Angiogenesis and morphogenesis of murine fetal distal lung in an allograft model

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Departments of Pediatrics and Cardiothoracic Surgery, Children's Hospital Los Angeles Research Institute, University of Southern California, Los Angeles, California 90027; Department of Pediatrics, University of Pennsylvania, Philadelphia 19104; Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; and Department of Pathology, Women's and Infant Hospital, Brown University School of Medicine, Providence, Rhode Island 02906

Schwarz, Margaret A., Fangrong Zhang, John E. Lane, Susan Schachtner, Yangsun Jin, Gail Deutsch, Vaughn Starnes, and Bruce R. Pitt. Angiogenesis and morphogenesis of murine fetal distal lung in an allograft model. Am J Physiol Lung Cell Mol Physiol 278: L1000–L1007, 2000.—Neovascularization is crucial to lung morphogenesis; however, factors determining vessel growth and formation are poorly understood. The goal of our study was to develop an allograft model that would include maturation of the distal lung, thereby ultimately allowing us to study alveolar development, including microvascular formation. We transplanted 14-day gestational age embryonic mouse lung primordia subcutaneously into the back of nude mice for 3.5–14 days. Lung morphogenesis and neovascularization were evaluated by light microscopy, in situ hybridization, and immunohistochemical techniques. Embryonic 14-day gestational age control lungs had immature structural features consistent with pseudoglandular stage of lung development. In contrast, 14 days after subcutaneous transplantation of a 14-day gestational age lung, the allograft underwent significant structural morphogenesis and neovascularization. This was demonstrated by continued neovascularization and cellular differentiation, resulting in mature alveoli similar to those noted in the 2-day postnatal neonatal lung. Confirmation of maturation of the allograft was provided by progressive type II epithelial cell differentiation as evidenced by enhanced local expression of mRNA for surfactant protein C and a threefold increase in vessel formation as determined by immunocytochemical detection of platelet endothelial cell adhesion molecule-1 expression. Using the tyrosine kinase Flk-1 receptor (flk-1) LacZ transgene embryos, we determined that the neovascularization within the allograft was from the committed embryonic lung endothelium. Therefore, we have developed a defined murine allograft model that can be used to study distal lung development, including neovascularization. The model may be useful when used in conjunction with an altered genetic background (knockout or knock in) of the allograft and has the further decided advantage of bypassing placental barriers for introduction of pharmacological agents or DNA directly into the lung itself.

Pulmonary microvasculature; lung development; embryonic vasculogenesis; surfactant protein C; platelet endothelial cell adhesion molecule-1

Almost all of our insight into the molecular mechanisms that affect lung organogenesis and patterning relate to events in the respiratory epithelium (4, 17). This in part reflects the availability of useful experimental model systems. Paradigms of epithelial-mesenchymal interaction (10) have led to staging of lung development primarily based on the histological appearance of proximal and distal airway epithelia (2). In this regard, embryonic period is apparent in which the lung develops from an outpouching of the foregut and critical interactions between epithelium and mesenchyme are needed for branching and cytodifferentiation. The developing lung then progresses through various fetal stages characterized by changes in airway epithelium, including pseudoglandular (airway tubes lined with high-columnar cells that become cuboidal in distal airway), canalicular (differentiation of airway epithelium leading to formation of air-blood barrier and surfactant synthesis system), and sacular (expansion of distal epithelium into prospective alveoli with type I and type II cells, thinning of mesenchymal septum, and an increase in the amount of extracellular matrix). A late-gestational and postnatal period of alveolarization then ensues with an increase in surface area, alveolar septation, and prominent interstitial and vascular remodeling. Our concepts of development of the pulmonary vasculature are considerably less clear, although it is generally accepted that changes in this portion of the lung are concurrent with events in the airway. In this regard, it is apparent that preacinar vessels (arteries and veins) accompany development of preacinar airways and intra-acinar vessels follow development of the alveoli (26). Formation of the pulmonary circulation is dependent on the invasion of lung parenchyma by extrapulmonary vessels via angiogenesis, and linkage of these vessels with distal capillary networks is formed via vasculogenesis of endothelial cell precursors present with the lung mesenchyme (9). Peripheral vasculogenesis and central angiogenesis occur concurrently, and communication of these vascular segments is established late in gestation (10).
nal interaction have been demonstrated for isolated fetal epithelial rudimentary cultures (30), and a role for soluble growth factors (11, 19), cell matrix interactions (integrins, proteoglycans), and extracellular matrix molecules (27) in branching morphogenesis has been facilitated by cultured embryonic lung explants (20). Morphometric assessment of airway branching that persists in short-term culture has revealed roles for growth factors, extracellular matrix molecules, hormones, and morphogens (22). A number of relatively lung epithelial cell-specific transcription factors have been shown to transactivate important respiratory epithelial cell-specific genes. Some of these factors (Nfk-1, HNF-4, HNF-3[b]) (4, 17, 34) and their targets (surfactant protein A, B, or C, Clara cell secretory protein) have been creatively manipulated in transgenic and/or null mutant mice to study their roles in airway epithelial development (5, 21, 39).

Neovascularization is dependent on endothelial cell differentiation, commitment, and migration that is dictated by a diverse group of molecules, including the extracellular matrix proteins, cell adhesion receptors, growth factors, and their receptors (1). During blood vessel development in the canalicular stage of murine lung epithelial development (16.5–17.4 days, also known as the vascular stage), the number of terminal sacs and vascularization increase, while type I and II epithelial cells differentiate (3, 23, 31). These forces result in vessel formation derived by either vasculogenesis or angiogenesis (8, 25). It is during the canalicular or vascular stage that the lung is canalized with the multiplication of capillaries. This is associated with the flattening out of the cuboidal, glycogen-rich peripheral epithelial precursor cells so that a thin air-blood barrier interface is formed (2). This stage of transition is pivotal in the normal progression of lung morphological and neovascular development.

From a recent report by Meneghetti et al. (22), it is apparent that the most commonly used experimental model, e.g., cultured embryonic lung, reproduces patterns of epithelial cell gene expression of normal lung and is particularly valuable for studies of early regulation of branching and modulation of epithelial gene expression. Nonetheless, among the limitations noted in this systematic study was the lack of angiogenesis, and the authors concluded that this model may not be suitable for studies aimed at later fetal events such as alveolarization. These latter limitations were only partly ameliorated by a heart-lung en bloc whole organ culture system (38). Attempts at creating an artificial environment for lung growth and maturation through the use of arteriovenous extracorporeal membrane oxygenation of a fetal goat were cumbersome while not defining microvascular development (36). Accordingly, there remains a critical need for a model that mimics in utero conditions of lung morphogenetic and vascular development while avoiding difficulties associated with the placental barrier. Therefore, we have generated a novel allograft (ex vivo) model of fetal lung neovascularization that allows us to evaluate lung vasculature without the presence of the placental barrier while maintaining blood flow within the lungs.

**EXPERIMENTAL PROCEDURES**

Ex vivo model of murine lung: transplantation of lungs into immunocompromised mice. Timed-pregnant Swiss-Webster mice, gestational age 12 days (based on the appearance of vaginal plug, day 0), were obtained, housed, and handled according to the animal care committee at Childrens Hospital Los Angeles Research Institute. In addition, embryonic lungs were transplanted from transgenic mice containing the reporter gene β-galactosidase in their endothelium via the endothelial cell-specific promoter flk-1 (a growth receptor of vascular endothelial growth factor). These mice were obtained from founders from Jackson Laboratories and were generated by Shalaby et al. (29). On gestational day 14, dams were killed and embryos were removed. After tracheal transection (6), the lungs and heart were removed en bloc and placed in PBS (4°C). The heart was then removed, and the lung was placed on an 0.80-µm Millipore filter disk and implanted into a dorsal skinfold chamber of a nude mouse using sterile technique (Fig. 1). The skin was closed with skin staples. A sibling (14 days gestational age) lung and a 2-day postnatal mouse lung were used for comparison of structural and biochemical changes that took place in the implanted lung.

Histological and immunohistochemistry analysis of the murine lung transplants. After 3.5, 7, 10.5, and 14 days, lung transplants were removed, separated from the carrier mouse skin, fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded (during all procedures, diethyl pyrocarbonate (DEPC)-treated water and precaution against RNases were taken). Fixed tissue was sectioned at 5-µm intervals, stained with hematoxylin and eosin, and examined at the light-microscopic level for structural indexes of lung maturation. For immunolocalization of platelet endothelial cell adhesion molecule 1 (PECAM-1) antigens (PharMingen; San Diego, CA) (32), we employed rat anti-murine PECAM-1 antibody (4 µg/ml). Tissues were deparaffinized and underwent peroxide quenching. With use of a histostain kit from Zymed (San

**Fig. 1.** Ex vivo model of fetal lung morphogenesis and neovascularization. Timed-pregnant Swiss-Webster mice (day 14 gestational dams) were killed according to institutional rules and embryos were removed. Lungs and heart were withdrawn as block, after which the heart was removed. Lung explants on top of a 0.80-µm Millipore filter disk were transplanted into a dorsal skinfold chamber of a nude mouse as denoted in caricature. Inset in right corner is 14-day gestational age lung on top of a Millipore filter disk.
Francisco, CA), after being blocked, the sections were exposed to rat anti-mouse PECAM-1 antibody overnight at 4°C. Sections were then incubated with secondary biotinylated antibody as per the manufacturer's protocol. A brief incubation with the streptavidin-horseradish peroxidase conjugate system (Zymed) was followed by development using the chromogen substrate 3-amino-9-ethylcarbazole. β-Galactosidase was revealed in the tyrosine kinase Flk-1 receptor (flk-1) LacZ-positive lungs using X-Gal staining and confirmed using a monoclonal β-galactosidase antibody (32.5 µg/ml; 5 Prime = 3 Prime; Boulder, CO). After fixation in 4% paraformaldehyde, lung allografts were exposed to an X-Gal solution (potassium ferricyanide, potassium ferrocyanide, MgCl₂, and X-Gal in a PBS solution) for 2 h at 37°C. Lungs were then paraffin embedded and sectioned. A separate group of lung allografts was fixed and directly paraffin embedded, and β-galactosidase was detected by immunohistochemistry.

With use of PECAM-1 for vessel identification, the lung transplant was examined microscopically. Vessel numbers within the transplant were determined by counting 10 high-power fields in every lung transplant. The mean vessel number for each lung transplant was compared with the transplants within the same group.

Construction of cDNA probes. Total RNA was extracted from 15-day gestational age mouse lung tissue by RNA STAT-60 (Tel-Test “B”; Friendswood, TX). RNA (3 µg) was incubated with oligo(dT) primer for 10 min at 70°C. First-strand cDNA synthesis was performed according to the manufacturer's instructions (GIBCO BRL; Grand Island, NY). After first-strand synthesis, cDNA was generated by PCR amplification with 10 pmol of specific primers for 30 cycles of amplification (94°C for 1 min, 62°C for 1 min, 72°C for 1 min). The primers for surfactant protein C (SP-C) (18) were sense, 5'-CAT ACT GAG ATG GTC CTT GAG-3'; and antisense, 5'-TCT GGA GCC ATC TTC ATG ATG-3'. The generated SP-C PCR product was subcloned into TA vector (Invitrogen; Carlsbad, CA) for the in vitro transcription of RNA.

Digoxigenin RNA probe labeling by in vitro transcription. DNA of the SP-C subclone, in good orientation for in vitro transcription of antisense RNA by T7 RNA polymerase, was linearized by Hind III digestion and used as a template for probe labeling. Antisense RNA probe labeling with digoxigenin (Dig)-UTP by in vitro transcription with T7 RNA polymerase was performed as per the manufacturer's instructions (Dig RNA labeling kit, Boehringer Mannheim; Indianapolis, IN).

RNA in situ hybridization using Dig-labeled cRNA probes. Murine embryo control lung day 14 gestational age and murine transplants (14 days gestational age + 3.5 days, 14

Fig. 2. Histological analysis shows structural progression and morphogenesis of transplanted embryonic lung. Lung transplants (hematoxylin and eosin staining) were analyzed for morphological and structural growth on 14 days gestational age + 3.5 days to 14 days gestational age + 14 days and compared with the control lungs at 14 days gestational age and 2-day-old mouse neonate. A: 14-day gestational age control lungs show well-defined features consistent with pseudoglandular stage. B: 7 days after transplantation, lungs progress into canalicular stage with respiratory duct formation (arrows). Further progression occurs after 14 days of transplantation with dilated bronchial epithelium (C, open arrow and top inset) and prospective alveolar formation (C, bottom inset). These structural and morphogenetic developments are consistent with late saccular stage, as seen in lungs of 2-day-old murine neonate (n = 5/group, 3 separate occasions; D). Bar: A–D, 500 µm; inset A–D, 250 µm.
days gestational age + 7 days, 14 days gestational age + 10.5 days, and 14 days gestational age + 14 days) were obtained for in situ hybridization. The Dig RNA antisense and sense (control) probes were made using the Dig RNA labeling kit (SP6-T7) from Boehringer Mannheim (Indianapolis, IN). RNA in situ hybridization was performed on 5-µm paraffin-embedded material sections according to nonradioactive in situ hybridization application manual (Boehringer Mannheim). With use of DEPC-treated equipment and solutions, paraffin-embedded specimens underwent sectioning, rehydration, and incubation in a prewarmed 5 µg/ml proteinase K solution. Slides were then immersed in 4% paraformaldehyde, treated with 0.25% acetic anhydride, and dehydrated. Sections were exposed to a hybridization solution containing 50% formamide, 10% dextran sulfate, 1 mg/ml tRNA, 1× Denhardt’s solution, 4× saline-sodium citrate (SSC), 50 mM Tris, and 5 mM EDTA that contained 150–300 ng/ml of Dig-labeled RNA probe at 50°C overnight. Slides were washed at 55°C in 2× SSC-50% formamide, 1× SSC, and 0.1 SSC for 30 min before being incubated with RNase A (20 µg/ml) for 30 min at 37°C. After a rinse with 2× SSC and Dig, nucleic acid detection was accomplished using the Genius 3 kit from Boehringer Mannheim. Briefly, slides were incubated in 0.1 M maleic acid-0.15 M NaCl, pH 7.5, for 5 min after which the slides were blocked in a 1% block reagent. After being blocked, slides were incubated with anti-Dig-alkaline phosphatase conjugate at 4°C overnight, rinsed, and incubated with a dilute nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution for 3 h at room temperature. Slides were then counterstained with a 0.02% fast green solution for 2 min, rinsed in water, air-dried, and mounted. Hybridization with sense probe or without probe was performed as negative control, and always showed no signals. All sections were examined and photographed under light microscopy.

Statistical analysis. Statistical analysis was performed using Student’s t-test on the program Statview. Differences were considered significant at \( P < 0.05 \).

RESULTS

Transplanted murine pseudoglandular lungs undergo growth, remodeling, and development. Murine embryonic lungs (14 days gestational age) were implanted subcutaneously into nude mice and evaluated for structural development by light microscopy and hematoxylin and eosin staining at 3.5, 7, 10.5, and 14 days after transplantation (n = 5 per group, 3 separate occasions). Findings were compared with a 14-day gestational age lung and a 2-day postnatal mouse neonate lung. In contrast to control 14-day gestational age lung in midpseudoglandular stage, seen in Fig. 2A, there were morphological features consistent with an apparent progression of the implanted lungs by 14-day gestational age + 7 days into the canalicular stage with respiratory duct formation (Fig. 2B, arrows). This was characterized by the extension and increase in the number of the epithelial tubules that represent the peribronchial and perivascular growth regions for the future gas-exchange parenchyma. As lung development continued with longer periods of transplantation (14-day gestational age + 14 days), we found time-dependent structural and morphological development. This was noted by a transition of the lung transplant into morphology consistent with the saccular stage where there is a formation of well-defined bronchi with ciliated epithelium (Fig. 2C, open arrow and higher magnification noted in the right top corner) and the distal spaces of lung parenchyma that contain attenuated epithelium consistent with alveoli (Fig. 2C, closed arrows and higher magnification noted in the right bottom corner). This is similar to those structures seen
in the lung of a 2-day postnatal mouse neonate (Fig. 2D, the high-power inset represents the alveolar sacs).

Morphological lung development was shown in this model through differentiation of type II alveolar cells consistent with that seen in 2-day postnatal mouse neonate. During the canalicular stage, the cuboidal cells differentiate into the epithelial cells of the future gas-exchanging region, including type I and type II epithelial cells (2). We analyzed the lung transplants for type II pneumocyte formation to indicate whether there was progression of the cellular differentiation seen within the allograft model. Our analysis of the lung transplant morphogenesis and alveolar formation through the use of in situ hybridization of SP-C showed that 14-day gestational age control lungs have limited distal epithelial expression of SP-C (Fig. 3A, arrows). After transplantation, 14-day gestational age + 7-day lung shows progressive respiratory duct formation, extension, and increase in number of the epithelial tubules, and the initiation of type II epithelial cell differentiation (Fig. 3B, arrow, high-power inset). Lung morphogenesis continued into the saccular stage with differentiation of type II cells seen within alveolar type structures of 14-day gestational age + 14-day transplants (Fig. 3C, arrow). Although there were less differentiated type II cells present in the lung implant, indicating less lung morphogenesis, these results are similar to that seen in the lungs of a 2-day postnatal mouse neonate (Fig. 3D).

Transplanted fetal lungs show neovascular development similar to that seen in fetal lung development. Formation of the thin air-blood barrier is a critical stage in the canalicular and saccular stages of lung development. This transition period allows interface
between the evolving alveolar cells and the neovascularization that is crucial to fetal survival. Assessment of vessel formation within the allograft model was performed using a PECAM-1 antibody. Similar to the progression of neovascularization within the embryonic lung, we found a time-dependent increase in vessel numbers. This was demonstrated by a statistically significant threefold increase in vessel counts per high-power field \((P < 0.008)\) (Fig. 4E) in the 14-day gestational age + 14-day transplanted lungs (Fig. 4C) compared with control 14-day gestational age lungs (Fig. 4A) \((10 \text{ high-power fields counted per lung transplant, } n = 5 \text{ per group, repeated on 3 separate occasions})\). Furthermore, 14-day gestational age + 7-day (Fig. 4B, arrows and high-power inset) and 14-day gestational age + 14-day (Fig. 4C, arrows and high-power inset) transplants show progressive vessel distribution throughout the lung in a manner consistent with alveolar type structures, indicating the formation of an air-blood barrier within the transplanted lung similar to that seen within a 2-day postnatal mouse neonate (Fig. 4D). This is consistent with the vascular expansion and distribution within lung development that allows the close approximation of vessels to epithelial cells, creating the alveolar air-blood barrier. In contrast, vessels within the control 14-day gestational age lungs follow the airways and the connective tissue septum between the dichotomously branching airways (Fig. 4A, arrow).

Examination of an allograft at 14 days after transplantation of a 14-day gestational age lung from a transgenic mouse containing flk-1 LacZ construct revealed expression of immunoreactive (Fig. 5B, high-power inset) \(\beta\)-galactosidase in the potential air-blood barrier compared with the 14-day gestational age pretransplanted lung where LacZ expression was limited to the connective tissue septum between the branching airways (Fig. 5A). Transgene expression was confirmed histochemically in Fig. 5B. There was no detectable LacZ expression in allografts from wild-type mice (data not shown). These data demonstrate that neovascularization within the transplant originated from the donor lung itself, rather than from vessel ingrowth from the host animal.

**DISCUSSION**

Vascular development within the lung is a highly regulated process involving carefully orchestrated formation of new vessels followed by cessation of further vascular proliferation. We know that formation of the pulmonary circulation appears to be dependent on the invasion of lung parenchyma by extrapulmonary vessels during angiogenesis. Linkage of these vessels with distal capillary networks is formed via vasculogenesis of endothelial cell precursors present within the lung mesenchyme (9). Furthermore, recent morphological studies by deMello et al. (10) demonstrate that peripheral vasculogenesis and central angiogenesis occur concurrently to form the lung vasculature. Communication between the two networks is rare early in lung development (13–14 days of gestation) but increases gradually with gestation until the full vascular circuit is established by day 17 in the mouse.

Pulmonary vascular formation is an active process involving not only proliferation and differentiation of vascular structures but also regression and stasis of these structures (28). It has been shown that flk-1 is one of the earliest markers for angioblast and is an indicator of vascularization with the developing embryo and lung (7, 29, 35). Our understanding of those processes that command vessel formation in the fetus is growing as indicated by recent studies implicating vascular endothelial growth factor (VEGF) (14, 15) and its receptors flk-1 and flt-1 (16, 35) as facilitators of vessel formation in the embryo. Simultaneous with epithelial differentiation is the vascularization of the

**Fig. 5.** Immuno (B, inset)- and histochemical (B) detection of LacZ 14 days after transplantation of 14-day gestational age lung from tyrosine kinase Flk-1 receptor (flk-1) LacZ transgenic mice compared with 14-day gestational age pretransplanted lungs (A). LacZ expression is detectable with either method in large vessels (B, arrow) and within future gas-exchanging regions of lung (B, high-power inset and asterisk) consistent with derivation of those vessels from committed differentiating embryonic endothelial cells. Bar: A and B, 500 \(\mu\)m; B, inset, 250 \(\mu\)m.
Evolving lung. Inhibition of vascular formation has led to gross abnormalities of lung morphogenesis in VEGF transgenic mice where there was an increase in peritubular vascularity with a decrease in acinar tubules and mesenchyme (37). In addition, transforming growth factor-β knockout mice exhibit vascular malformations (13). These studies suggest that inhibition of neovascularization in the developing lung might also influence lung morphology through an alteration in epithelial-mesenchymal interactions.

Despite marked advances in the understanding of lung morphogenesis, little is known regarding factors influencing embryonic lung neovascularization. This is secondary to the lack of reproducible models that exhibit continued pulmonary microvascular development and allow manipulation of this process without the influence of the placental barrier. Recent work by Deutsch et al. (12) showed that transplanted human fetal lung tissue in severe combined immune deficiency (SCID) mice continues to grow, differentiate, and revascularize, forming morphologically advanced lung structures for gestational age at transplantation. Adult human bronchial xenografts transplanted in SCID mice have been shown to revascularize within 1 wk of transplantation with microvasculature that retains a human phenotype (24). Despite the existence of these well-defined human models, murine tissue offers the advantages of lung growth that occurs in a compressed period of time. Furthermore, with use of genetically modified lungs from transgenic or null mutant mice as the source of the allograft may facilitate identification of critical molecular pathways in lung airway and vascular development. Therefore, the goal of the current study was to create a murine allograft lung model that would allow for the study of distal lung and microvascular development.

As a source of transplant material, we started with fetal murine lungs in the mid pseudoglandular stage (14-day gestational age). This particular stage of lung development coincides with a point in time when there is minimal vessel formation. These immature lungs were subcutaneously transplanted into immunocompromised nude mice. Inspection of the lung histology throughout the 2-wk posttransplantation period showed advanced structural development defined by the appearance of an extension and increase in numbers of the epithelial tubules at 14 days gestational age + 7 days (Fig. 2B). This was followed by the transition of the transplanted lung to morphology compatible with the saccular stage as indicated by the distal spaces of lung parenchyma containing attenuated epithelium consistent with alveoli and the formation of defined bronchi with dilitated epithelium in the 14-day gestational age + 14-day allograft (Fig. 2C). The level of allograft morphological evolution at 14 days gestational age + 14 days was consistent with that seen in 2-day-old postnatal mouse neonate shown in Fig. 2D whose lungs were in the late saccular stage of lung development.

Endogenous SP-C expression was used to assess distal lung morphogenesis in the allograft. Corresponding to the findings of Wert et al. (33), SP-C expression is initially detected in the distal epithelial elements of the bronchial tubules (Fig. 3A) before a marked increase in production at 15 days gestational age localized to the distal epithelial elements of the bronchial tubules. This is similar to the marked increase in SP-C expression noted in the 14-day gestational age + 7-day transplanted lung (Fig. 3B) where we see the initiation of type II epithelial cell differentiation in the extending respiratory tubules. Furthermore, at the time of birth, SP-C mRNA was restricted to the epithelial cells of the terminal alveolar sacculles and appeared to define a subset of epithelial cells that were type II alveolar cells (22, 33) noted in the 2-day postnatal mouse neonate (Fig. 3D). Accordingly, the similar pattern of distribution of SP-C in the 14-day gestational age + 14-day transplant indicated a continued cellular maturation and differentiation similar to that noted in native, nontransplanted murine lung development.

Having demonstrated that morphologically this murine model developed similarly to the fetal lung, we then explored whether there was continued neovascular development consistent with that seen in utero. We used PECAM-1, an antibody expressed on endothelial cells to identify the vasculature within the fetal lung allograft, because PECAM-1 is also expressed in the endothelium of the developing embryo (32). The localization of PECAM-1 to the endothelial cells of blood vessels also has been demonstrated in murine fetal heart-lung and bloc organ cultures (38). We noted progressive vessel distribution throughout the lung in a manner consistent with alveolar-type structures, indicating the formation of an air-blood barrier within the transplanted lung. The vascular expansion and distribution that allow the close approximation of vessels to epithelial cells, creating the alveolar air-blood barrier noted within the allograft at 14 days gestational age + 14 days, were similar to those seen within a 2-day postnatal mouse neonate (Fig. 4D). This is in sharp contrast to the vessels within the control 14-day gestational age lungs that are restricted to accompanying branching conducting airways and are localized to the poorly defined, disorganized connective tissue septum between the dichotomously branching airways. Vessel counts per high-power field revealed a threefold statistically significant increase (P < 0.008) in the number of vessels present from 14 days gestational age through 14 days gestational age + 14 days (Fig. 4E). Furthermore, utilizing the lungs of murine embryos that contain the transgene for flk-1 LacZ, we determined that the origin of the vasculature within the lung is from committed differentiating embryonic endothelial cells within the allograft and not from vessel ingrowth (Fig. 5, A and B).

Therefore, as demonstrated by morphological advancement and progressive neovascularization, we have generated a novel allograft model of fetal lung neovascularization that allows us to evaluate pulmonary vascular formation in the presence of air-blood interface but without the interference of the placental barrier.

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EX VIVO MODEL OF LUNG MORPHOGENESIS

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