passage 4–5) were then exposed to all-trans-retinoic acid (ATRA), VEGF, or 1,3-dihydro-[3-(3,5-dimethyl-1H-pyrrol-2-yl)methylene]-2H-indol-2-one (SU5416), as described in the experiments below.

Fetal PAECs were plated in triplicate at 50,000 cells/well in DMEM with 10% FBS into 24-well plates and allowed to adhere overnight. The following day (day 0), the cells were washed twice with PBS. DMEM with 5% FBS with ATRA (50 nM, Sigma, St. Louis, MO) or SU5416 (5 μM Sigma, St. Louis, MO) was then added, and cells were incubated in 1% oxygen. Experimental media was changed daily, and cells were cultured at 37°C for 96 h followed by 48 h under in room air. Branch-point counting was performed in blinded fashion under ×10 magnification from each of three wells, with three to four fields of view per well, as previously described (18). Wells were imaged using an Olympus IX71 fluorescence microscope (Olympus).

Table 1. Sequences for primers/probes used in quantitative real time RT-PCR

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tr>
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<td>Fgf18</td>
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<td>Raldh-1</td>
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<tr>
<td>Raldh-3</td>
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Table 2. Sequences for primers used in RT-PCR

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<tr>
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<td>CCC AGA GTC TGG TCC ATG AA</td>
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<tr>
<td>β-actin</td>
<td>TAC CAG AGG CAT TGT GAT GGA</td>
<td>CAA GAT CAG ACT TCA TGA TGG</td>
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pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Study design. Three-day-old rats received a single dose of SU5416 (20 mg/kg) or its vehicle (carboxymethylcellulose) by subcutaneous injection. The selection of SU5416 and its dose was based on the results of our laboratory’s previous studies, in which a single dose of SU5416 (20 mg/kg) in newborn rats caused pulmonary hypertension and impaired lung growth throughout infancy (25, 49). Following the single dose of SU5416, rats were randomized to the following treatment groups, untreated control, SU5416, SU5416 + ATRA (0.005 mM), or SU5416 + ATRA + retinyl palmitate (RP; 0.05 mM). ATRA or ATRA + RP was delivered orally from days 3–14 of life in an oil suspension. The combination of ATRA and RP (10:1) has previously been shown to be necessary for increasing the storage and delivery of vitamin A, as previously described (22, 41).

Animals were killed by IP pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated with 4% paraformaldehyde and maintained at 20-cmH2O pressure for 60 min. A ligature was tightened around the trachea to maintain pressure, and the tracheal cannula was removed. Lungs were immersed in 4% paraformaldehyde at room temperature overnight for fixation. A 2-mm-thick transverse section was taken from the midplane of the left lobe of the fixed lungs per animal. Two sections from each animal were processed and embedded in paraffin wax for study.

Immunohistochemistry. Slides with 5-mm paraffin sections were stained with H&E for assessing alveolar structures and with von Willebrand factor-stained vessels with external diameter <100 μm per high-power field. The fields containing large airways or vessels were avoided. At least five pulmonary vessels were measured.

Radial alveolar counts. Alveolarization was assessed by the radial alveolar count (RAC) method of Emery and Mithal, as previously described and applied (50). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective tissues or septum or pleura, and the number of septae intersected by this line was counted. In each animal, at least five measurements were obtained.

Pulmonary vessel density. Pulmonary vessel density was determined by counting von Willebrand factor-stained vessels with external diameter <100 μm per high-power field. The fields containing large airways or vessels were avoided. At least five pulmonary vessels were measured.

Right ventricular hypertrophy. Hearts were dissected and weighed at 14 days of age. The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed, and the ratio of RV to LV+S weights was determined.

Western Blot Analysis for Retinaldehyde Dehydrogenase

PAECs were grown on 150-mm cloning plates with DMEM and 10% FBS. When the cells reached 90% confluence, PAECs were treated with 2.5% FBS DMEM ± VEGF (50 ng/ml) for 24 h, and cell lysates were collected, as described previously (2). Protein content of samples was determined using the bicinchoninic acid protein assay and lysates were collected as described above. Total protein was normalized by bicinchoinic acid protein assay, and 8 ng/well were assayed in triplicate utilizing a RA ELISA kit (Mybiosource, San Diego, CA). Optical density measurements were collected using model 680XR microplate reader (Bio-Rad Laboratories, Hercules, CA) and expressed as nanograms per milliliter.

RESULTS

Timed Deletion of the Vegf-A Gene during Early Postnatal Life Impairs Alveolarization

A role for VEGF-A signaling in alveolar development is suggested by its increased expression in the postnatal period in the mouse (5, 13). Our own data are consistent with the published data, showing that Vegf-A mRNA increases ~10-fold from embryonic day 16 (E16) to E18, and peaks during alveolarization (data not shown). To study the role of VEGF-A during alveolar development, we utilized the conditional Cre/loxP system to delete the Vegf-A gene in respiratory epithelial cells, as previously reported (56). Our laboratory has previously shown that inactivation of the Vegf-A gene in this transgenic system during fetal lung development by treatment of pregnant dams with Dox from E6.5 to birth impairs saccular development and causes perinatal lethality (56). To inactivate Vegf-A expression during alveolar development, we treated litters of newborn pups with Dox from P1 up to P20.

Postnatal inactivation of the Vegf-A gene did not cause lethality, and the mice were viable, but alveolarization was markedly reduced (Fig. 1). Histological analyses of lungs of littermate non-triple transgenic mice (hereafter designated as VegfA+/−) and of mice with homozygous inactivated Vegf-A alleles in lung epithelium (hereafter designated as VegfSP.CΔ/Δ) at P20 showed that the VegfA+/− lungs contain numerous alveoli, whereas the VegfSP.CΔ/Δ lungs show enlarged distal air spaces not divided by secondary septae (Fig. 1, B and C). Quantification of air space size by MLI measurements confirmed a significant increase in air space size in the VegfSP.CΔ/Δ lungs (Fig. 1D). Not all of the triple transgenic lungs exhibit a phenotype. In general, only ~20% do so, and not all areas of the lungs are equally affected. We determined the efficiency of Vegf-A gene inactivation by quantification of Vegf-A mRNA levels in the lungs by real-time RT-PCR and found that the efficiency of Vegf-A gene inactivation was variable. In the most severe cases, Vegf-A mRNA level in VegfSP.CΔ/Δ lungs was as low as 5% of that in VegfA+/− lungs (Fig. 1A). In these studies, we focused our analyses on VegfSP.CΔ/Δ lungs with the more severe phenotype.

Disrupted Capillary Patterning at the Alveoli in VegfSP.CΔ/Δ Lungs

Deficient VEGF-A expression in the VegfSP.CΔ/Δ lungs would be expected to cause disruption of the vascular compartment. PECAM-1 immunostaining showed no apparent abnormalities in the larger pulmonary arteries and veins (data not shown). However, capillary formation at the alveoli in the affected areas of the VegfSP.CΔ/Δ lungs was severely disrupted. In contrast to control lungs that show an extensive network of endothelial cells within alveolar walls, the VegfSP.CΔ/Δ lungs

ELISA for RA Measurements

PAEC were treated with VEGF (50 ng/mL) or disulfiram (10 μM), and lysates were collected as described above. Total protein was normalized by bicinchoinic acid protein assay, and 8 ng/well were assayed in triplicate utilizing a RA ELISA kit (Mybiosource, San Diego, CA). Optical density measurements were collected using model 680XR microplate reader (Bio-Rad Laboratories, Hercules, CA) and expressed as nanograms per milliliter.
showed few endothelial cells in the walls of the enlarged distal air spaces (Fig. 1, E and F). In unaffected areas of the VegfSP-C–/H9004/H9004 lungs, the pattern of endothelial cells appears normal (data not shown). Quantitative real-time RT-PCR (qRT-PCR) confirmed decreased mRNA expression of the endothelial markers Pecam-1 and Flk-1 (Fig. 1G). These results demonstrate the dependence of alveolar capillary development on VEGF-A derived from the epithelium and the necessity of normal endothelial cell development for normal alveolar formation.

Disrupted Elastin Deposition in VegfSP-C–/H9004/H9004 Lungs

Elastin constitutes the most abundant extracellular matrix component in the lungs, forming elastic fibers that allow the tissue to stretch and recoil with respiration. Deposition of elastin by alveolar myofibroblasts is coupled to secondary septation and is critical for alveolar formation (7). We examined whether impaired alveolarization in the VegfSP-C–/H9004/H9004 lungs is associated with abnormality in elastin deposition. Staining for elastin in P21 lungs demonstrated that, in contrast to the normal pattern of elastin deposition in alveolar walls and at the tips of alveolar septae in control lungs, elastin staining is significantly decreased in VegfSP-C–/H9004/H9004 lungs. Elastin was reduced in the walls of the enlarged distal air spaces, and few secondary septae contained elastin at the tips (Fig. 2, A and B). Deposition of elastin around blood vessels and airways is present in both control and VegfSP-C–/H9004/H9004 lungs (data not shown). qRT-PCR confirmed decreased expression of tropoelastin mRNA in the VegfSP-C–/H9004/H9004 lungs (Fig. 2C). These data demonstrate that the alveolarization defect in VegfSP-C–/H9004/H9004 lungs is associated with impaired elastin deposition.

FGF-18 and RA-Synthesizing Enzyme Expression Is Reduced in VegfSP-C–/H9004/H9004 Lungs

Impaired elastin deposition in VegfSP-C–/H9004/H9004 lungs suggests that expression of factors that regulate elastogenesis might be
disrupted following Vegf-A gene inactivation. One candidate is FGF-18, which has been shown to regulate elastin expression in lung fibroblasts in vitro and in the lungs in vivo (10). We, therefore, determined whether FGF-18 expression is altered in the VegfSP-C-ΔΔ lungs. qRT-PCR showed that the level of Fgfl8 mRNA in the VegfSP-C-ΔΔ lungs is decreased to ~25% of that in control lungs (Fig. 3A), suggesting that the impaired elastin deposition in the VegfSP-C-ΔΔ lungs might be due to deficiency in FGF-18 expression. We next determined whether endothelial cells might be a source of FGF-18. Using flow cytometry, we isolated early postnatal endothelial cells from the lungs of Tie2-EGFP mice, which express EGFP in endothelial cells under the Tie2 promoter, and compared the expression of FGF-18 in EGFP-positive endothelial cells with that in the EGFP-negative nonendothelial cell fraction by qRT-PCR. We found higher expression of FGF-18 in EGFP-positive endothelial cells than in the EGFP-negative fraction (data not shown), indicating that endothelial cells are not a significant source of FGF-18 in the lungs, and thus impaired endothelial cell development caused by epithelial Vegf-A gene inactivation is unlikely to directly account for the observed decreased FGF-18 expression in the VegfSP-C-ΔΔ lungs.

Another factor that has been found to regulate elastin expression in the lungs is RA (28, 32, 33). RA is a potent regulator of alveolar development, maintenance, and regeneration (29, 31). We, therefore, asked whether RA synthesis is disrupted in the VegfSP-C-ΔΔ lungs. We examined the expression of enzymes regulating the last step of RA synthesis, the RALDH-1, RALDH-2, and RALDH-3. qRT-PCR showed that the expression of all three of these RA-synthesizing enzymes is significantly decreased in VegfSP-C-ΔΔ lungs compared with control lungs (Fig. 3B). We then asked whether lung endothelial cells express these enzymes and thus are a source of RA. We isolated early postnatal endothelial cells from the lungs of Tie2-EGFP mice by FACS and assayed for the expression of RA-synthesizing enzymes by RT-PCR. As shown in Fig. 3C, all three RALDH enzymes are expressed at higher levels in Tie2-EGFP-positive endothelial cells than in the EGFP-negative nonendothelial cell fraction. These results indicate that endothelial cells are a potentially significant source of RA in the developing lung.

VEGF-A Regulates the Expression of RALDHs and RA in Pulmonary Endothelial Cells

We next determined whether the expression of RA-synthesizing enzymes in endothelial cells is regulated by VEGF-A. We treated cultured lung endothelial cells isolated from the immortomouse with vehicle or VEGF-A at the nonpermissive temperature and found that VEGF-A induced the expression of Radlh-3 mRNA to approximately sixfold with vehicle-treated cells (Fig. 3D). Radlh-1 and Radlh-2 mRNA expression was not significantly induced by VEGF-A. To further test this hypothesis, we studied RALDH isoform protein expression in primary fetal sheep PAEC. As shown in Fig. 3E, VEGF-A treatment upregulated expression of RALDH proteins in fetal PAEC. Induction of these enzymes correlated with increased RA production as measured by ELISA (Fig. 3F). Our data suggest that deficiency in VEGF-A expression in the VegfSP-C-ΔΔ lungs can reduce RALDH and RA production through direct effects of VEGF-A on RALDH expression in lung endothelial cells, as well as through VEGF-A effects on endothelial cell number.

RA Regulates FGF-18 Expression and Endothelial Cell Proliferation

We next explored the mechanisms whereby reduced RA production might lead to impaired alveolar development. We first determined RA effects on endothelial cell proliferation. As shown in Fig. 4, A and B, treatment with RA increased fetal sheep PAEC proliferation and tube formation, indicating that RA can directly stimulate angiogenesis. We asked whether this effect might be partly due to upregulation of VEGF expression by RA. As shown in Fig. 4C, treatment of PAECs with RA increased VEGF protein content, suggesting that VEGF may mediate the effects of RA on angiogenesis. To test this, we treated PAECs with a combination of RA and SU5416, an inhibitor of VEGF receptor tyrosine kinase. Treatment of PAECs with SU5416 alone decreased PAEC proliferation and tube formation, suggesting basal production of VEGF by PAECs (Fig. 4, D and E). Treatment of fetal PAEC with both RA and SU5416 attenuated the increase in PAEC cell proliferation and tube formation induced by RA, but does not reduce cell numbers.
to values observed in SU5416-treated cultures, indicating that RA likely acts through an additional mechanism besides induction of VEGF (Fig. 4, D and E).

We next asked whether the decreased FGF-18 expression in the VegfSP-C^{-/-} lungs might be related to decreased RA. Isolated lung fibroblasts have been found to express FGF-18 in vitro (10), but mechanisms underlying FGF-18 expression are unknown. To determine whether RA might regulate the expression of FGF-18 in lung fibroblasts, we isolated and cultured primary lung fibroblasts from early postnatal mice and found that these cells express FGF-18 at baseline (Fig. 5A). Treatment with RA dramatically induced FGF-18 expression in neonatal mouse lung fibroblasts (Fig. 5B). These results suggest that the decreased FGF-18 expression found in VegfSP-C^{-/-} lungs might be due to deficient RA synthesis secondary to decreased expression of RA-synthesizing enzymes.
Inhibition of RA Synthesis Impairs Alveolarization and Decreases FGF-18 and Elastin Expression

Our data suggest that RA is produced by pulmonary endothelial cells in response to VEGF and acts to regulate endothelial cell proliferation and elastin expression in fibroblasts through the induction of FGF-18. A prediction of this model is that inhibition of RA synthesis would decrease lung FGF-18 and elastin expression. To test this prediction, we treated newborn mice with disulfiram, a known RALDH inhibitor, during the period of alveolar development and assayed lung FGF-18 and elastin expression. Disulfiram has previously been shown to block RA synthesis and impair limb growth in the developing chick, which was reversed with exogenous RA (47). As shown in Fig. 5, disulfiram treatment impairs alveolar development, causing enlarged distal air spaces (Fig. 5, C and D), consistent with a previous report (19). As predicted, qRT-PCR showed reduced FGF-18 and elastin mRNA levels in disulfiram-treated lungs (Fig. 5, E and F). Overall, these results demonstrate that RA synthesis is required for normal FGF-18 and elastin expression during alveolar development.

Vitamin A and RA Treatment Reverses the Effects of VEGF Inhibition on Alveolar Development. Another prediction of our model is that RA should reverse the effects of VEGF-A inhibition on alveolar development. We tested this prediction by administering RA or RA with RP (vitamin A) to rats that have impaired alveolar development caused by inhibition of VEGF-A signaling with SU5416, a VEGF-A receptor kinase inhibitor. As shown in Fig. 6, SU5416 treatment of newborn rat pups caused reduced lung vessel density, decreased alveolar complexity with reduced RACs, and RV hypertrophy (Fig. 6, A, B, and D–F). Treatment with RA alone did not improve lung structure in the SU5416-treated mice, but did preserve vessel density (Fig. 6, D and E). Treatment with both RA and vitamin A (RP) preserved vessel density, caused a modest increase in the RAC, and prevented RV hypertrophy (Fig. 6, C–F). These data indicate that the effects of VEGF-A inhibition can be partially reversed by vitamin A and RA supplementation, further supporting our concept that RA can act downstream of VEGF-A and mediate some of its effects on lung growth.

DISCUSSION

In this study, we identified several paracrine mediators associated with VEGF activity that might serve to coordinate the development of the different lung tissue components. We show that, during alveolarization, epithelium-derived VEGF-A is necessary for endothelial cell proliferation and tube formation, and that the pulmonary vasculature may regulate mesenchymal processes essential for secondary septation through the production of RA, which stimulates endothelial cell proliferation and tube formation and modulates elastin expression by regulation of FGF-18 expression in fibroblasts.

We found that inactivation of the Vegf-A gene in respiratory epithelial cells during the period of postnatal alveolar development results in defective endothelial cell development and enlarged distal air spaces. This finding is consistent with previous studies, which by using pharmacologic inhibitors of angiogenesis, inhibitors of VEGF-A signaling, or genetic ma-
Manipulation of VEGF-A expression, have shown that inhibition of endothelial development impairs alveolar formation and suggested that angiocrine factors derived from the developing endothelium may contribute to normal distal air space growth (16, 17, 21, 25, 35, 39, 48). Our study identified the epithelium as an essential source of VEGF-A and RA as a specific lung angiocrine that contributes to alveolar development.

Our studies show that RA may act by regulating two essential processes of alveolarization: lung vascular development and elastin expression. Similar to findings by Cho et al. (11), our data show that RA induces pulmonary endothelial cell proliferation. In addition, we show that RA also supports pulmonary endothelial cells tube formation, and that the effects of RA on lung endothelial cells are partly mediated by induction of VEGF. To our knowledge, this action of RA on lung endothelial cells has not been previously reported. RA has been reported to induce human umbilical vein endothelial cell tube formation through paracrine induction of VEGF in fibroblasts in cocultures (44). RA may also regulate alveolar development through regulation of elastin expression (28, 32, 33). RA induces elastin expression in cultured neonatal lung fibroblasts and fetal lung explants (28, 33). However, it is not known whether RA induces elastin directly or through intermediate molecular targets. FGF-18 has also been shown to regulate elastin expression in lung fibroblasts in vitro and in the lungs in vivo (10). Our studies show induction of FGF-18 expression by RA, thus linking the action of these two factors on elastin expression. This helps to elucidate the mechanisms of RA action in previous studies, whereby treatment of postnatal mice or of fetal sheep with impaired alveolar development secondary to induced congenital diaphragmatic hernia with RA or with its precursor vitamin A results in increased lung expression of FGF-18 and elastin and corrects the alveolarization defect (6, 10).

Based on our data, we propose a model for the role of the pulmonary vasculature in alveolar formation (Fig. 7). Epithelium-derived VEGF-A activates receptors on endothelial cells in the mesenchyme to stimulate capillary formation, which in turn produces RA that acts both as an autocrine factor regulating endothelial development (proliferation and tube formation) and as a paracrine factor to induce the expression of FGF-18 by lung fibroblasts. FGF-18 then acts either in an
autocrine or a paracrine fashion on other lung fibroblasts to modulate elastin deposition that is necessary for secondary septation. Our model does not rule out potential direct effects of VEGF-A on other cell types or other effects of the vasculature, such as the production of other paracrine factors by endothelial cells. Future studies will be needed to further define the complex molecular interactions among the different cell types involved in alveolar formation.

In summary, our study identified novel roles of the pulmonary vasculature in alveolarization, implicating the endothelial cell as a source of RA signaling in the developing lungs and identifying FGF-18 from lung fibroblasts as a downstream target of RA. These findings further our understanding of the regulation of lung vascular development and elastogenesis and of the intricate cellular cross-talk controlling alveolar generation. Our study underscores the need for further investigations of the roles of these signaling molecules in diseases of impaired alveolar development, such as BPD, to determine their potential as therapeutic targets.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


