VEGF and Endothelium-Derived Retinoic Acid Regulate Lung Vascular and Alveolar Development

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ABSTRACT

Prevention or treatment of lung diseases caused by the failure to form, or destruction of, existing alveoli, as observed in infants with bronchopulmonary dysplasia (BPD) 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 FGF18, respectively. Inhibition of RA synthesis in newborn mice decreased FGF18 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 FGF18 in mesenchymal cells.
INTRODUCTION

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 histologic 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, we
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 pharmacologic 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,
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.

Fibroblast growth factor-18 (FGF18) belongs to the FGF family, which has more than 20 members with diverse roles throughout embryonic life, including early lung development. FGF signaling is necessary for normal alveolar formation, as mice deficient in both FGF receptor (FGFR) -3 and FGFR -4 exhibit impaired alveologenesis (53). FGF18 can bind and activate both of these receptors and Fgf18 null mice die at birth with abnormal saccular development (14, 59). However, since Fgf18 null mice die at birth, the role of FGF18 during alveolar development remains unknown. Past studies suggest that FGF18 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 FGF18 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 FGF18 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-rtTA and (tetO)7-Cre mice to obtain SpC-rtTA (+/-);Vegf-A /loxP (+/-) or (+/-) and (tetO)7-Cre (+/-); Vegf-A /loxP (+/-) progenies, which were further intercrossed to generate offspring including the desired triple transgenic, SpC-rtTA (+/-);(tetO)7-Cre (+/-); Vegf-A /loxP (+/-) 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 three days, from P1 until sacrifice. Littermates that are not triple transgenic were used as controls.

Tissue Collection and Histology

Littermate control and triple transgenic mice were sacrificed 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 mean ± SD. Statistical analysis was done using the student’s t-test.

Immunohistochemistry

Sections were deparaffinized and rehydrated and endogenous peroxidase quenched with 3%
H$_2$O$_2$ in methanol for 15 min. After blocking with 5% normal serum, sections were incubated with primary antibodies (rat anti-mouse 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 a 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 DAB substrate and lightly counterstained with methyl green.

**RNA Isolation and Quantitative Real time RT-PCR (qRT-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 (C$_T$) of fluorescence detection was calculated for each sample and the results were standardized to the mean C$_T$ of murine glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for each sample. The relative
level of mRNA abundance was determined by comparative \( C_T \) 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-week-old mice as previously described (8, 27). Briefly, lungs from 1 week old mice were dissected, rinsed in \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) 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\(^\circ\)C for 60 minutes. During digestion the tissues were pipetted through a 25mL pipette at 20-minute 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, the cells were pelleted by centrifugation, then resuspended in fresh culture medium and plated. For RA treatment of lung fibroblasts, cells were seeded at 3.3x10\(^3\) cells/cm\(^2\) and treated with RA at 1nM concentration for 48 hours. The sequences for the primer pairs used in RT-PCR are shown in Table 2.

**Isolation of P6 Embryonic Lung Endothelial Cells**

Lungs from postnatal day 6 Tie2-EGFP mice were dissected, rinsed in PBS, minced, and
digested in 1 mg/ml collagenase/dispase (Roche Applied Science, Indianapolis, IN) in 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 GFP⁺ 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 MHC class I promoter. At the permissive temperature (32°C) the SV40 Large T antigen is stable but 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 endothelial cell mitogen (Biomedical Technologies, Stoughton, MA), 100 μg/ml heparin (Sigma, St. Louis, MO), 20 u/ml of IFNγ (R&D Systems, Minneapolis, MN), and Pen/Strep at the permissive temperature (32°C). For VEGF treatment, the cells were cultured in media without IFNγ at the non-permissive temperature (37°C).

**Disulfiram Treatment**
To determine the effects of RA on early postnatal lung development, we treated newborn CD1 mouse pups with disulfiram on postnatal days 2 through 14. 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/d. Control animals received ip injections of corn oil only.

Fetal PAECs isolation, cultures, and treatment

To further examine VEGF-RA interactions during lung development, 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 ATRA, VEGF or 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 2.5% FBS with ATRA (50nM, Sigma, St. Louis, MO) or SU5416 (5μM Sigma, St. Louis, MO) was then added, and cells were incubated in 21% oxygen. Experimental media was changed daily, and cells were counted on day 3 after removing cells with 0.25% trypsin and counted with a hemocytometer. Growth studies with treatment were performed in DMEM with 2.5% FBS, based on previous studies that
determined that this was the lowest serum concentration that supported fetal PAEC survival with some proliferation (18).

**PAEC tube formation assay**

To assay in vitro angiogenesis, we cross-linked rat-tail collagen using 0.2% flavin mononucleotide and a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Cells (50,000 cells/well) were added in serum-free DMEM media supplemented with ATRA and SU5416, and each condition was tested in triplicate. PAECs were then incubated for 18 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).

**Treatment of rat pups with SU5416, ATRA, and Vitamin A**

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver's altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 week before birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 hours. Rats were killed with an ip injection of 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 SU-5416 (20 mg/kg) or its vehicle (carboxymethylcellulose) by subcutaneous injection. The selection of SU-5416 and its dose were based on the results of our previous studies, in which a single dose of SU-5416 (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 (0.005mM) or SU5416+ATRA+retinyl palmitate (0.05mM). ATRA or ATRA+RP was delivered orally from day of life 3 to 14 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 with 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 hematoxylin and eosin for assessing alveolar structures and with von Willebrand Factor (vWF), an endothelial cell-specific marker, for assessing vascular density and vascular wall thickness.

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 (PVD) was determined by counting vWF-stained vessels with external diameter less than 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 RALDH

PAEC were grown on 150-mm cloning plates with DMEM and 10% FBS. When the cells
reached 90% confluence, PAEC were treated with 2.5% FBS DMEM +/-VEGF (50ng/ml) for 24 hours and cell lysates were collected, as described previously (2). Protein content of samples was determined using the BCA protein assay (catalog no. 23225; Pierce Biotechnology, Rockford, IL), using bovine serum albumin as the standard. A 25-μg protein sample was added to each lane and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins from the gel were then transferred to a PVDF membrane. RALDH 1, 2, 3, 4 (Abcam, San Francisco, CA) and β-actin (Sigma, St. Louis, MO) were detected as previously described (3, 26) using appropriate controls and molecular weight as identified by the manufacturer for the protein of interest. Densitometry was performed using Image Lab (version 4.0.1; Bio-Rad Laboratories, Hercules, CA). Changes in protein expression were analyzed after normalizing for β-actin expression.

**ELISA for Retinoic Acid Measurements**

PAEC were treated with VEGF (50ng/mL) or disulfiram (10uM) and lysates were collected as described above. Total protein was normalized by BCA protein assay and 8ng/well was assayed in triplicate utilizing a retinoic acid ELISA kit (Mybiosource, San Diego, CA). Optical density measurements were collected using model 680XR microplate reader (Bio-Rad Laboratories, Hercules, CA) and expressed as ng/ml.

**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 approximately 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). We have 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 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 Vegf+/+) and of mice with homozygous inactivated Vegf-A alleles in lung epithelium (hereafter designated as VegfSP-C-∆/∆) at P20 showed that the Vegf+/+ lungs contain numerous alveoli, whereas the VegfSP-C-∆/∆ lungs show enlarged distal airspaces not divided by secondary septae (Fig. 1B&C). Quantification of airspace size by mean linear intercept measurements confirmed a significant increase in airspace size in the VegfSP-C-∆/∆ lungs (Fig. 1D). Not all of the triple transgenic lungs exhibit
a phenotype. In general only approximately 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-C-∆/∆ lungs was as low as 5% of that in Vegf+/+ lungs (Fig. 1A). In these studies, we focused our analyses on VegfSP-C-∆/∆ lungs with the more severe phenotype.

**Disrupted capillary patterning at the alveoli in VegfSP-C-∆/∆ lungs**

Deficient VEGF-A expression in the VegfSP-C-∆/∆ lungs would be expected to cause disruption of the vascular compartment. Platelet-endothelial cell adhesion molecule-1 (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-C-∆/∆ lungs was severely disrupted. In contrast to control lungs that show an extensive network of endothelial cells within alveolar walls, the VegfSP-C-∆/∆ lungs showed few endothelial cells in the walls of the enlarged distal airspaces (Fig. 1E and F). In unaffected areas of the VegfSP-C-∆/∆ 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 Vegf$^{SP. C-\Delta/\Delta}$ 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 Vegf$^{SP. C-\Delta/\Delta}$ 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 Vegf$^{SP. C-\Delta/\Delta}$ lungs. Elastin was reduced in the walls of the enlarged distal airspaces and few secondary septae contained elastin at the tips (Fig. 2A and B). Deposition of elastin around blood vessels and airways is present in both control and Vegf$^{SP. C-\Delta/\Delta}$ lungs (data not shown). qRT-PCR confirmed decreased expression of troпоelastin mRNA in the Vegf$^{SP. C-\Delta/\Delta}$ lungs (Fig. 2C). These data demonstrate that the alveolarization defect in Vegf$^{SP. C-\Delta/\Delta}$ lungs is associated with impaired elastin deposition.

FGF18 and RA-synthesizing enzyme expression is reduced in Vegf$^{SP. C-\Delta/\Delta}$ lungs

Impaired elastin deposition in Vegf$^{SP. C-\Delta/\Delta}$ lungs suggests that expression of factors that regulate elastogenesis might be disrupted following Vegf-$A$ gene inactivation. One candidate is FGF18, which has been shown to regulate elastin expression in lung fibroblasts
in vitro and in the lungs in vivo (10). We therefore determined if FGF18 expression is altered in the \( \text{Vegf}^{\text{SP-C-} \Delta/\Delta} \) lungs. qRT-PCR showed that the level of \( Fgf18 \) mRNA in the \( \text{Vegf}^{\text{SP-C-} \Delta/\Delta} \) lungs is decreased to approximately 25% of that in control lungs (Fig. 3A), suggesting that the impaired elastin deposition in the \( \text{Vegf}^{\text{SP-C-} \Delta/\Delta} \) lungs might be due to deficiency in FGF18 expression. We next determined whether endothelial cells might be a source of FGF18. 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 FGF18 in EGFP-positive endothelial cells with that in the EGFP-negative non-endothelial cell fraction by qRT-PCR. We found higher expression of FGF18 in the non-endothelial fraction (data not shown), indicating that endothelial cells are not a significant source of FGF18 in the lungs, and thus impaired endothelial cell development caused by epithelial \( \text{Vegf-A} \) gene inactivation is unlikely to directly account for the observed decreased FGF18 expression in the \( \text{Vegf}^{\text{SP-C-} \Delta/\Delta} \) lungs.

Another factor that has been found to regulate elastin expression in the lungs is retinoic acid (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 \( \text{Vegf}^{\text{SP-C-} \Delta/\Delta} \) lungs. We examined the expression of enzymes regulating the last step of RA synthesis, the retinaldehyde dehydrogenase-1, -2, and -3 (Raldh-1, Raldh-2, and Raldh-3). qRT-PCR showed that the expression of all these three RA-synthesizing enzymes is significantly
decreased in Vegf<sup>SP-CΔ</sup> lungs compared to 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 qRT-PCR. As shown in Fig. 3C, all three Raldh enzymes are expressed at higher levels in Tie-2 EGFP-positive endothelial cells than in the EGFP-negative non-endothelial 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 if 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 non-permissive temperature and found that VEGF-A induced the expression of Raldh-3 mRNA to approximately 6-fold compared to vehicle-treated cells (Fig 3D). Raldh-1 and Raldh-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 up-regulated 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 Vegf<sup>SP-CΔ</sup> 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 FGF18 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. 4A&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 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. 4D&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. 4D&E).

We next asked whether the decreased FGF18 expression in the *Vegf*<sup>SP-CΔΔ</sup> lungs might be related to decreased RA. Isolated lung fibroblasts have been found to express FGF18 in
vitro (10), but mechanisms underlying FGF18 expression are unknown. To determine whether RA might regulate the expression of FGF18 in lung fibroblasts, we isolated and cultured primary lung fibroblasts from early postnatal mice and found that these cells express FGF18 at baseline (Fig. 5A). Treatment with RA dramatically induced FGF18 expression in neonatal mouse lung fibroblasts (Fig. 5B). These results suggest that the decreased FGF18 expression found in Vegf<sup>SP-C-ΔA</sup> lungs might be due to deficient RA synthesis secondary to decreased expression of RA-synthesizing enzymes.

**Inhibition of RA synthesis impairs alveolarization and decreases FGF18 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 FGF18. A prediction of this model is that inhibition of RA synthesis would decrease lung FGF18 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 FGF18 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 airspaces (Fig. 5C&D), consistent with a previous report (19). As predicted, qRT-PCR showed reduced FGF18 and elastin mRNA levels in disulfiram-treated
lungs (Fig. 5E&F). Overall, these results demonstrate that RA synthesis is required for normal FGF18 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 retinyl palmitate (RP, vitamin A) to rats that have impaired alveolar development caused by inhibition of VEGF-A signaling by treatment 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 radial alveolar counts (RAC) and right ventricular hypertrophy (RVH) (Fig. 6 A&B, D, E&F). Treatment with RA alone did not improve lung structure in the SU5416 treated mice but did preserve vessel density (Fig. 6 D&E). Treatment with both RA and vitamin A (RP) preserved vessel density, caused a modest increase in the RAC and prevented RVH (Fig. 6 C&D, E&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 development, 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 FGF18 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 airspaces. This finding is consistent with previous studies, which by using pharmacologic inhibitors of angiogenesis, inhibitors of VEGF-A signaling, or genetic 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 airspace 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 HUVEC tube formation through paracrine induction of VEGF in fibroblasts in co-cultures (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. FGF18 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 FGF18 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 FGF18 and elastin and correct 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 FGF18 by lung fibroblasts. FGF18 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 FGF18 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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25. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, and Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal


FIGURE LEGENDS

Figure 1. Conditional inactivation of the Vegf-A gene in respiratory epithelial cells during alveolarization causes impaired alveolar development. A: Expression of Vegf-A mRNA by qRT-PCR analysis showing decreased expression in VegfSP-C-∆/∆ lungs to approximately 10% of that in control lungs. B&C: H&E stained sections of control (B) and VegfSP-C-∆/∆ lungs (C) showing enlarged distal airspaces in the VegfSP-C-∆/∆ lungs. Arrows in B show examples of secondary septae in the control lungs that are absent in the VegfSP-C-∆/∆ lungs. D: Measurement of alveolar size by mean linear intercept showing increased airspace size in the VegfSP-C-∆/∆ lungs. The difference between control and VegfSP-C-∆/∆ lungs is statistically significant (P < 0.05). E&F: PECAM-1 immunostaining of tissue sections of control (E) and VegfSP-C-∆/∆ lungs (F) showing relatively few endothelial cells in the walls of the enlarged distal airspaces in the VegfSP-C-∆/∆ lungs. G: Expression of Flk-1 and Pecam-1 mRNAs by qRT-PCR analysis showing decreased expression of these endothelial cell markers in the VegfSP-C-∆/∆ lungs to approximately 20-30% of that in control lungs. qRT-PCR data are shown as mean and SEM of 3 samples. Bar: B&C, 200 µm; D&E, 50 µm.

Figure 2. Impaired alveolarization in the VegfSP-C-∆/∆ lungs is associated with decreased elastin deposition. A&B: Elastin staining of tissue sections of control (A) and VegfSP-C-∆/∆ lungs (B) from mice given Dox from P2 to P21 showing abundant elastin fibers in the alveolar walls (A, arrows) as well as dense elastin deposits at the tips of secondary septae in
the control lungs (A, arrowheads), whereas in the \( Vegf^{SP-C\Delta/\Delta} \) lungs the distal airspaces are enlarged with few elastin fibers in the walls (B, arrows) and few secondary septae with elastin deposits at the tip (B, arrowheads). C: Expression of \( Tropoelastin \) mRNA by qRT-PCR showing decreased expression in \( Vegf^{SP-C\Delta/\Delta} \) lungs to approximately 30% of that in control lungs. qRT-PCR data are shown as mean and SEM of 4 samples. Bar: A&B, 50 \( \mu m \).

**Figure 3.** Expression of FGF18 and RA-synthesizing enzymes in the lungs and in lung cell populations. A: Expression of \( Fgf18 \) mRNA by qRT-PCR showing decreased expression in \( Vegf^{SP-C\Delta/\Delta} \) lungs to approximately 25% of control lungs. Mean and SEM of 3 samples are shown. B: Expression of mRNAs of the three RA synthesizing enzymes, \( Raldh-1 \), -2, and -3 is decreased in \( Vegf^{SP-C\Delta/\Delta} \) lungs compared to control lungs as assayed by qRT-PCR. Mean and SEM of 4 samples are shown. C: EGFP-positive endothelial cells isolated from one-week old Tie2-EGFP mice express higher levels of \( Raldh-1 \), -2, and -3 mRNA than EGFP-negative non-endothelial cells. D: qRT-PCR showing highly induced expression of \( Raldh-3 \) and modestly induced expression of \( Raldh-1 \) and \( Raldh-2 \) in lung endothelial cells by VEGF-A treatment. Mean and SEM of 3 samples are shown. E: Western blots showing induction of Raldh-1, -2 and -4 proteins by VEGF-A in fetal sheep pulmonary endothelial cells. F: Induction of Vitamin A in fetal sheep pulmonary endothelial cells is inhibited by disulfiram (p<0.05 between NoTx and VEGF, p> 0.05 between NoTx and disulfiram).

**Figure 4.** Retinoic acid regulates endothelial cell proliferation and tube formation. A&B:
Treatment of fetal sheep pulmonary endothelial cells (PAEC) with all trans retinoic acid (ATRA) increases cell number (A) and tube formation indicated by increased number of branch points (B). Differences between CTL and ATRA are statistically significant (p<0.001). C: Treatment of PAEC with ATRA induces VEGF-A expression as assayed by Western blot (p<0.001). D&E: Treatment of PAEC with SU5416 decreases cell proliferation (D) and tube formation (E) both at baseline and with stimulation with ATRA. p<0.001 between CTL and ATRA, p<0.001 between CTL and SU5416, p < 0.001 between ATRA and ATRA + SU5416, p<0.001 between SU5416 and ATRA + SU5416.

**Figure 5.** Inhibition of RA synthesis causes impaired alveolar development and decreased FGF18 and elastin expression.  A: RT-PCR showing expression of Fgf18 mRNA in primary lung fibroblasts (fibro) from one-week old postnatal mice. Myofibroblasts (myo) (fibroblasts treated with TGF-β) also express Fgf18 mRNA, whereas lung epithelial cells (epi) do not. B: RA treatment induces the expression of Fgf18 mRNA over 8 fold in primary lung fibroblasts as assayed by qRT-PCR. Mean and SEM of 3 samples are shown. C&D: H&E stained sections of control (C) and disulfiram-treated lungs (D) showing enlarged distal airspaces in the disulfiram-treated lungs. E&F: qRT-PCR showing reduced expression of FGF18 and tropoelastin in disulfiram-treated lungs. Mean and SEM of 3 samples are shown. Bar in C&D, 100 μm.

**Figure 6.** Retinoic acid and retinyl palmitate (vitamin A) rescue the impaired alveolar
development caused by inhibition of VEGF. A-C: vWF immunostained sections of control (A), SU5416 treated (B) and SU5416+ATRA+RP treated (C) rat lungs. Bar, 100 μm. D: Radial alveolar count is reduced in rat lungs following treatment with SU5416, this effect is partially reversed by combined treatment with ATRA and retinyl palmitate (RP). E: Vascular development in the lungs as indicated by number of vessels per high power field is impaired in rat lungs following treatment with SU5416, this effect is partially reversed by ATRA and further reversed by combined treatment with ATRA + retinyl palmitate (RP). F: SU5416 (Sugen) treatment results in right ventricular hypertrophy, which is partially reversed by combined treatment with ATRA and RP. (A: p<0.05 between CTL and SU5416 p>0.05 between SU5416 and ATRA, p<0.05 between SU5416 and ATRA+RP; B: p<0.01 between CTL and SU5416 p>0.05 between SU5416 and ATRA, p<0.15 between SU5416 and ATRA+RP; C: p<0.05 between CTL and SU5416 p>0.05 between SU5416 and ATRA, p<0.05 between SU5416 and ATRA+RP).

**Figure 7.** Model of cell-cell interactions during alveolar secondary septation. At the site of secondary septation, respiratory epithelium produces VEGF-A that stimulates pulmonary capillary formation in the mesenchyme. Endothelial cells in turn produce RA that acts as in a paracrine manner to induce the expression of FGF18 by lung fibroblasts and in an autocrine manner to regulate proliferation and tube formation in endothelial cells. FGF18 then acts either in an autocrine or a paracrine manner on other lung fibroblasts to induce elastin
deposition.
Table 1. Sequences for primers/probes used in quantitative real time RT-PCR

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SEPTATION

Epithelial cells

Elastin

FGF18

Fibroblasts

HGF

VEGF

RA

Endothelial cells