Engineering *De Novo* Assembly of Fetal Pulmonary Organoids

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Induction of morphogenesis by competent lung progenitor cells in a 3D environment is a central goal of pulmonary tissue engineering, yet little is known about the microenvironmental signals required to induce *de novo* assembly of alveolar-like tissue *in vitro*. In extending our previous reports of alveolar-like tissue formation by fetal pulmonary cells stimulated by exogenous fibroblast growth factors (FGFs), we identified some of the key endogenous mediators of FGF-driven morphogenesis (organoid assembly), for example, epithelial sacculatation, endothelial network assembly, and epithelial–endothelial interfacing. Sequestration of endogenously secreted vascular endothelial growth factor-A (VEGF-A) potently inhibited endothelial network formation, with little or no effect on epithelial morphogenesis. Inhibition of endogenous sonic hedgehog (SHH) partially attenuated FGF-driven endothelial network formation, while the addition of exogenous SHH in the absence of FGFs was able to induce epithelial and endothelial morphogenesis, although with distinct morphological characteristics. Notably, SHH-induced endothelial networks exhibited fewer branch points, reduced sprouting behavior, and a periendothelial extracellular matrix (ECM) virtually devoid of tenascin-C (TN-C). By contrast, focal deposition of endogenous TN-C was observed in the ECM-surrounding endothelial networks of FGF-induced organoids, especially around sprouting tips. In the FGF-induced organoids, TN-C was also observed in the clefts of sacculated epithelium and at the epithelial–endothelial interface. In support of a critical role in the formation of alveolar-like tissue *in vitro*, TN-C blocking inhibited endothelial network formation and epithelial sacculatation. Upon engraftment of *in-vitro*-generated pulmonary organoids beneath the renal capsule of syngeneic mice, robust neovascularization occurred in 5 days with a large contribution of patent vessels from engrafted organoids, providing proof of principle for exploring intrapulmonary engraftment of prevascularized hydrogel constructs. Expression of proSpC, VEGF-A, and TN-C following 1 week *in vivo* mirrored the patterns observed *in vitro*. Taken together, these findings advance our understanding of endogenous growth factor and ECM signals important for *de novo* formation of pulmonary tissue structures *in vitro* and demonstrate the potential of an organoid-based approach to lung tissue augmentation.

Introduction

CHRONIC LUNG DISEASES of prematurity, such as bronchopulmonary dysplasia often occurring secondary to pulmonary hypoplasia and respiratory distress with premature birth, remain major clinical challenges.¹ In adults, chronic lung diseases, for example, chronic obstructive pulmonary disease (COPD) and emphysema, continue to increase in prevalence.² Currently, extensive research efforts are aimed at engineering whole lungs for implantation, with the majority of attention focused on the use of decellularized cadaveric lungs recellularized with pulmonary derivatives of various stem/progenitor cell sources.^{3–5} Over the past few years, significant progress has

been made in understanding specific pathways that drive the directed differentiation of anterior foregut endoderm and lung epithelia from murine and human embryonic stem cells^{6–10} and human induced pluripotent stem cells.^{9,11–13} In addition, there have been several recent reports that identify diverse resident lung stem/progenitor cells in humans^{14,15}; however, very little is known about the growth factor and extracellular matrix (ECM) cues required to drive *de novo* tissue formation by competent lung progenitor cells *in vitro*.

One potential application of directed differentiation of distal lung progenitor cells is the construction of organotypic microtissue models for application in high-throughput screening of candidate therapeutics and biologics, as is currently

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being undertaken in other organ and tissue systems, such as cardiac muscle.¹⁶ The “lung on a chip” concept is promising for applications in pharmaceutical testing.¹⁷ To date, however, there are only few high-fidelity models that allow for the study of the complex cellular and molecular interactions during formation of distal alveoli. Besides providing basic mechanistic insights pertaining to lung development, such models could provide the basis for designing informed biologic/therapeutic and cell-based or engineered-construct-based therapies that might stimulate postnatal lung development in premature infants, and/or regeneration of functional alveoli in adults. Such cell-based, tissue-engineered models have the added advantage of potential miniaturization, increases in throughput, and the ability to develop human-cell-based systems.

In recent years we have been studying the effects of key microenvironmental parameters, such as soluble paracrine factors and ECM composition, both exogenously supplied and endogenously elaborated, on the spatial organization of competent fetal lung progenitor cell populations.^{18–20} In these studies we used a mixed population of embryonic day 17.5 (E17.5) murine fetal pulmonary cells (FPCs) (onset of the saccular stage) cultured under serum-free conditions in 3D collagen type I hydrogels. In this setting, the use of a serum-free medium containing fibroblast growth factor (FGF)-10, -7, and -2 (FGF-10/7/2)–stimulated formation of 3D histiotypic fetal lung structures comprised of sacculated distal (proSpC-expressing) epithelial structures enrobed in and interfaced with capillary-like endothelial networks. These morphogenetic processes did not occur with serum-free medium devoid of FGFs or in the presence of fetal bovine serum.¹⁸ In extending these studies, we now sought to identify endogenous paracrine and ECM mediators of *in vitro* pulmonary tissue assembly driven by exogenous FGF10/7/2. We specifically focused on elucidating the role of endogenous factors, such as vascular endothelial growth factors (VEGFs),^{21–23} sonic hedgehog (SHH),^{23–26} and the ECM protein tenascin-C (TN-C),^{27,28} based on their known and implicated roles in distal lung morphogenesis *in vivo*.

Materials and Methods

Preparation and culture of murine fetal pulmonary tissue constructs

All animal procedures were carried out in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved protocol as previously described.¹⁸ Briefly, E17.5 murine FPCs were isolated by enzymatic digestion, and admixed to a collagen type I solution (1.2 mg/mL, pH 7.2) at 5×10^6 cells per mL. Gels were then cast at 900 μ L in 24-well plates or 400 μ L in 48-well plates (depending on the experiments to be carried out) and cultured overnight in 10% FBS medium to aid in recovery from tissue dissociation. The following morning culture media was changed to a serum-free formulation containing a 50/50 mixture of F-12 and DMEM 1% ITS+ (BD Biosciences), L-glutamine, antibiotics, and 10 U/mL heparin (base culture medium) and supplemented with various combinations of FGFs as previously described.¹⁸ Additional media supplements such as growth factors and blocking reagents/inhibitors specific to a particular set of experiments were added to the base culture medium as described below. Switching to

defined media was considered the zero time point; for example, the time points in the results reflect the time elapsed from the addition of the serum-free media. All experiments were carried out for either 4 or 7 days.

Recombinant human FGF-10, FGF-7, and FGF-2 (Sigma; Cat. Nos. F8924, K1757, and F0291) were added at concentrations of 25, 12.5, and 25 ng/mL, respectively.^{18–20} For VEGF-A loss-of-function experiments, a soluble VEGFR1-Fc chain chimeric protein (sVEGFR1; R & D Systems; Cat. No. 321-FL-050) was added to culture media at concentrations ranging from 500 ng/mL to 2 μ g/mL. VEGF-A inhibition experiments were stopped at 96 h. The sVEGFR1 approach was chosen to sequester VEGF-A protein in the extracellular space without manipulating intracellular signaling activities in VEGFR-expressing cells, since small-molecule VEGFR tyrosine kinase inhibitors such as SU-5416 can cause apoptosis in target cells.²⁹ For SHH loss-of-function studies, cyclopamine (Sigma; Cat. No. C4116) was added at 5 μ M using DMSO as a vehicle³⁰ when switching to serum-free medium (zero time point). In all SHH inhibition experiments, an equal volume of DMSO alone was added to untreated control wells to account for the effect of the vehicle. For SHH addition, recombinant human SHH (R & D Systems; Cat. No. 1845-SH-025) was added at 500 ng/mL to the 1% ITS basal medium alone or in the presence of FGF-10/7/2 or cyclopamine.³⁰ For TN-C-blocking experiments, a neutralizing rabbit polyclonal antibody against TN-C (Chemicon; Cat. No. AB19013) was added to both the gel and the medium at 20 μ g/mL³¹ and the medium supplemented with 20 μ g/mL anti-TN-C IgG was replenished at 48 h. TN-C-blocking experiments were stopped at 96 h. Normal rabbit IgG (20 μ g/mL) was applied to all other conditions as a control. For TN-C addition studies, exogenous, purified human TN-C protein (Chemicon; Cat. No. CC065) was added to the gel and the medium at 10 μ g/mL. The medium was replaced at 48 h and experiments were stopped at 96 h.

Whole-mount immunohistochemistry

In the past we described a whole-mount immunohistochemistry (IHC) protocol optimized for application in collagen type I gel-based constructs, which facilitates 3D visualization of developing tissue structures by laser scanning or multiphoton confocal microscopy.¹⁸ We used this approach to visualize cellular proteins and ECM molecules of interest in our 3D constructs. Constructs were fixed with 4% paraformaldehyde (diluted from ultrapure 16%; Electron Microscopy Sciences) for 2–3 h at room temperature with gentle agitation, and then overnight at 4°C. Constructs were then washed several times with sterile 1 $\times$ PBS and stored in sterile 1 $\times$ PBS containing 0.1% sodium azide at 4°C until processing for whole-mount IHC according to our published procedures.¹⁸ Here we utilized antibodies against pro-surfactant protein C (Millipore; Cat. No. AB3786) to identify distal lineage epithelial cells, anti-VEGF-A (Thermo Scientific; Cat. No. RB-222) to visualize cellular sources, and anti-TN-C (Millipore; Cat. No. AB19011) and anti-pan-laminin (Abcam; Cat. No. ab14055) to visualize ECM deposition. Endothelial cells were routinely labeled with the highly specific isolectinB4 (Life Technologies; Cat. No. I32450) as previously described.^{18–20}

Quantitative image analysis

Quantification of isoB4 staining in laser-scanning confocal micrographs was carried out using NIH ImageJ, as previously described.¹⁸ Briefly, for each experimental condition, we analyzed at least 20 randomly acquired fields (200× magnification) at comparable z-positions taken from at least two whole-mount-stained constructs per experiment analyzed (at least $n=3$ for each series). Individual images were binarized, and the total area of isoB4-stained pixels per 200× microscopic field was calculated. To quantify endothelial network formation, we developed a morphogenetic index, termed the index of endothelial interconnectivity (IEI), defined as the fraction of EC staining area in a micrograph contributed by interconnected ECs (e.g., tubules) out of the total area of EC staining (e.g., tubules plus individual non-networked EC):

$$IEI = \frac{A_{IEC}}{A_{IEC} + A_{sEC}}$$

Where A_{IEC} = area of interconnected ECs and A_{sEC} = area of single EC.

IEI values were typically <0.05 for 1% ITS and 10% FBS cultures. Branch point counts for endothelial networks in select conditions of the SHH modulation experiments were performed manually, with a branch point being defined as any common node in a network from which at least 3 tubules extend.

VEGF-A ELISA

At each media change (excluding removal of the initial 10% FBS-containing medium), ~500 µL of medium from each culture condition was collected and frozen at -80°C. Media samples were thawed gradually on ice to minimize protein degradation, mixed by gentle agitation, and centrifuged to remove any precipitated or solid material. VEGF-A protein levels in cell culture media across conditions were then quantified using a commercially available ELISA kit (Quantikine; R & D Systems; Cat. No. MMV00). In preliminary studies we determined that dilutions between 1:20 and 1:100 were optimal to ensure that the samples fall within the linear range of the assay (<500 pg/mL). For each experiment the measured VEGF-A concentration in the 1% ITS medium samples (serum-free medium devoid of growth factors or inhibitors; considered the baseline in our system) was set equal to 1, and the VEGF-A levels for other experimental conditions are reported as fold increase relative to the 1% ITS baseline.

Statistical analysis

Statistical analysis of ELISA data, IEI values, and endothelial network branch counts was carried out by one-way ANOVA with Tukey's posttest for individual comparisons between area values for the various media supplementation conditions. *p*-Values for individual comparisons were calculated by Student's *t*-test, when applicable. $p < 0.05$ was regarded as statistically significant, with further significance assigned for $p < 0.01$.

In vivo engraftment model

For *in vivo* engraftment we utilized the renal capsule model, which was originally developed for xenografting of

human carcinoma cells in cancer research.³² Constructs were prepared essentially as described for all *in vitro* experiments, with some modifications to generate a smaller construct with sufficient mechanical integrity to handle with forceps and place beneath the renal capsule. FPCs were isolated per protocol and suspended in type I collagen solution (1.2 mg/mL, pH 7.2) at 5×10^6 FPCs/mL, as described previously. Constructs were cast in a 48-well plate at initial volumes of 300 and 400 µL. Both volumes were utilized due to variations in the degree of gel contraction in each experiment, thus allowing for use of constructs with a consistent final size for the implantations. To facilitate gel contraction/compaction, in deviation from the protocol for standard *in vitro* culture, gels were carefully detached from the well plate following the first night of culture (e.g., upon switching from 10% FBS "recovery" medium to the FGF10/7/2-containing medium), and cultured in 60-mm Petri dishes on an orbital shaker (Belly Dancer, Stovall). In addition, to facilitate compaction of the constructs, serum was kept in the medium at a concentration of 5% in addition to FGF10/7/2 at the previously described concentrations.

In select experiments, FPCs were tracked by labeling with CMTPIX CellTracker™ dye (Life Technologies; Cat. No. C34552) according to manufacturer's protocol. Briefly, cells were incubated for 30 minutes in serum-free medium containing 25 µM CMTPIX. The labeled cells were then washed with several changes prior and suspended in collagen gels, as described earlier. In some experiments, GFP-expressing transgenic mice [Charles River; C57BL/6-Tg(UBC-GFP) 30Scha/J] were utilized as hosts. In these experiments, tetramethylrhodamine isothiocyanate (TritC) dextran (TritC-dextran; 2,000,000 MW, prepared as a 5% w/v solution in PBS) was injected via the tail vein immediately prior to sacrifice and harvest, allowing for visualization of patent, perfused vessels within the constructs. This approach, in combination with the use of GFP-expressing hosts, enabled us to differentiate patent vessels of graft/donor origin (perfused vessels lined by GFP-negative cells) from angiogenic ingrowth of host vessels (perfused vessels lined by GFP-positive cells).

Upon completion of the experiments, constructs were harvested and fixed in 4% paraformaldehyde overnight and then stored in PBS at 4°C until processing for paraffin embedding, sectioning, and histological and immunohistochemical staining. Sections were stained with hematoxylin and eosin (H&E), and with the same antibodies against proSpC, VEGF-A, and TN-C, as utilized for whole-mount IHC.

Results

VEGF-A expression in 3D constructs

Previously we described the *in vitro* effects of FGF-10, -7, and -2 (FGF10/7/2) on concomitant epithelial and endothelial morphogenesis, as well as on the establishment and maintenance of distal epithelial differentiation (pro-surfactant protein C-positive, proSpC) within a mixed population of E17.5 fetal lung cells.^{18,20} In this study, we routinely used phase-contrast microscopy and whole-mount IHC with confocal visualization of 3D tissue assembly to confirm FGF10/7/2-driven formation of sacculated epithelial structures (Fig. 1A); maintenance of distal phenotype

was confirmed by proSpC expression (Fig. 1B), and histiotypic interfacing of nascent epithelial structures with developing vascular networks (isolectinB4; Fig. 1B, magenta staining). Next we sought to identify potential endogenous mediators of FGF10/7/2-driven morphogenesis, focusing first on VEGF-A.

VEGF-A is expressed in the pulmonary epithelium³³ and mesenchyme in a temporally and spatially controlled manner, the pattern of which regulates distal capillary plexus formation.²³ VEGF-A also affects mesenchymal proliferation and differentiation,²² as well as that of epithelial cells,^{21,34} although the latter is a topic of debate.³⁵ Whole-mount IHC demonstrated cytoplasmic localization of VEGF-A in epithelial cells (Fig. 1C) and interstitial mesenchymal cells throughout the constructs (Fig. 1D), as well as in perivascular cells directly contacting endothelial tubules (Fig. 1E). Little, if any, staining was observed in the endothelial cells themselves, supporting the notion that nonendothelial sources of VEGF-A are critical in the mediation of endothelial network formation driven by exogenous FGFs.

Secreted VEGF-A was measured in tissue culture supernatants using a commercially available ELISA assay (Fig. 1F). VEGF-A levels in 10% FBS cultures were not significantly different from serum-free controls (ITS). Both FGF-2 alone, and FGF-10/7, induced statistically significant increases of greater than twofold in the secreted levels of VEGF-A at 6 days compared with 1% ITS controls. The combination of FGF-10/7/2 induced the highest levels of secreted VEGF-A, with a >3-fold increase relative to 1% ITS control cultures after 6 days (Fig. 1F). Similar relative increases were observed earlier in the experiments at 2 and 4 days (not shown). Taken together with our previous report of an approximately three- to fourfold increase in the number of epithelial and mesenchymal cells with FGF10/7/2 versus 1% ITS controls, the increase in secreted VEGF-A likely reflects an expansion of VEGF-A-expressing populations of cells.

VEGF-A sequestration ablates FGF-induced endothelial network formation, but not epithelial sacculation

To determine the functional significance of increased VEGF-A production observed in FGF10/7/2-treated constructs, we attenuated VEGF signaling using a soluble VEGFR1-Fc chimeric protein (sVEGFR1) that sequesters VEGF-A in the extracellular space without directly influencing VEGFR expression and/or function of the cells expressing these receptors. As seen in Figure 2A–D, endothelial network formation was inhibited by sVEGFR1 in a concentration-dependent manner. In the presence of 500 ng/mL of sVEGFR1, network interconnectivity was decreased com-

pared with untreated controls (Fig. 2B vs. A), but only few rounded endothelial cells were typically observed. When exposed to 1–2 μ g/mL sVEGFR1, most individual endothelial cells appeared rounded (Fig. 2C, D), suggesting disruption of cell adhesion, spreading/elongation, and inhibition of the formation of multicellular endothelial assemblies. In all VEGF-A sequestration experiments, we also assessed epithelial growth and sacculation over the experimental time course by phase-contrast microscopy (Fig. 2E, F). Remarkably, and in contrast to the effects on endothelial cell tubular morphogenesis, exposure to sVEGFR1 did not disrupt FGF10/7/2-driven assembly, expansion, and sacculation of developing epithelial structures (Fig. 2E vs. F).

Exogenous SHH induces epithelial and endothelial morphogenesis in the absence of FGFs

SHH is expressed exclusively in the endoderm-derived epithelium^{25,26} and coordinates, in collaboration with FGF-9, the expression of VEGF-A in the distal mesenchyme, thus establishing a functional link between FGFs, SHH, and VEGF-A in the patterning of the distal lung capillary network.²³ Therefore, we tested the effects of the addition of exogenous SHH (in the absence of exogenous FGFs) on epithelial and endothelial morphogenesis in our constructs, while also testing the effect of cyclopamine (11-deoxojervine), a well-defined alkaloid inhibitor of SHH signaling,²⁴ on FGF-10/7/2-induced construct development.

Epithelial growth (e.g., expansion of assembled structures) and sacculation was monitored throughout the duration of the experiments by phase-contrast microscopy (Fig. 3A–D). Exogenous SHH alone (Fig. 3B) stimulated epithelial growth and sacculation relative to 1% ITS controls (Fig. 3A), although not to the degree typically observed in FGF10/7/2-treated constructs (Fig. 3C). Addition of cyclopamine to FGF10/7/2-treated cultures did not appreciably inhibit growth or sacculation (Fig. 3D), suggesting that, similarly to VEGF-A sequestration, inhibition of SHH signaling does not significantly attenuate epithelial morphogenesis in the presence of exogenous FGF10/7/2.

Our main focus was to examine the effects of SHH manipulations on endothelial network formation in 3D constructs using our whole-mount staining approach with isolectinB4 (Fig. 3E–J). As previously described,¹⁸ serum-free basal medium (1% ITS) alone did not support network formation (Fig. 3E), while FGF10/7/2 induced robust endothelial network formation (Fig. 3H). Addition of exogenous SHH to 1% ITS promoted endothelial network formation (Fig. 3F), although SHH-induced networks were morphologically distinct from those observed in FGF10/7/2-treated constructs. The SHH-induced networks contained appreciably thicker tubules and fewer branch points (compare Fig. 3F

FIG. 2. Extracellular sequestration of VEGF-A with a soluble VEGFR1-Fc chimeric protein inhibits endothelial, but not epithelial, morphogenesis in distal lung tissue constructs. Confocal micrographs were obtained following 96 h of culture for all conditions in this series of experiments. (A–D) Z-stacks (75–100 μ m) of endothelial cells and structures (isolectinB4, magenta) in FGF10/7/2-treated constructs with increasing concentrations of sVEGFR1-Fc, as labeled in the panels. Representative fields from conditions in a single experiment. Scale bars = 150 μ m. (A) 0 μ g/mL; (B) 0.5 μ g/mL; (C) 1 μ g/mL; (D) 2 μ g/mL. (E, F) Phase-contrast images showing similar levels of FGF-10/7/2 induced expansion and sacculation of epithelial structures and sacculation with (1 μ g/mL, E) and without (0 μ g/mL, F) sVEGFR1-Fc treatment. Images taken at 96 h, original magnifications = 100 $\times$.

FIG. 1. VEGF-A expression in FGF10/7/2-treated distal lung tissue constructs. All images are taken from FGF10/7/2-treated constructs cultured for 7 days as described in the “Materials and Methods” section. **(A)** Sacculated epithelial structure with typically observed morphology; phase-contrast image (original magnification 200 $\times$). Endothelial tubules in the surrounding spaces are faintly visible but difficult to resolve. **(B)** Whole-mount immunohistochemistry for pro-surfactant protein C (green staining) revealed the distal phenotype of sacculated epithelial structures seen interfacing with tubular endothelial networks (isolectinB4, magenta). Confocal z-stack, thickness = 100 μ m, scale bar = 75 μ m. **(C)** Robust VEGF-A localization in sacculated epithelial structures (inferred based on established morphological-phenotypic correlation¹⁸) interfaced with endothelial tubules (isolectinB4, magenta). Single optical section, scale bar = 12 μ m. **(D)** VEGF-A localization (green staining) in interstitial and perivascular cells surrounding endothelial networks (isolectinB4, magenta). Single optical section, scale bar = 75 μ m. **(E)** Robust VEGF-A (green) localization in perivascular cells directly contacting tubular endothelial networks (isolectinB4, magenta). Single optical section, scale bar = 9.38 μ m. **(F)** Quantification of secreted VEGF-A by ELISA. Culture media was collected following 48-h feeding periods and assayed for mouse VEGF-A (Biosource). Data are expressed as relative amount of immunoreactive VEGF normalized to 1% ITS in media collected following 6 days of culture (secreted over a 48-h period from previous feeding, i.e., 97–144 h). * p < 0.05 compared with 1% ITS at the same time point, ** p < 0.01 for FGF10/7/2 versus FGF10/7. FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

FIG. 3. Effects of SHH modulation on endothelial tubular network morphogenesis in the presence and absence of FGF-10/7/2. (A–D) Phase-contrast images depicting the morphology of epithelial structures across conditions in SHH modulation experiments (media composition indicated in each panel). All images taken at 7 days, original magnifications = $100\times$. (E–J) Three-dimensional (z-stacks, thickness = 75–100 μm) visualization of endothelial network formation in SHH gain- and loss-of-function experiments, isolectinB4 (green) whole-mount staining. Scale bars = 150 μm . These micrographs depict parallel samples from a single experiment, which was repeated several times with similar outcomes. Experimental conditions are indicated in each panel. (E) 1% ITS; (F) 1% ITS + 500 ng/mL SHH; (G) 1% ITS + 500 ng/mL SHH + 5 μM Cyclopamine (Cy); (H) 1% ITS + FGF-10/7/2 (25/10/25 ng/mL); (I) 1% ITS + FGF-10/7/2 + 500 ng/mL SHH; (J) 1% ITS + FGF10/7/2 + 5 μM Cy. (K) Quantification of secreted VEGF-A levels across conditions of SHH modulation by ELISA. Data are expressed as fold increase relative to measured values for 1% ITS in culture media collected at 6 days (changed every 2 days, so these data reflect VEGF-A secreted from 97 to 144 h); *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; significance shown for each condition versus 1% ITS at the same time point unless otherwise indicated by brackets. (L) Index of endothelial interconnectivity across conditions of SHH modulation. For details see “Materials and Methods” section. ** $p < 0.01$ versus 1% ITS baseline, * $p < 0.05$ for individual comparison of FGF10/7/2 versus FGF10/7/2 + Cy. (M) Average number of branch points per field in SHH- versus FGF-10/7/2-treated networks from representative micrographs of isolectinB4-stained networks following 7 days of culture. * $p < 0.05$. SHH, sonic hedgehog.

vs. H). Details of these effects were further quantified using our previously described IEI, which is the fraction of EC staining area in a micrograph contributed by interconnected ECs (e.g., tubules) from the total area of EC staining (e.g., tubules plus individual non-networked ECs). As seen in Figure 3L, the IEI values for both SHH-induced and FGF10/7/2-induced networks were more than an order of magnitude above that for the base culture medium (ITS, $p < 0.01$), but not significantly different from one another ($p > 0.05$). Cyclopamine blocked SHH-induced endothelial network formation (Fig. 3G), returning IEI values to baseline levels. Interestingly, cyclopamine partially, but significantly, lowered FGF10/7/2-induced IEI values ($p < 0.05$, Fig. 3J). IEI allows us to measure the degree to which fetal pulmonary ECs present within our constructs are forming networks, but does not give a measure of network complexity. As a measure of complexity, we quantified the number of branch points (defined as points/nodes from which three or more endothelial tubules connect and/or extend from), which revealed a statistically significant ($p < 0.05$), ~50% decrease in the number of branch points in SHH-induced networks compared with FGF10/7/2-induced networks (Fig. 3M).

Combining exogenous FGF10/7/2 and SHH (Fig. 3I) yielded results essentially identical to those for FGF10/7/2, suggesting that morphologies promoted by FGFs predominate even when exogenous SHH is added in supraphysiologic concentrations. Cyclopamine attenuated SHH-induced endothelial morphogenesis to near 1% ITS control levels (Fig. 3G). By comparison, addition of cyclopamine to FGF10/7/2-treated constructs visibly attenuated endothelial network formation (Fig. 3J vs. H), although not to the degree observed with VEGF-A sequestration (Fig. 2).

To connect the effects of SHH modulation on endothelial network formation with established roles of VEGF-A, we measured VEGF-A levels in cell culture supernatants across conditions of SHH modulation (Fig. 3K). Exogenous SHH alone, FGF10/7/2, and FGF10/7/2 + SHH stimulated very significant increases in secreted VEGF-A levels compared with 1% ITS baseline controls (Fig. 3K, vs. ITS; $p < 0.001$). Exogenous SHH alone induced an approximately eightfold increase in VEGF-A secretion above baseline, compared with approximately sixfold for FGF10/7/2, although this difference was not statistically significant. Cyclopamine significantly attenuated SHH-induced VEGF-A secretion (Fig. 3K, SHH vs. SHH + Cy; $p < 0.01$), while the inhibitory effect of cyclopamine on FGF10/7/2-induced levels of secreted VEGF-A was statistically not significant (Fig. 3K, FGF10/7/2 vs. FGF10/7/2 + Cy; $p > 0.05$).

Our results so far establish that endogenous VEGF-A is a critical mediator of FGF10/7/2-induced endothelial network formation (Fig. 2), and confirm a role for SHH as part of the FGF/SHH/VEGF-A axis of signals that is active *in vivo*²³ and in our constructs (Fig. 3). However, these results do not provide an explanation for the spatial patterning of key steps in endothelial morphogenesis, such as network formation, sprouting, and interfacing with epithelium. We hypothesized that spatial patterning of these events in our constructs is regulated by deposition of ECM molecules, several of which have been shown to regulate fetal lung morphogenesis.^{27,28,36}

Focal deposition patterns of TN-C indicate a role in FGF10/7/2-driven morphogenesis

Given the known and implicated roles of TN-C in lung development,^{37–44} we hypothesized that focal deposition of TN-C could provide pivotal cues for the spatial patterning of histiotypic endothelial and epithelial morphogenesis. Since the starting ECM is a simple, homogeneous type I collagen gel, the presence of TN-C and other ECM constituents, such as laminin (LN), is reflective of endogenous production by fetal pulmonary cells. Examination of focal TN-C deposition in FGF10/7/2-treated constructs by whole-mount IHC and confocal microscopy revealed that TN-C was consistently localized in epithelial clefts with a marked predominance over LN deposition (Fig. 4A–C). The predominance of TN-C in the internal clefts of sacculated epithelial structures is apparent when examining 3D z-stack reconstructions of entire epithelial units (Fig. 4A), as well as in single optical sections through such structures (Fig. 4B). In addition to the predominance of TN-C in clefts, TN-C was associated with LN basement membranes along the perimeter of epithelial structures (Fig. 4C).

Assemblies of TN-C were observed surrounding endothelial networks (Fig. 4D), and, importantly, in the perivascular matrix surrounding sprouting endothelial tip cells (Fig. 4E), indicating a potential role in the spatial guidance of sprouting processes. Though we have not performed *in situ* hybridization studies to confirm lineage-specific expression, TN-C, as expected, appears to be produced by mesenchymal/interstitial cells (Fig. 4F), which is line with data from *in vivo* studies.⁴³ An interesting dichotomy in the distribution of TN-C and LN was observed at the epithelial–endothelial interface (Fig. 4G–I). LN was primarily located on the “stromal” side of endothelial structures not directly in contact with the epithelium (Fig. 4G), whereas TN-C was localized in the thin ECM layer filling the intervening space between epithelial and endothelial cells, that is, the shared epithelial–endothelial basement membrane, as well as in underlying clefts (Fig. 4B, H). At higher magnification, we consistently observed thin layers of TN-C in the intervening space, for example, the shared basement membrane, between an epithelial sacculle and apposed endothelial cells (Fig. 4I), suggesting a potential role in facilitating the formation of the epithelial–endothelial interface.

TN-C blocking disrupts endothelial and epithelial morphogenesis

Based on the previous correlative observations between focal TN-C deposition, capillary-like morphogenesis, and the formation of sacculated epithelial structures, we performed a series of TN-C modulation experiments to elucidate the functional significance of TN-C deposition patterns. Neutralizing anti-TN-C antibodies,^{31,40} which block access of cell surface receptors to TN-C in the ECM, disrupted FGF10/7/2-induced epithelial sacculatation (Fig. 5). In constructs treated with TN-C antibodies, epithelial structures displayed a more spherical, dilated morphology with less budding and sacculatation compared with nontreated controls (Fig. 5A vs. B).

Further, TN-C-blocking antibodies significantly attenuated endothelial network formation typically observed at 96 h, although formation of some interconnected tubules

FIG. 4. TN-C localization in the clefts of sacculated epithelial structures, in the periendothelial ECM surrounding sprouting tips, and at the epithelial–endothelial interface. FGF10/7/2-treated constructs cultured for 7 days in all panels. (A) TN-C and LN localization surrounding a sacculated epithelial structure. Overlay of TN-C (red) and LN (green) with DAPI counterstaining (blue) taken through a sacculated epithelial structure; Z-stack, thickness = 100 μm ; scale bar = 37.5 μm . (B) Single optical section taken through a sacculated epithelial structure depicting the preferential localization of TN-C (red, arrows) in the intervening clefts between saccules; scale bar = 46.5 μm . (C) High-magnification view of a single epithelial cleft, depicting the association of TN-C (red) and LN (green) in the basement membrane, with TN-C predominating in the cleft area. Single optical section, scale bar = 9.38 μm . (D) Focal deposition of TN-C (green) in the ECM surrounding endothelial (isolectinB4, magenta) network branch points; scale bar = 23.8 μm . (E) Focal deposition of TN-C (green) in the ECM surrounding the tips of endothelial tubules (isolectinB4, magenta); scale bar = 11.9 μm . (F) TN-C localization (red) surrounding an endothelial tubule (isolectinB4, green), with surrounding apparent mesenchymal cells (inferred here by nuclear staining, blue); scale bar = 23.8 μm . (G) Predominance of LN localization (green) on the outside (stromal-facing) of an elongated endothelial structure (isolectinB4, magenta, arrow) directly contacting an epithelial structure (inferred here by morphology); scale bar = 18.75 μm . (H) Comparison of TN-C (red) and LN (green) localization in the same optical section as in (G) reveals the presence of TN-C, and absence of LN, in the intervening space (basal surface of the epithelium), as well as the underlying epithelial cleft (arrow). (I) High-magnification view of the thin TN-C layer (red), and virtual absence of LN (green), at the interface between epithelium and an enrobing endothelial structure (white arrows, inferred here by morphology; see DAPI counterstaining). Note the paucity of TN-C staining on the stromal-facing side of the endothelial structure. Single optical section, scale bar = 9.38 μm . ECM, extracellular matrix; LN, laminin; TN-C, tenascin-C.

was consistently observed (Fig. 5C vs. D). Individual endothelial cells (not contributing to network formation) did not display a rounded morphology as observed with VEGF-A blockade (Fig. 2D). Rather, the cells appeared more spread in the surrounding matrix (Fig. 5D). These observations suggest a spatial derailment of endothelial cell networking, but not adhesion to the ECM upon disruption of TN-C function.

Addition of exogenous TN-C protein to the type I collagen hydrogel at the beginning of the experiment saturates the matrix with TN-C, creates a spatially homogeneous distribution, and disrupts the spatially defined endogenous TN-C deposition. In the presence of exogenous TN-C, the pattern of FGF-induced endothelial network formation and epithelial–endothelial interfacing were disrupted (Fig. 5E, F).

Altered endothelial network morphology, decreased sprouting, and lack of periendothelial TN-C deposition in SHH-treated constructs

Exogenously supplemented FGFs and SHH elicited distinct morphogenic effects in developing endothelial networks (Fig. 6). Tip cells of extending endothelial tubules in FGF-treated constructs (Fig. 6A, B) displayed filopodia-like morphologies reminiscent of angiogenic sprouting.⁴⁵ By contrast, cells at the tip of endothelial tubules in SHH-treated constructs displayed a relative lack of filopodia (Fig. 6C, D), which corresponds to our finding that treatment with

SHH yields a 50% reduction in network complexity (compared with FGF10/7/2-treated constructs), as assessed by counting branch points (Fig. 3M). We hypothesized that these differences in network complexity and sprouting behavior, despite equally high levels of VEGF-A, may be due to local variations in ECM composition. In support of this hypothesis, we observed distinct local variations in the TN-C content of the ECM in FGF- versus SHH-treated constructs. In the presence of FGF2/7/10, TN-C is found in and around the basement membrane of endothelial tubules (Fig. 6F) and in the ECM surrounding sprouting tip cells (Fig. 6E). Importantly, TN-C protein is also observed in the cytoplasm of periendothelial mesenchymal cells in FGF-treated constructs (Fig. 6E, F). By contrast, TN-C is barely detectable in the areas surrounding endothelial tubules in SHH-treated constructs or in interstitial cells surrounding the capillary tips (identified by nuclei adjacent to endothelial cells, Fig. 6G, H). These differences in morphology and local ECM content with FGF versus SHH treatment highlight the ability to fine-tune vascular network assembly in engineered tissue constructs.

Phenotypic maintenance and establishment of a perfused vasculature in fetal pulmonary organoids engrafted in vivo following in vitro preculture

One of the rationales for engineering distal pulmonary organoids is their potential use in lung augmentation.⁴⁶ To test the capacity of our collagen-gel-based constructs to

FIG. 5. Blocking of endogenous TN-C attenuates epithelial sacculatation (cleft formation) and endothelial network formation in fetal distal lung tissue constructs. Ninety-six hours of culture for all conditions. (A, B) Standard fluorescent micrographs of whole mounts stained for cytokeratin (pan-epithelial marker, green), depicting the effect of TN-C blockade (polyclonal antibodies) on epithelial morphology and sacculatation. (A) FGF-10/7/2, original magnification = 400 $\times$; (B) FGF-10/7/2 + TN-C antibodies, original magnification = 400 $\times$. (C, D) Z-stacks (confocal microscopy, thickness = 75–100 μm) depicting the effects of TN-C blockade (polyclonal antibodies) on endothelial network formation (isolectinB4, magenta). (C) FGF-10/7/2, scale bar = 150 μm . (D) FGF-10/7/2 + TN-C antibodies, scale bar = 150 μm . Note the attenuation of endothelial network formation, but maintenance of an adhered/spread morphology of individual endothelial cells with TN-C blockade. (E, F) Addition of exogenous TN-C protein to the collagen type I matrix disrupts endothelial tubular network formation and interfacing with epithelial structures. Z-stacks (thickness = 75–100 μm) depicting the effect of adding exogenous TN-C protein (10 $\mu\text{g}/\text{mL}$) at the time of hydrogel formation on endothelial tubular network formation (isolectinB4, green; A, scale bar = 150 μm) and interfacing with epithelial structures (cytokeratin, red; B, scale bar = 75 μm) following 96 h of culture.

integrate into a host tissue while maintaining their differentiated characteristics and rapidly establishing a perfused circulation, we utilized the renal capsule model in syngeneic C57/BL6 mice. In extending our previous studies,¹⁹ this setup allowed for combinatorial experiments implanting

organoids generated using wild-type FPCs into GFP-expressing hosts, which in combination with rhodamine-dextran infusion and cell tracer labeling of FPCs within the implanted constructs allowed us to determine donor versus host origin of perfused vascular structures.

FIG. 6. Different morphology, sprouting behavior, and local TN-C deposition in SHH- versus FGF-induced endothelial tubular networks. FGF10/7/2-treated (A/B, E/F) and SHH-treated (C/D, G/H) constructs cultured for 7 days, confocal micrographs for all conditions. (A) IsolectinB4 (magenta) labeling of FGF-10/7/2-treated constructs depicting filopodia of tip ECs, scale bar = 20 μ m. (B) Enlarged crop of boxed area in (A) highlighting filopodia. (C) IsolectinB4 (green) labeling of SHH-treated constructs, depicting attenuated EC morphology, lumen formation, and lack of sprouting tubule, scale bar = 40 μ m. (D) Enlarged crop of boxed area in (C). (E) TN-C (red) is seen in the endothelial basement membrane (BM) as yellow colocalization with LN (green), in the periendothelial matrix beyond the BM, and in the cytoplasm of neighboring mesenchymal/stromal cells, scale bar = 12 μ m. (F) TN-C (red) and LN (green) localization with DAPI counterstaining, scale bar = 10 μ m. Note the presence of TN-C in areas surrounding sprouting endothelial tips, and in the cytoplasm of surrounding mesenchymal and perivascular cells. (G) Low levels of TN-C (red) are seen as faint yellow colocalization with LN (green), and there is an absence of TN-C protein in the periendothelial matrix beyond the BM, DAPI nuclear stain (blue). Scale bar = 9.38 μ m. (H) TN-C (red) and LN (green), scale bar = 18.75 μ m. Note the absence of TN-C surrounding SHH-treated vessels and also from the cytoplasm of surrounding mesenchymal and perivascular cells.

As described in the “Materials and Methods” section, constructs were harvested after 5 days of *in vivo* culture beneath the renal capsule of syngeneic mice (9 days total including 4 days of *in vitro* preculture) and analyzed with routine histological stains and IHC of paraffin sections. Gross examination of a typical construct at the time of harvest showed the establishment of a perfused vasculature connected via a source emerging from the perinephric fat (Fig. 7A, inset). Constructs were easily excised from beneath the capsule, separated from the underlying kidney, and processed for histological examination. H&E staining demonstrated the presence of pulmonary-like tissue constructs containing glandular/sacculated epithelial structures, a loose mesenchyme/stroma, evidence for patent blood vessels, and the absence of a noticeable host inflammatory response (Fig. 7A).

The presence of a robust internal vascular supply is shown throughout a construct in H&E-stained sections taken from the upper region of the tissue block (Fig. 7B), which corresponds to a lateral side of the construct as viewed in the gross photograph inset of Figure 7A. Similarly, robust vascularization was observed in sections taken after sectioning through most of the tissue block. For example, the image shown in Figure 7C corresponds to tissue located at the contralateral side of the tissue section shown in image 7B, thus demonstrating uniform vascularization throughout

the entire disc-shaped constructs. Numerous red-blood-cell-containing vessels, ranging in size from arterioli (arrowheads in Fig. 7B, C) to capillaries (arrows in Fig. 7B, C), were observed throughout the constructs, as were vessels interfaced with epithelial structures, giving rise to structures reminiscent of nascent “capillary–alveolar interfaces” (Fig. 7B, C insets). Maintenance of epithelial differentiation during the engraftment period was demonstrated by proSpC expression in the cells lining alveolar-like glandular structures (Fig. 7D). In addition, we observed preservation of VEGF-A expression in epithelial and mesenchymal cells (Fig. 7E), as well as widespread localized deposition of TN-C in the ECM, especially surrounding glandular structures and tracks of apparent blood vessel growth (Fig. 7F).

To discern the donor versus host origin of cells lining the vascular structures, we first utilized a commercially available cell tracking dye CMTPX. Preliminary *in vitro* experiments demonstrated maintenance of fluorescence for up to 14 days in culture (data not shown). We consistently observed CMTPX signal in the cells lining perfused vessels (Fig. 7G, arrows). To further differentiate donor versus host origin of endothelial structures, without the caveat of possible uptake of leaked CMTPX dye during the engraftment period, we implanted constructs generated with wild-type cells into GFP-expressing C57/BL6 mice and infused

FIG. 7. *In vivo* engraftment of *in vitro* precultured fetal pulmonary organoids. All organoids were precultured in FGF10/7/2-containing medium for 4 days *in vitro* before implantation, maintained for 5 days *in vivo* before harvest, and processed for histological and immunohistochemical staining as described in the Materials and Methods section. (A) Hematoxylin and eosin (H&E) staining, scale bar = 200 μ m, 100 $\times$ original magnification; inset: gross photograph of engrafted organoid with visible surface vascular supply. (B and C) H&E staining of sections taken from opposite (contralateral) sides of the same construct illustrating vascularization throughout the entire thickness. Arteriolar-sized (arrowheads) and capillary-sized perfused vessels (arrows) as well as the presence of perfused capillaries interfaced with saccular epithelial structures (insets) are observed throughout in both sections, scale bars = 50 μ m, 400 $\times$ original magnification. (D) Immunohistochemical staining of prosurfactant protein C (proSpC, red) depicting the distal lung epithelial phenotype of cells lining saccular structures, scale bar = 50 μ m, 400 $\times$ original magnification. (E) Immunohistochemical staining of VEGF-A (green) demonstrating the maintenance of VEGF-A expression in epithelial and mesenchymal cells *in vivo* during the 5-day engraftment period, scale bar = 50 μ m, 400 $\times$ original magnification. (F) Immunohistochemical staining of tenascin-C (TN-C, red) depicting the subepithelial and subendothelial deposition patterns typically observed in our *in vitro* experiments (Figure 4), scale bar = 50 μ m, 400 $\times$ original magnification. (G) H&E staining and CellTracker™ CMTPX dye in the same section of a construct. The section was rehydrated and imaged for CMTPX fluorescence (orange-red) without mounting and subsequently processed for H&E staining. The donor origin of cells lining red blood cell-containing capillary structures is confirmed by the presence of CMTPX fluorescence in these cells (arrows), scale bar = 50 μ m, original magnification = 400 $\times$ (cropped). (H) Visualization of patent (perfused) vessels by the presence of TritC-dextran (DEX, orange-red) in the lumen of tubular structures; the donor (arrows) or host (arrowhead) origin of which was discerned by the presence or absence of green fluorescent protein (GFP, host mouse cells, green) expression in the cells lining patent vessels, scale bar = 50 μ m, 400 $\times$ original magnification. (I) GFP channel only from micrograph in (H) clearly showing the presence of invading host cells and an apparent capillary structure (arrowhead), scale bar = 50 μ m, 400 $\times$ original magnification.

fluorescent dextran (TritC-dextran) immediately prior to sacrifice to identify perfused vascular structures by fluorescence microscopy. The majority of the TritC-dextran-perfused vessels were not lined by GFP-expressing cells of host origin (Fig. 7H, arrows), although some host-derived vessels were observed (Fig. 7H, I, arrowhead). We were not able to unequivocally identify chimeric vessels containing both host and donor-derived endothelial cells in the plane of sectioning. This may be due to the fact that tubular endothelial networks may have fully assembled during the 96 h of preculturing *in vitro* (Figs. 2A and 5C) prior to implantation underneath the renal capsule. It is intriguing to speculate that the rapid establishment of perfused vascula-

ture in these constructs *in vivo* is at least partially achieved through anastomosis of endothelial networks preformed *in vitro* with the host circulation upon engraftment.

Discussion

Successful engineering of pulmonary tissue that is suitable for efficacious pharmaceutical/biologic screening or toxicity testing *in vitro*, and potentially for implantation/engraftment *in vivo*, will require integrating multiple areas of research, such as directed stem cell differentiation,^{6,7,9} cell bioprocessing,^{47,48} scaffold/matrix design and engineering,³ and factor-mediated approaches.^{18,20} Each of these

areas is part of the interdisciplinary mosaic that promotes our increased understanding of pulmonary regenerative biology.^{49,50} The major contribution of the current article to this mosaic is a deeper understanding of how exogenous FGF signals drive *de novo* fetal pulmonary alveolar tissue morphogenesis *in vitro*.

In vivo, the blood-air barrier is formed by interfacing of the distal capillary network and the sacculated terminal epithelial buds⁵¹; a process that requires temporal and spatial regulation of multiple overlapping signaling pathways.^{25,52–57} The central role of VEGF-A among these signals has been demonstrated in numerous experimental contexts.^{21,23,33,34,58–60} In line with findings from embryological studies, endothelial network morphogenesis in our *in vitro* constructs is critically dependent on VEGF-A, as evidenced by near-complete ablation of network formation with 2 µg/mL of sVEGFR1-Fc (Fig. 2D). Other experimental models have correlated decreased VEGF-A levels with impaired lung maturation and associated vascular abnormalities.^{34,61–63} Increased sVEGFR1 levels in the amniotic fluid of fetal rats resulted in impaired lung development characterized by reduced capillary density and increased endothelial cell apoptosis.⁶⁴ Taken together with data from the literature, our VEGF-A sequestration studies (Fig. 2) establish an essential role for parenchymal VEGF-A production in the orchestration of vascular development in engineered distal lung tissues *in vitro*.

The role of SHH in lung vascular development *in vivo* has largely been described in the context of regulating VEGF-A expression as a reciprocal mediator of FGF signaling.^{23,65} In limb bud development, SHH was shown to be downstream of FGFR2b,⁶⁶ the high affinity receptor for FGF-10 and -7. Both these FGFs are also expressed in lung epithelium *in vivo*^{25,67–69} as well as in our fetal pulmonary cell constructs,²⁰ suggesting that SHH signaling is a mediator of FGF-driven events. In support of this notion, the SHH pathway antagonist cyclopamine partially attenuated FGF10/7/2-induced endothelial morphogenesis in our 3D constructs (Fig. 3J, L), although not to the level of near-complete ablation observed with VEGF-A sequestration (Fig. 2), perhaps due to the continued presence of elevated levels of VEGF-A despite SHH inhibition (Fig. 3K).

Evidence that FGFs and SHH regulate vascular development *in vivo* through the induction of VEGF-A signaling, and not by directly affecting endothelial cells, comes from experiments in which endothelial-cell-restricted conditional deletion of FGFR1 and FGFR2 (loss of FGF-2 and -9 signaling⁷⁰), as well as Patched-1 (loss of SHH signaling), did not disturb lung vascular development.²³ Therefore, we posit that exogenous FGF10/7/2- and SHH-driven endothelial network morphogenesis in our constructs is mediated through endogenous VEGF-A production, rather than direct effects in endothelial cells. This notion further stresses the importance of an appropriate mixture of epithelial, mesenchymal, and endothelial cells in order to facilitate organotypic lung tissue development. In the context of our engineered tissue constructs, we speculate that exogenous FGFs (FGF10/7/2) act as “first domino” factors that facilitate a cascade of heterocellular interactions involving the action of diverse endogenous mediators, such as SHH, VEGF-A, and TN-C.

The critical role of TN-C in lung development *in vivo* has largely been deduced from histological studies^{37,38,43} and

embryonic lung explant cultures in the context of hypoxia²⁸ and TN-C gene deletion,³⁹ all of which demonstrated a positive regulatory role of TN-C in lung epithelial branching morphogenesis. Jones and colleagues demonstrated an indirect functional connection between TN-C and distal lung vascular development through the homeodomain transcription factor Prx-1, which is expressed in the mesenchyme and is required for pulmonary vascular development and TN-C gene expression.⁴⁰ In a model of developmental pulmonary hypoplasia, which leads to significant vascular abnormalities, TN-C was downregulated along with MMP-9.⁴¹ To the best of our knowledge, ours is the first study to demonstrate dual roles for TN-C in epithelial sacculization and endothelial network morphogenesis (Figs. 4–6) in engineered fetal distal lung tissue.

The functional role of TN-C in endothelial (and parallel epithelial) morphogenesis becomes evident upon neutralization of TN-C in the extracellular space with polyclonal antibodies, resulting in reduced epithelial sacculization (Fig. 5B), and a marked reduction in endothelial network morphogenesis (Fig. 5D), although the latter effect was less pronounced than that seen with VEGF-A sequestration (Fig. 2). The observation that individual ECs remained well spread and were viable under conditions of TN-C blocking (Fig. 5D) suggests that TN-C is a spatial refiner of VEGF-A-dependent endothelial tubular morphogenesis and network formation. TN-C has been implicated in guiding the extension of secondary septa during alveolarization,^{37,43} but our correlative observations suggest that it may also guide the apposition of epithelial and endothelial cells at the nascent alveolar-capillary interface (Fig. 4G–I). The importance of localized/focal TN-C deposition in our engineered constructs was further highlighted by the derailment of endothelial network formation observed upon exogenously and homogeneously admixing TN-C protein to the collagen type I hydrogel (Fig. 5E, F) at the time of cell embedding, thereby disrupting spatial TN-C gradients and patterns.

One of our most noteworthy findings is the observation that both SHH alone and the FGF10/7/2 cocktail potently induce endothelial network formation, however, with markedly different morphologies. Specifically, FGF10/7/2 induces the formation of networks with more branch points (Fig. 3), a distinctly “angiogenic” phenotype in extending tubules as evidenced by characteristic filopodia (Fig. 6), and the presence of copious amounts of TN-C in the perivascular ECM (Fig. 6)—all of which are markedly reduced or absent in SHH-induced endothelial networks. Taken together, these differential EC responses to SHH and FGF10/7/2 demonstrate the ability to fine tune vascular development in engineered tissues, and highlight the importance of selecting the right set of exogenous factors that elicit specific states of endogenous ECM synthesis, which in turn promote desired patterns of morphogenesis, that is, degree of angiogenic sprouting.

Following some significant breakthroughs in past few years, the focus of pulmonary tissue engineering has shifted to the use of decellularized lungs as scaffolds for the generation of lung tissue, with reports demonstrating proof of concept for whole lung organ engineering.^{3,71,72} While the use of cadaveric lung scaffolds may be a promising approach, there are numerous technical hurdles to overcome with the process of recellularization⁷³ and the resultant mechanical and physiological functionality of the engineered lungs,⁷⁴ as

well as potential immune reactions and some of the same donor availability issues facing organ transplantation. It is currently not known whether “old” or “sick” lungs can provide matrices suitable for the regeneration of healthy lungs.

As an alternative approach worthy of further investigation, especially in light of recent reports of compensatory lung growth in adult animal models⁵⁰ and humans,⁴⁹ we envision lung augmentation strategies aimed at providing a template for growth and functional assembly of healthy lung tissue prior to the progression of chronic lung disease to the point of necessitating a transplant to sustain life. Subpleural injections of hydrogel mixtures with or without cells could potentially accelerate and/or amplify expansion of the pre-existing, healthy alveolar epithelial and microvascular components. Our data from the renal capsule implantation model provide an early proof of concept that such hydrogel/cell-based constructs could be engineered to interface with the host circulation and maintain phenotypic characteristics established *in vitro* (Fig. 7).

These findings build on our previous report of *de novo* pulmonary-like tissue formation by freshly isolated FPCs injected subcutaneously in Matrigel plugs. In those studies we observed relatively little vascularization, and virtually no interfacing of patent vessels and epithelial structures, when FPCs were injected in Matrigel without the incorporation of an FGF2-loaded polyvinyl sponge in the plug.¹⁹ In this article we now demonstrate that implantation of *in-vitro*-precultured, collagen type I hydrogel-based organoids facilitates robust vascularization, including interfacing with epithelial structures, after 5 days *in vivo* without the need to incorporate exogenous FGF2. Rapid construct vascularization was aided by a large contribution of graft-derived patent vessels that anastomosed with the host vasculature and augmented host neovascularization. Importantly, the implants were able to maintain patterns of proSpC, VEGF-A, and TN-C expression established during *in vitro* culture in fetal pulmonary constructs interfaced with a living host. Evidence of angiogenic ingrowth from the host demonstrates that these implants could be exploited to modulate host tissue development and/or regeneration, since the only inductive signals, that is, from continued VEGF-A production (Fig. 7E), are provided by the cells within the constructs. In addition, the ability to induce the *de novo* formation of individual alveolar subunits with heterogeneous mixtures of cells and hydrogels lays the foundation for the engineering microtissue models (organoids) for applications in high-throughput screening.

In this article we provide some mechanistic understanding of the signals required for *de novo* distal pulmonary tissue formation *in vitro* (i.e., parallel epithelial and endothelial development) in engineered constructs comprised of an organotypic mixture of fetal lung cells. At the same time we also demonstrate the persistence of phenotypic characteristics observed *in vitro* and the establishment of a perfused vascular supply largely comprised of donor-derived endothelial structures upon *in vivo* engraftment. Similar system parameters could be applied to stem-cell-derived mixtures of progenitor cells in the development of pulmonary tissue constructs,⁴ or in the construction of “disease-in-a-dish” models from iPSC-derived cells,¹¹ which will require the ability to generate morphologically correct 3D lung tissue

structures, rather than monolayer cultures of cells. Our approach demonstrates that with an appropriate mixture of heterotypic cell populations and the use of defined factors that promote ECM synthesis, it may be possible to use simple scaffold designs that focus on providing permissive spatio-mechanical cues to facilitate cell-mediated tissue assembly.

At the same time, we also envision the development of more complex composite biomaterials for pulmonary tissue engineering that will encompass instructive spatial patterning of biochemical cues such as matrix ligands, rather than the homogeneous incorporation of an ECM component within a 3D matrix, to sculpt desired morphogenesis. In this context, fetal-pulmonary-cell-based systems will continue to be beneficial for building an understanding of the microenvironmental cues required to drive alveolar morphogenesis *in vitro*, while the stem cell field continues to work toward the derivation of the multiple epithelial, mesenchymal, and endothelial cell lineages required to build the distal lung.^{12,13,75,76}

In conclusion, we believe that harnessing crosstalk between endogenous networks of growth factor signals, such as the FGF/SHH/VEGF-A network, as well as focal patterns of synthesis and deposition of specific ECM molecules, such as TN-C, in concert with advances in scaffolding technologies and further progress in directed pulmonary differentiation of multifarious progenitor cells, will be essential stepping stones on the way to achieving the interdisciplinary goal of engineering complex functional human lung tissue for therapeutic and research purposes.

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Disclosure Statement

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