TISSUE ENGINEERING
Volume 00, Number 00, 2007

DOI: 10.1089/ten.2007.0041

In Vivo Pulmonary Tissue Engineering: Contribution of Donor-Derived Endothelial Cells to Construct Vascularization*

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ABSTRACT

Intrapulmonary engraftment of engineered lung tissues could provide a potential therapeutic approach for the treatment of pediatric and adult pulmonary diseases. In working toward this goal, we report here on *in vivo* generation of vascularized pulmonary tissue constructs utilizing the subcutaneous Matrigel plug model. Mixed populations of murine fetal pulmonary cells (FPCs) containing epithelial, mesenchymal, and endothelial cells (ECs) were isolated from the lungs of embryonic day 17.5 fetuses. FPCs were admixed to Matrigel and injected subcutaneously into the anterior abdominal wall of adult C57/BL6 mice to facilitate *in vivo* pulmonary tissue construct formation. Vascularization was enhanced by placing fibroblast growth factor 2 (FGF2)–loaded polyvinyl sponges into the hydrogel. After 1 week, routine histology and immunohistochemical staining for donor-derived epithelial cells and ECs as well as analysis of patent vasculature in the constructs following tail vein injection of fluorescein isothiocyanate–conjugated dextran were performed. In the Matrigel-only controls, some level of host infiltrate, but no measurable vascularization, was detected. In the presence of FPCs, the constructs contained ductal epithelial structures and patent vasculature. In the absence of FPCs, exogenous FGF2 induced the formation of numerous patent blood vessels throughout the entire constructs; in combination with FPCs, it resulted in enhanced capillary density and abundant interfacing between developing epithelial and vascular structures. The significant findings of this study are that distal pulmonary epithelial differentiation (as assessed by the expression of prosurfactant protein C) can be maintained *in vivo* and that donor-derived ECs contribute to the formation of patent vessels that interface tightly with ductal epithelial structures.

INTRODUCTION

PULMONARY HYPOPLASIA IS FOUND in as many as 15–20% of all neonatal autopsies.[1] The pathology of pulmonary hypoplasia and resultant pediatric pulmonary conditions, such as bronchopulmonary dysplasia, are hallmarked by aberrant vascular and epithelial development.[2] In addition, adult pulmonary diseases, such as emphysema, are characterized by destruction of epithelial and vascular tissues, culminating in respiratory distress.[3] We hypothesize that underdeveloped/diseased lungs may be augmented by implantation of engineered pulmonary tissue constructs. To recapitulate the organotypic cell–cell interactions that give rise to the complex lung tissue architecture during embryonic development, we have focused on the use of embryonic day 17.5 murine fetal pulmonary cells (FPCs).[4,5] Our

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*Part of this work was presented at the 8th Annual Meeting of Tissue Engineering Society International, Shanghai, PR China, October 2005, and has been published in abstract form (see references 6 and 11). This paper constitutes part of the Ph.D. thesis by M.J.M.

goal is to facilitate appropriate interactions between developing epithelium and vasculature, as they occur *in vivo* during fetal alveolar development.[6]

We have recently demonstrated that under appropriate *in vitro* culture conditions epithelial cells present within the organotypic mixture of FPCs maintain expression of surfactant protein C (SpC), a marker of the distal lung epithelial lineage, and form three-dimensional (3D) branching structures reminiscent of sacculated terminal airways, when cultured in 3D extracellular matrix gels made of Matrigel[4] and collagen type I.[5] In addition, under these *in vitro* conditions, a microvascular-like tubular network is formed by the endothelial cells (ECs) in both Matrigel (unpublished observations) and collagen type I gels.[5] Endothelial tubular morphogenesis in collagen gels *in vitro* depended on the addition of exogenous fibroblast growth factors (FGFs), specifically of FGF2.[5] FGF2 is a heparin-binding growth factor that has both mitogenic and differentiative effects on lung epithelial cells,[7] as well as proangiogenic effects *in vitro*[8] and *in vivo*.[9] The Matrigel/sponge angiogenesis assay[10] is a convenient *in vivo* model for investigating the angiogenic effects of paracrine signals (originating from embedded cells and tissues) and exogenous growth factors stored in and released from a polyvinyl sponge also embedded in the plug. In extension of our previously reported preliminary work on incorporation of FPCs and exogenous FGF2 into the Matrigel plug system,[11] we now demonstrate that it is possible to engineer alveolar-like structures comprised of distal epithelium interfaced with patent blood vessels *in vivo*.

MATERIALS AND METHODS

Isolation of FPCs

Unless mentioned otherwise, all cell culture materials were purchased from Fisher Scientific (Rome, Georgia). All animal procedures were carried out in accordance with a protocol approved by the Institutional Animal Care and Usage Committee (IACUC #16150). Timed-pregnant Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Fetal lungs were harvested from pups at gestational day 17.5 as previously described.[4,5] Briefly, isolated lungs were rinsed in 1× phosphate-buffered saline (PBS) (Cellgro, Herndon, VA), minced, and digested with prewarmed 0.5% trypsin in 1× PBS for 20–25 min at 37°C. Following the trypsin digestion, the enzymatic activity was quenched by addition of two volume equivalents of Dulbecco's modified Eagle's medium (DMEM) (Cellgro) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), followed by extensive trituration using a Pasteur pipette. The resultant homogenates were filtered through a nylon mesh (70 μm; BD Falcon, San Jose, CA) and centrifuged at 800 rpm for 5 min. The cell pellet was resuspended in 900 μL of distilled water for 30–45 s to lyse red blood cells, followed by addition of 100 μL 10× PBS. The cells were then pelleted again, resuspended in a defined volume of DMEM containing 10% FBS, and counted in a hemocytometer; viability was assessed by trypan blue exclusion. For cell tracking experiments, freshly isolated FPCs were loaded with 25 μM CMTPX CellTracker dye (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol before admixing to liquid Matrigel (BD Biosciences, San Jose, CA), as described below.

Preparation of Matrigel plugs and surgical implantation

Matrigel™ plugs were prepared in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC #02662), as described previously by Akhtar *et al*.[10] In brief, syngeneic C57/BL6 mice (Jackson Labs, Bar Harbor, ME) were injected subcutaneously in the anterior abdominal wall with 500 μL of Matrigel (BD Biosciences) containing 5 million FPCs per milliliter at a volume ratio of one part cell suspension to nine parts Matrigel (MG + FPCs). Upon solidification of the Matrigel (~5 min), an FGF2-soaked polyvinyl sponge (polyvinyl sponges were a generous gift from Dr. Robert Auerbach, University of Wisconsin, Madison, WI) preloaded with 100 ng FGF2 (Sigma, St. Louis, MO) was introduced (MG + FPCs + FGF2) into the construct via a small skin incision over the injection site and a second incision into the solidified constructs. Matrigel without cells (MG) and with FGF2-loaded polyvinyl sponges only (MG + FGF2) were prepared as controls. Animals were humanely killed, and the constructs were harvested at 7 days.

Characterization of Matrigel plug vascularization

Perfused vasculature on the surface of the constructs was visualized by tail vein injection of fluorescein isothiocyanate (FITC)–conjugated dextran [FITC-dextran, 2,000,000 MW, 5% (w/v), in PBS; Sigma] immediately before killing, as previously described.[10] These high-molecular-weight "fixable" dextrans contain lysine residues that allow cross-linking and fixation by aldehydes. Five minutes after tail vein injection, the animals were humanely killed, and the implants were excised and fixed in 10% buffered formalin for 2 h at room temperature and then overnight at 4°C. The FITC-dextran–labeled vasculature was viewed by low-power fluorescence microscopy of entire constructs. In addition, paraffin sections of the FITC-dextran–perfused constructs were prepared for quantification of vascular density within the constructs via the persistence of the fixable dextran within the lumina of patent blood vessels. Upon deparaffinization and rehydration of the sections, patent vessels were readily visible under the fluorescent microscope. In double-staining experiments, using the CMTPX CellTracker dye, both engrafted donor FPCs and patent host vessels were readily

visible in the sections. The total area of FITC-dextran–positive pixels was quantified as described below (see "Statistical analysis" section) using NIH ImageJ software.

Histology and immunohistochemistry

Excised constructs were prepared for routine histology and immunohistochemistry in paraffin sections as previously described.[4] General construct morphology was assessed by hematoxylin and eosin staining. Expression of specific proteins was probed by indirect immunohistochemistry. Primary antibodies used included cytokeratin (polyclonal rabbit; DAKO, Carpinteria, CA) to identify epithelial cells, and prosurfactant protein C (proSpC) (polyclonal rabbit; Chemicon, Temecula, CA) to label alveolar type II lineage epithelial cells. For antigen detection, we used either immunoperoxidase staining (using the DAKO AEC+ HRP kit, red reaction product) according to manufacturer's protocol or fluorescent Alexa488–conjugated goat anti-rabbit secondary antibody (Invitrogen). Fluorescent secondary antibodies were used for analyzing only those constructs that had not been perfused with FITC-dextran. Negative controls were processed identically, except that the primary antibodies were omitted. An endothelial-specific lectin,[12,13] Alexa488-conjugated *Griffonia simplicifolia* isolectinB4 (isolectinB4; Invitrogen), was used for EC identification in select whole mount preparations. In our hands, paraffin processing disrupted the sugar-binding interactions of isolectinB4, necessitating a whole mount approach to utilize this marker for EC identification. Briefly, after fixation, constructs were dissected into pieces approximately 5 mm^3 in size, permeabilized with 0.25% Triton-X prepared in 1% bovine serum albumin containing 1× PBS for 2 h at room temperature, and incubated overnight with a 10 µg/mL solution of Alexa488–isolectinB4. The stained samples were washed in three to four changes of 0.1% Triton-X containing 1× PBS over 4–6 h, whole mounted on a microscope slide, and examined by laser scanning confocal microscopy, as previously described.[5]

Statistical analysis

Quantitative image analysis using NIH ImageJ software was employed to measure relative levels of vascularization by measuring the area of FITC-dextran–positive pixels. For quantifying vascularization, fields of paraffin sections (200× magnification) from FITC-dextran–perfused constructs were binarized and the percentage of the total pixel area contributed by FITC-dextran signal was calculated. These measurements were performed from a minimum of 10 sections of constructs harvested from a minimum of 12 animals for Matrigel + FPCs and Matrigel + FPCs + FGF2, and 6 animals for Matrigel + FGF2. The statistical significance in individual comparisons between the aforementioned conditions was determined by Student's *t*-test, with $p < 0.05$ being statistically significant.

RESULTS

Histology of in vivo*–engineered pulmonary tissue constructs*

Histological analysis of control MG constructs without FPCs or FGF2 revealed considerable host infiltrate with little or no internal vascularization (Fig. 1A, B). In the absence of FPCs (Matrigel + FGF2), incorporation of FGF2-loaded polyvinyl sponges led to abundant internal vascularization in addition to host infiltrates (Fig. 1C, D). Incorporation of

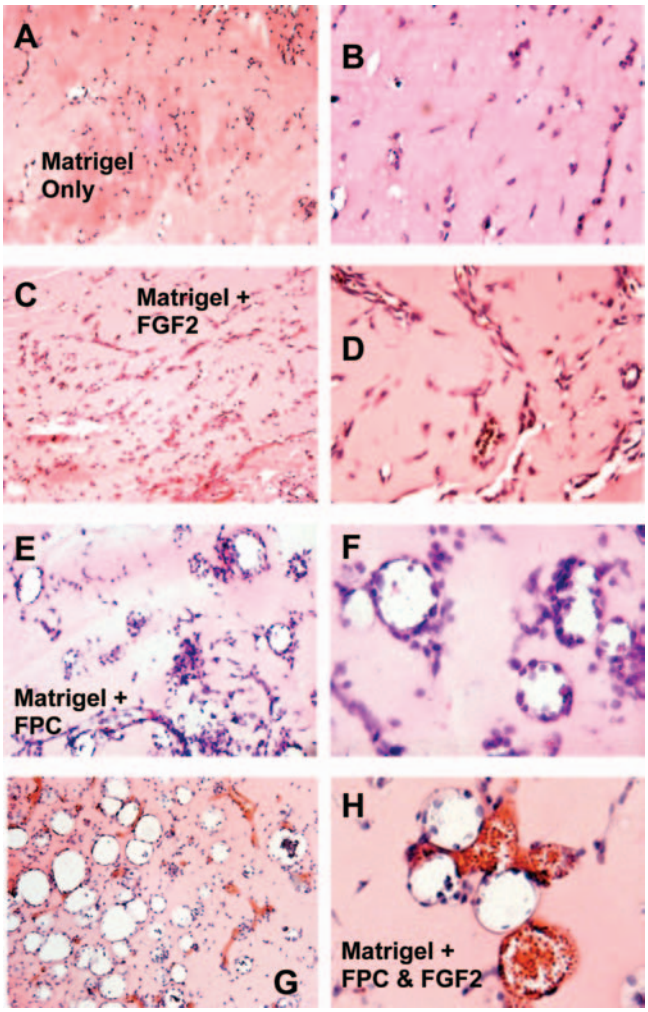

FIG. 1. Hematoxylin and eosin staining of Matrigel constructs following 7 days *in vivo*. (**A**) Matrigel only, magnification = 200×; (**B**) high-power view of Matrigel only, magnification = 400×; (**C**) Matrigel + FGF2–loaded polyvinyl sponge, magnification = 200×; (**D**) high-power view of Matrigel + FGF2–loaded sponge, magnification = 400×; (**E**) Matrigel + FPCs, magnification = 200×; (**F**) high-power view of Matrigel + FPCs, magnification = 400×; (**G**) Matrigel + FPCs + FGF2, magnification = 200×; (**H**) high-power view of Matrigel + FPCs + FGF2, magnification = 400×. Color images available online at www.liebertpub.com/ten.

FPCs alone in the absence of FGF2 (Matrigel + FPCs) resulted in the formation of FPC-derived ductal structures, as well as the appearance of some internal blood vessels (Fig. 1E, F). Constructs generated using FPC- and FGF2-loaded polyvinyl sponges (MG + FPCs + FGF2) demonstrated a significant increase in the number of patent blood vessels (Fig. 1G), which were often found juxtaposed with ductal epithelium (Fig. 1H).

Immunohistochemical analysis of engrafted FPCs

The epithelial nature of the cells lining ductal structures in hematoxylin and eosin–stained, FPC-containing constructs was confirmed by immunoperoxidase staining for the epithelial intermediate filament cytokeratin (Fig. 2A). The donor origin of the engrafted FPCs and their distal lung epithelial differentiation in the ductal structures was confirmed, respectively, by CellTracker labeling (orange) and fluorescent immunostaining for proSpC (green), the SpC gene product, which is expressed exclusively in cells of the type II alveolar epithelial lineage (Fig. 2B). As seen in Figure 2B, most of the cells lining the ductal epithelium stain positive for proSpC (green/yellow).

ECs present within the constructs were also identified by immunoperoxidase staining for von Willebrand factor, which highlights tubular endothelial structures amidst ductal epithelial structures (Fig. 2C). To assess the donor versus host origin of ECs, we stained whole mounts of constructs containing CellTracker-labeled FPCs with the endothelial marker isolectinB4 and evaluated the double-stained whole mounts by laser scanning confocal microscopy.[5] As seen in Figure 2D, some of the cells lining vessel-like structures in the constructs are host derived (isolectinB4-positive and CellTracker-negative, arrowhead). However, other endothelial structures contain cells apparently of donor origin (CellTracker-positive, Fig. 2D, arrows).

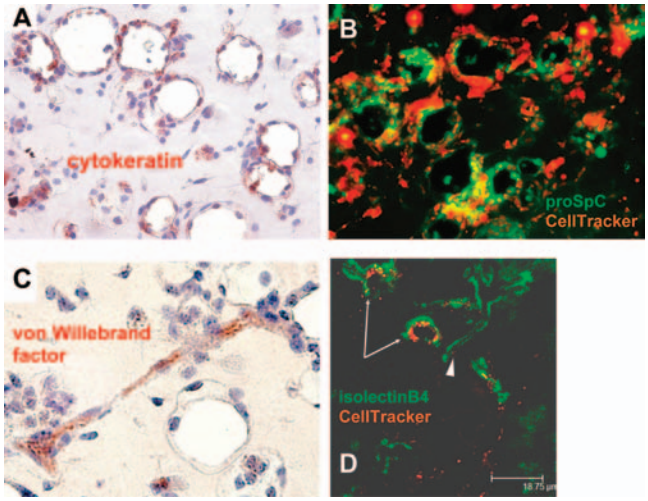

FIG. 2. Immunohistochemical staining of Matrigel + FPCs + FGF2 constructs generated for 7 days *in vivo*. (**A**) Immunoperoxidase staining for cytokeratin (AEC substrate chromogen reaction product appears in red) confirms the epithelial phenotype of the abundant ductal structures observed within Matrigel + FPCs + FGF2 constructs, magnification = 400×. (**B**) ProSpC immunostaining (green) confirms the distal lung differentiation status of the cells lining the ductal epithelium, the graft origin of which is highlighted by CMTPX labeling (orange) before admixing in Matrigel, magnification = 400× (see "Materials and Methods" section for details). (**C**) Immunoperoxidase staining for von Willebrand factor (vWF, AEC substrate chromogen reaction product appears in red) illustrates the presence of vWF-positive ECs within the constructs, original magnification = 400×; (**D**) Confocal optical section (250 nm thickness) of an isolectinB4 whole mount–stained MG + FPCs + FGF2 construct, identifying ECs (green), along with CMTPX labeling to identify ECs derived from engrafted FPCs (orange) reveals mixed graft (arrows) and host (arrowhead) origin of endothelial structures, scale bar = 18.75 μm. Color images available online at www.liebertpub.com/ten.

Analysis of patent construct vascularization

Fluorescent microscopy was used to assess the degree of vascularization in Matrigel plugs across experimental conditions. FITC-dextran tail vein injection allowed for visualization of patent, perfused vasculature both on the surface of freshly dissected constructs by gross fluorescent microscopy (not shown) and, subsequently, in transverse sections of paraffin-embedded samples (Fig. 3). Only sparse, small vessels were observed in the Matrigel-only controls (Fig. 3A). Matrigel + FGF2 constructs (Fig. 3B) and Matrigel + FPCs constructs (Fig. 3C) displayed similar levels of patent vessels, as seen qualitatively and confirmed by quantification of FITC-dextran pixel area (Fig. 3E). Matrigel + FPCs + FGF2 elicited an apparent additive effect, with significant increases in FITC-dextran pixel area (Fig. 3E), as well as a visually denser vascular network with more capillary-size vessels visible amidst larger diameter vessels (Fig. 3D).

Donor FPC–derived ECs contribute to patent vascularization

To evaluate whether donor-derived ECs present within the FPC mixture contribute to establishment of patent vasculature, we prepared constructs using FPCs prelabeled with CMTPX CellTracker dye to study the fate of donor-derived ECs. Figure 4A (transverse section of a Matrigel + FPCs + FGF2 construct after FITC-dextran perfusion) shows CMTPX-labeled graft-derived cells, some of which form small lumen-containing structures, reminiscent of blood vessels (arrows). Merging with the FITC-dextran exposure of the same field reveals that the cells in these tubular structures are indeed ECs of FITC-dextran–perfused blood vessels, indicating that donor-derived ECs are part of a patent vasculature in the constructs that assembles into and/or anastomoses with the host circulation (Fig. 4B, arrows).

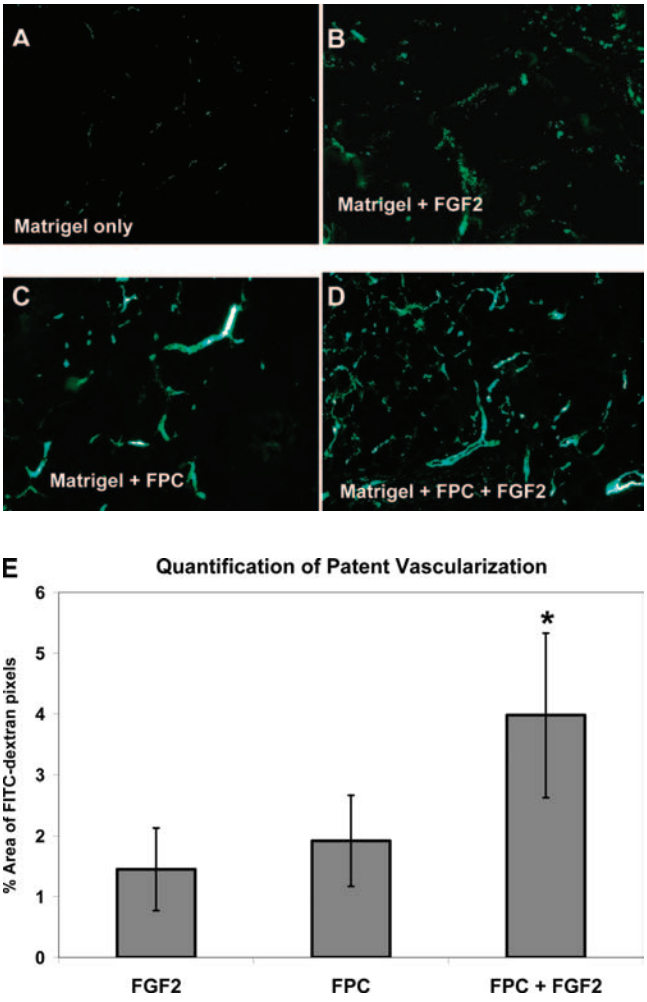

FIG. 3. Visualization and quantification of patent vasculature within Matrigel plugs. Images in panels (**A–D**) are paraffin sections of FITC-dextran–perfused constructs across conditions following 7 days *in vivo*. Perfused vessels appear green. (**A**) Matrigel-only control, magnification = 200×; (**B**) Matrigel + FGF2, magnification = 200×; (**C**) Matrigel + FPCs, magnification = 200×; (**D**) Matrigel + FPCs + FGF2, magnification = 200×. (**E**) Quantification of patent vascularization within constructs by measuring the total area of FITC-dextran pixels (expressed as percentage of total image area) in paraffin sections of tail vein–injected animals across conditions. Error bars indicate standard deviation. Minimum $n = 12$ animals for FPCs and FPCs + FGF2; $n = 6$ animals for FGF2. *$p < 0.05$, indicating a significant increase with FPCs + FGF2, relative to FPCs or FGF2 alone (see "Materials and Methods" section for details). Color images available online at www.liebertpub.com/ten.

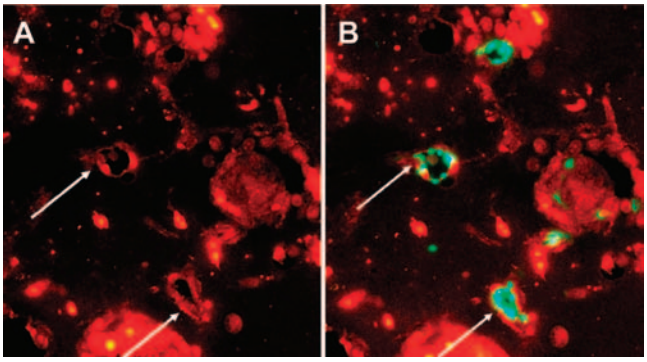

FIG. 4. E18 (gestational day 17.5) FPC-derived ECs contribute to the formation of FITC-dextran–perfused vessels within MG + FPCs + FGF2 construct generated over 7 days *in vivo*. CMPTX-labeled FPCs (orange) were used to ascertain ECs of graft origin. (**A**) CMPTX-labeled FPCs within the constructs form lumen structures (arrows) (cropped image, original magnification = 400×). (**B**) Merged image with FITC-dextran perfusion illustrating that the graft-derived lumen structures observed in panel (**A**) are indeed blood-perfused vessels (arrows) (cropped image, original magnification = 400×). Color images available online at www.liebertpub.com/ten.

DISCUSSION

Success in the budding field of distal lung tissue engineering will require the ability to generate a complex, 3D lung architecture and maintain lung epithelial differentiation in engineered systems. In addition to the importance of maintaining epithelial differentiation, *in vivo* vascularization of engineered pulmonary tissues upon implantation and connection to the host circulation will be a prerequisite for graft survival and integration. In contrast to prior *in vitro* work in which purified alveolar epithelial cells were used to generate 3D cultures,[14–16] we are using mixed populations of fetal lung cells containing mesenchymal cells and ECs in addition to epithelium.[4,5,11] To translate our prior *in vitro* work into an *in vivo* model of pulmonary tissue construct development, we adopted the Matrigel plug assay[10] as a system for investigating *in vivo* formation and vascularization of distal pulmonary tissue. Based on our *in vitro* findings,[4,5] we specifically hypothesized that grafting of a mixed FPC population would support epithelial differentiation (SpC expression) and morphogenesis (formation of glandular structures) in an *in vivo* environment. Further, we hypothesized that graft vascularization could be enhanced by exogenous FGF2, via increased host angiogenesis, and that graft ECs will contribute to neovascularization in the constructs.

FPCs significantly enhanced neovascularization compared to Matrigel-only controls (Figs. 1 and 3), as reported previously for various tumor cells.[10] Addition of FPCs alone promotes significant neovascularization, most likely as a result of angiogenic paracrine signals and/or contribution of donor-derived ECs. Previous studies indicated that distal vascular development and patterning in the lung is governed in part by vascular endothelial growth factor (VEGF) family ligands elaborated by epithelial[17] and mesenchymal cells,[18] both of which are present in our organotypic FPC mixture.[4,5] In separate *in vitro* experiments, we have determined that our FPCs secrete physiological levels of VEGF-A (data not shown). Therefore it is likely that FPC-derived VEGF and other paracrine factors contribute to graft vascularization

via influencing both donor-derived and host ECs. In line with previous studies using the Matrigel plug model,[10] incorporation of FGF2-soaked polyvinyl sponges in the absence of FPCs significantly enhanced vascularization relative to Matrigel only, to a similar degree as FPCs alone (Fig. 3). Interestingly, FPCs + FGF2 elicits an additive effect, with a significant twofold increase relative to both FPCs and FGF2 alone (Fig. 3E). Since we are using a syngeneic, not an immunodeficient, mouse model, a host inflammatory response to transplanted cells, Matrigel, and polyvinyl sponges likely contributes to enhanced vascularization. Indeed, there is a known correlation between inflammation due to the innate immune response and angiogenesis.[19,20] In preliminary studies (not shown), we established that ~20% of all cells present within FPC-containing plugs following 7 days *in vivo* were $CD3^+$ lymphocytic infiltrate, a number that did not change in FPCs + FGF2 conditions, despite an approximately twofold increase in patent vascularization. At this point we cannot determine the specific contribution of inflammatory signaling to angiogenesis in response to FPC transplantation; however, we can conclude that the augmentation of neovascularization by exogenous FGF2-loaded sponges appears to be specific and does not result from increased inflammation.

Exogenous FGF2 significantly enhances construct vascularization above Matrigel-only controls (Fig. 3); however, at this stage, determining the exact mechanism is beyond the scope of this paper. FGF2 is a pleiotropic factor that elicits effects on lung epithelial cells, ECs, and mesenchymal cells via FGF receptors expressed by all these cell types. FGF2 has been reported to influence lung epithelial differentiation[7] and is also a potent angiogenic factor.[8,9] In addition to its well-elucidated role in promoting sprouting angiogenesis both *in vitro*[8] and *in vivo*,[9] FGF2 is also known to play a major role in vasculogenesis. Exogenous FGF2 induced *in vitro* hemangioblast differentiation of dissociated blastodisc cells that do not normally form blood islands,[21] and mediated vascular development in the embryonic chick chorioallantoic membrane.[22] We have demonstrated that exogenous FGF2 potently stimulates vascular plexus formation in 3D collagen gel cultures of FPCs *in vitro*.[5] In addition, we have demonstrated that exogenous FGF2 significantly enhances proliferation of mesenchymal cells present within the FPC mixture,[5] which reciprocally enhances epithelial and endothelial development. Therefore, it is likely that exogenous FGF2 may manifest its effects in our system based on a combination of (i) stimulating sprouting of host vessels (angiogenesis), (ii) promoting by donor-derived ECs the formation of a primitive vascular plexus (vasculogenesis) that anastomoses with the host vasculature, and (iii) enhancing mesenchymal and epithelial growth/proliferation, which positively impacts neovascularization via increased paracrine signaling. The elucidation of these mechanisms is currently ongoing in our laboratory.

There have been several successful attempts at engineering endothelial-lined microvessels *in vitro* and *in vivo*.[23–26] Recently, many groups have focused on engineering tissues containing differentiated, functional parenchyma and functional vascular structures *in vitro* and *in vivo*.[27–32] The generation of engineered vascularized bone,[27] hepatic,[28] skeletal muscle,[29,30] cardiac muscle,[31] and pancreatic tissues[32] by *in vivo* implantation of organ-specific parenchymal cells in the absence of grafted ECs has been previously reported. In all these cases, neovascularization is therefore likely mediated by angiogenesis from the host blood supply. For example, in experiments aimed at generating vascularized pancreatic islet tissue, Brown *et al*.[32] transplanted pancreatic beta cells in Matrigel within polycarbonate chambers that contained a surgically created AV loop, relying on host angiogenesis to develop the microvascular network of the graft.

There is increasing evidence that tissue construct vascularization may be enhanced by mixed vasculogenesis/angiogenesis, provided that exogenously incorporated ECs can be coaxed to form vascular structures.[33] A study by Levenberg *et al*.[29] reported that graft-derived endothelial structures present within *in vitro*–engineered skeletal muscle tissue constructs contribute to patent vessels *in vivo*. The enhanced *in vitro* vascularization and subsequent translation into function *in vivo* in the system described by Levenberg *et al*.[29] were attributed to their coculture conditions, in which ECs were coseeded with fibroblasts and skeletal muscle myoblasts. In extending our previous *in vitro* work,[4,5] our *in vivo* model employs a similar coculture approach, focusing on the role of heterotypic cell–cell interactions as a means of generating tissue constructs with an appropriately patterned vasculature, found in direct proximity to developing glandular epithelial structures (Figs. 1H and 2C). This is significant to lung tissue engineering, where the developing circulation must interface with developing alveolar structures to establish the required architecture for efficient gas exchange. In addition to paracrine angiogenic activity resulting from coculture, contribution of FPC-derived ECs to neovascularization also accelerates establishment of patent vasculature throughout the 3D constructs in 7 days (Fig. 4). Our FPCs contain approximately 15–20% ECs following brief 2D *in vitro* culture,[4] and we have demonstrated that these ECs undergo vascular morphogenesis *in vitro* with exogenous FGF2.[5] Therefore, we conclude that optimization of combinatorial approaches employing addition of exogenous ECs, coculture with organ-specific parenchymal cells, and provision of exogenous proangiogenic factors to influence both donor-derived ECs and host angiogenesis will be required to generate tissue constructs with robust, appropriately patterned vasculature.

Recently, the field of lung tissue engineering has made significant progress, as evidenced by several recent studies on the *in vivo* and *in vitro* generation of pulmonary tissue constructs.[4,5,11,34–36] With recent advances in stem cell biology indicating the potential of generating lung epithelial lineages,[37] it has become increasingly relevant to understand the contributions of specific extracellular matrix

molecules and growth factors that promote organization of pulmonary tissue in engineered systems. Cortiella *et al*.[35] described tissue-engineered lung constructs generated using polyglycolic acid and Pluronic F-127 hydrogels *in vivo*. In these studies, somatic lung cell progenitors were implanted *in vivo* on these matrices and evidence of Clara cell and alveolar type II lineage differentiation was presented, without addressing the differentiation of these progenitors into ECs or characterization of host angiogenesis. Andrade *et al*.[36] have recently reported intrapulmonary implantation of FPCs on a Gelfoam (collagen) matrix utilizing an injection-based approach. In this study, differentiated Clara cell and alveolar cell lineages following long-term *in vivo* engraftment were observed; however, the grafted tissue was not functionally contributing to gas exchange.[36] While Andrade *et al*.[36] also demonstrated graft vascularization, the graft versus host origin of the ECs comprising the vessels was not reported. We believe that our study is the first to report the formation of histiotypic alveolar-like structures *in vivo*, comprised of differentiated distal epithelial cells (proSpC expressing) forming ductal structures that are interfaced with a patent vascular network containing donor-derived ECs.

In summary, we have demonstrated the ability to generate vascularized pulmonary tissue constructs *in vivo* utilizing Matrigel as a venue for transplantation of freshly isolated FPCs. Significantly, distal epithelial differentiation (proSpC expression) can be maintained *in vivo* in organotypic culture, and pulmonary ECs present in the organotypic mixture contribute to the formation of patent blood vessels. As a result, our model recapitulates the formation of structures reminiscent of alveolar forming units comprised of ductal epithelium tightly interfaced with the host circulation. Therefore, we conclude that this model will be useful for testing the effects of parameters such as exogenous growth factors, genetic modifications to engrafted cells, and addition of specific extracellular matrix molecules, as well as the utility of stem cell–derived populations of pulmonary cells in the process of distal lung tissue formation *in vivo*.

ACKNOWLEDGMENTS

We are grateful to Drs. Nasim Akhtar and Robert Auerbach (University of Wisconsin, Madison, WI) for their help with setting up the Matrigel plug assay and the generous gift of the polyvinyl sponges. This work was supported by grants from the National Institutes of Health (5R21EB003520-02, to C.M.F. and P.I.L.) and the St. Christopher's Foundation (to C.M.F.).

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