Self-Assembly of Prevascular Tissues from Endothelial and Fibroblast Cells Under Scaffold-Free, Nonadherent Conditions

Caitlin A. Czajka, PhD, and Christopher J. Drake, PhD

To advance the emerging field of bioengineered prevascularized tissues, we investigated factors that control primary vascular network formation in scaffold-free, high-density cell suspension-derived tissues. Fabricating primary vascular networks in a scaffold-free system requires endothelial cells (ECs) and extracellular matrix (ECM)-producing cells that act together to elaborate a permissive matrix. We report findings on the effects to vascular patterning induced by altering the ratio of human endothelial to human fibroblast cells. Analysis revealed that a 1:4 ratio of ECs to fibroblasts resulted in the synthesis of an ECM permissive for organization of primary vascular networks that recapitulated the pattern of primary vascular networks observed *in vivo*. Importantly this work highlighted the significance of tension in the organization of vascular networks in prevascularized tissues. To our knowledge our *in vitro* studies are the first to demonstrate the formation of two distinct vascular patterns in an initially homogenous culture system. Specifically, we demonstrate that within our constructs, vascular networks formed with distinct directional orientations that reflect self-assembly-mediated tension. Further, our studies demonstrate that treatment of prevascularized tissues with matrix-promoting factors such as transforming growth factor beta 1 (TGF β 1) increases tissue strength without altering vascular network patterning. Together, the ability to generate prevascularized tissues from human cells in scaffold-free systems and the ability to enhance the strength of the constructs with matrix-promoting factors represent advances to the potential translational utility of prevascularized tissues both as subcutaneous implants and in surgical scenarios requiring the application of tension to the tissue construct.

Introduction

MULTIPLE RECENT INVESTIGATIONS have evaluated the anastomotic ability of prevascular tissue constructs (tissues containing vascular networks or cells capable of forming networks). These works showed that prevascular tissues can rapidly anastomose with host vasculature upon subcutaneous implantation in an immune-compromised mouse model.^{1–5} These findings are important because without access to a vascular network, tissue engineering is limited by the inability to support the metabolic needs of tissues exceeding the limits of diffusion (2 mm).⁶ Although these studies used a variety of cell types, collectively the results indicate that stromal cells are required in combination with endothelial or endothelial-progenitor cells to establish vascular networks in Matrigel, fibrin, and collagen scaffolds. Each study reported successful anastomosis of the tissue constructs to host vasculature upon subcutaneous implantation into immune-compromised mice. Whereas these studies highlighted the utility of prevascularized tissue constructs, the use of scaffolds

such as Matrigel, collagen, and/or fibrin pose potential issue when considering translational use of prevascularized tissues in humans. To overcome this we investigated whether prevascularized tissues could be generated from human endothelial and fibroblast cells in a scaffold-free *in vitro* system.

Previously we have described principles that govern scaffold-free modular and cell-based tissue assembly *in vitro*.⁷ These findings established that the tissue cortical cytoskeleton serves as the dynamic backbone, integral to subsequent tissue morphogenesis, and that tissues derived from high-density cell suspensions form shape-appropriate tissue cortical cytoskeletons. With respect to small-caliber vascular engineering we focused on identifying a ratio of ECs to fibroblasts that provided a permissive matrix for the assembly of primary vascular networks. Primary vascular networks are the first vascular networks formed in the embryo as part of vasculogenesis and consist of both cords of angioblasts and lumenized capillary vessels.⁸ Concomitantly, we sought to produce tissues with sufficient strength to render constructs useful for subcutaneous implantation. To further explore the translational utility of

prevascularized constructs for surgical scenarios requiring the application of tension (i.e., sutures) we investigated whether treatment with growth and matrix-promoting factors (vascular endothelial growth factor [VEGF], transforming growth factor beta 1 [TGF β 1], retinoic acid [RA], and transglutaminase 2) could increase tissue strength without compromising vascular patterning.

Materials and Methods

Human cell subculture

Human umbilical vein endothelial cells (HUVECs) and adult normal human dermal fibroblasts (NHDFs) were purchased from Lonza. Human adipose microvascular endothelial cells (HAMECs) were purchased from ScienCell. Cells were cultured in 75 cm² BioLite vented culture flasks (ThermoFisher Scientific) in 37°C 5% CO₂ humidified incubators. HUVECs and HAMECs were grown in the Medium 200 (M200; Gibco) supplemented with low serum growth supplement (LSGS; Gibco) and 1% penicillin streptomycin solution (PS; Cellgro). NHDFs were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Corning), and 1% PS. Cells were passaged using 0.05% Trypsin/0.53 mM EDTA (Cellgro) and harvested when confluent.

Generation of agarose molds

Two percent w/v agarose (Fisher Scientific) was dissolved in MilliQ water, heated until molten, and nonadherent molds prepared by allowing agarose to harden around various (nonagarose) shapes within 3.5 cm Petri dishes. Linear molds were $\sim 0.9 \times 0.1 \times 0.5$ cm; toroidal molds were ~ 0.4 cm outer diameter $\times 0.2$ cm inner diameter $\times 0.2 \times 0.3$ cm high. Molds were equilibrated in media at 37°C and 5% CO₂ in a humidified incubator for 24 h before use.

Generation of tissue constructs

Approximately 900,000 cells were seeded into equilibrated linear molds and 750,000 cells into equilibrated toroidal molds. Ratios of cell types consisting of 0:1, 1:7, 1:4, 1:3, and 1:1 HUVECs:NHDFs or HAMECs:NHDFs were used such that the total cell number was held constant (e.g., $\sim 750,000$ cells per toroid). Molds were fully immersed in culture media (1:2 M200 + LSGS + PS:DMEM + FBS + PS) and cultures maintained in a 37°C 5% CO₂ humidified incubator for 3 days. For one set of experiments 1 mg/mL rat tail type I collagen (BD Biosciences) was added to the initial 1:3 HUVEC:NHDF cell suspension at the time of placement into the equilibrated agarose mold and cultures maintained for 3 days in a 37°C 5% CO₂ humidified incubator (data not shown). For sheet-like shapes, an approximately equivalent total cell density per unit volume was used according to the dimensions of the mold.

Treatment of toroids with matrix-promoting factors and transglutaminase 2

Initial 1:4 HAMEC:NHDF cell suspensions were treated with 300 ng/mL recombinant human vascular endothelial growth factor (VEGF₁₆₅; R&D Systems), 5 ng/mL or 25 ng/mL recombinant human TGF β 1 (R&D Systems), or 1 μ g/mL all-

trans RA (Sigma) in media at the time of seeding into equilibrated toroid molds. Media and supplemental factors were refreshed daily for the duration of culture (3 days). For one set of experiments, 1:4 HAMEC:NHDF cell suspension-derived tissues were treated with 300 ng/mL VEGF on day three of culture to assess ability to promote the remodeling of primary vascular networks (data not shown, see Discussion). For transglutaminase experiments: 50 μ g/mL transglutaminase 2 (gift from W.S. Argraves lab) was added to the culture media supplemented with 5 mM CaCl₂·2H₂O (Fisher Scientific) of day 3 1:4 HAMEC:NHDF-derived toroids for 6 h immediately before uniaxial tensile testing.

Uniaxial tensile testing

Toroids were removed from agarose molds after 3 days in culture and mounted onto a modified Bose ElectroForce 3100 testing instrument as previously described.⁷ In brief: a 0.1 mm/s vertical ramp displacement was used to stretch the inner diameter of toroids mounted on two cantilevers until failure. Load data was recorded using a 50g force transducer at 100 data points per second (WinTest software). Plots of load-displacement data were analyzed to obtain engineering stress σ and strain ϵ as follows: $\sigma_{eng} = F/2A_{cs}$ and $\epsilon_{eng} = \Delta L/L_o$, where F is the load the toroid exerts on the lower cantilever, A_{cs} is the initial cross-sectional area, ΔL is the change in specimen length (corresponding to cantilever displacement), and L_o is the initial specimen length (corresponding to the initial state stretch length). Young's modulus E in the linear elastic ($E = \sigma_{eng}/\epsilon_{eng}$) was calculated from stress-strain plots. Average modulus (kPa), ultimate tensile strength (kPa), and displacement at failure (mm) were reported with graphical data constructed using Microsoft Excel; error bars represent standard deviations.

Immunofluorescence labeling

Tissue constructs were rinsed with phosphate buffered saline (PBS; Sigma) before fixation in 4% paraformaldehyde (Sigma). Fixed tissue was rinsed thoroughly in PBS supplemented with Sodium Azide (EMD) (PBSA; NaN₃, 0.01% v/v) before 20 min incubation in Triton X-100 (Bio-Rad) permeabilization solution (Triton X-100, 0.1% v/v in PBSA). Permeabilized tissue was incubated in a blocking solution (Donkey Serum [Jackson ImmunoResearch], 5% v/v; bovine serum albumin [Sigma], 3% v/v in permeabilization solution) for 1 h at room temperature on an orbital shaker. Blocked tissue was incubated in primary antibodies diluted in blocking solution overnight at 4°C on rocker. After rinsing at room temperature in PBSA, tissue was incubated in secondary antibodies and Hoechst 33342 (1:1000; Invitrogen), diluted in blocking solution overnight at 4°C on rocker. After rinsing at room temperature in PBSA, samples were mounted whole on glass slides (Fisher Scientific) using Fluoro-Gel (Electron Microscopy Science). Primary antibodies used: rabbit anti-fibronectin (15 μ g/mL; Array Genetics), rat anti-human anti-CD31/PECAM (1:40; BD Pharmingen), rabbit anti-vimentin (1:100; abcam), rabbit anti-laminin (1:300; Sigma), and rabbit anti-collagen I (1:250; abcam). Secondary antibodies used: anti-rabbit and anti-rat cy3-, cy5-, and FITC-conjugated IgG produced in donkey (7:1000; Jackson ImmunoResearch). Other fluorescent-conjugated antibodies

used: Texas Red-X-conjugated Phalloidin (1:50; Molecular Probes).

Imaging

Stereo microscopic images of tissue formation were captured daily using a Dage-MTI CCD300-RC camera system and Scion ImagePC software. Z-stack (1.5 μ m step size) image arrays were acquired on a Leica TCS SP5 AOBS Confocal Microscope System (Leica Microsystems, Inc.) in sequential multichannel mode to minimize fluorescence signal mixing. Image stacks and subsets are presented as maximum projection images.

Statistical analysis

One-way analysis of variance and Bonferroni post-test calculations using Microsoft Excel, GraphPad, and VassarStats software were performed for uniaxial tensile testing data at a significance level of 0.05 (Supplementary Tables S1–S9; Supplementary Data are available online at www.liebertpub.com/tea). Error bars represent standard deviations on all graphs.

Results

In vitro generation of vascularized tissue constructs

In keeping with principles that govern scaffold-free modular and cell-based tissue assembly *in vitro*,⁷ cocultures of human fibroblasts and human ECs within spatially defined, nonadherent agarose molds (rods, toroids, squares) resulted in the formation of tissue constructs within 24 h (Fig. 1). Over the course of 3 days, rod-shaped tissues exhibited progressive lateral condensation (i.e., became shorter in the long-axis direction). Similarly, toroidal tissues exhibited progressive radial condensation and square tissue constructs decreased proportionately while retaining a mostly square shape. By day 3 in culture the fibroblast/EC constructs, regardless of shape, had formed tissues with sufficient mechanical properties to allow them to retain their

integrity upon removal from agarose molds and during manipulation with forceps.

Based on studies indicating a relationship between EC numbers and vascular patterning,^{8–10} we next evaluated the effects of varying the ratio of endothelial to fibroblast cells within high-density cocultures. Analysis of 1:4 EC to fibroblast rod constructs cultured for 3 days and then immunolabeled with antibodies to CD31 showed the formation of *in vivo*-like primary vascular networks extending throughout the entirety of the tissue construct and exhibiting directional alignment (Fig. 2).

To further evaluate the effects of altering the ratio of ECs to fibroblasts we generated 1:4 EC:fibroblast toroids and 1:1 EC:fibroblast toroids using nonadherent molds. As seen in Figure 3, increasing the ratio from 1:4 ECs:fibroblasts to 1:1 ECs:fibroblasts resulted in ECs organizing into a sheet-like endothelium with a concomitant absence of avascular spaces that define primary vascular networks. ECs within 1:1 EC:fibroblast toroids had a rounded or cobblestone appearance (Fig. 3A), whereas those in 1:4 EC:fibroblast toroids had a taut, elongated morphology and appeared to form polygonal networks (Fig. 3B). Changes in network patterning due to increasing the ratio of ECs:fibroblasts were reflected by concomitant changes in cytoskeleton organization of intermediate filaments and f-actin, as indicated by vimentin and phalloidin staining (Fig. 3, right).

Inherent to our approach to engineer prevascularized tissues using a scaffold-free system was the hypothesis that the correct ratio of ECs to extracellular matrix (ECM)-producing cells (i.e., fibroblasts) would lead to the synthesis of matrix permissive for primary vascular network assembly. Immunofluorescence analysis of 1:4 EC:fibroblast-derived rods after 3 days of culture showed presence of key ECM proteins, including fibronectin and laminin (Fig. 4). As shown in Figure 5, toroids immunolabeled with antibodies to type I collagen showed ratio-dependent differences. Type I collagen immunolabeling highlighted the cobblestone morphology of cells in 1:1 EC: fibroblast toroids, whereas in 1:4 EC:fibroblast and fibroblast-only toroids collagen had an elongated, fibrillar appearance.

FIG. 1. Stereo microscopic images showing tissues formed from high-density cell suspension within non-adherent molds. (A, B) Linear rod-shaped tissue, 1:4 HAMEC:fibroblast; 1 $\times$. (C) Sheet-like tissue, 1:4 HUVEC:fibroblast; 1.6 $\times$, insets 8 $\times$. (D) Toroid tissue, 1:4 HAMEC:fibroblast; 1.6 $\times$. Scale bars 1 mm. HAMEC, human adipose microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell.

FIG. 2. Confocal image projections of whole-mount 1:4 HUVEC:fibroblast-derived rod tissue illustrating primary vascular networks (CD31, green). Arrows denote directional orientations. Left and middle panels: 10 $\times$; right panel: 20 $\times$. Scale bars 100 μ m.

Biomechanical properties of prevascularized tissues generated in a scaffold-free system

The utility of prevascularized tissues generated in a scaffold-free system with respect to use as surgical tissue implants is related to their tissue strength. To investigate whether alterations in cell type ratios affected tissue biomechanical properties, we subjected toroidal constructs to a uniaxial tensile testing paradigm previously described.⁷ We found ratio-dependent differences in ultimate tensile strength and Young's modulus in toroids generated from differing cell ratios. Prevascularized 1:4 EC:fibroblast toroids were stronger, as determined from greater average ultimate tensile strength, than 1:1 EC:fibroblast and 1:7 EC:fibroblast toroids (Fig. 6A). Similarly, average Young's modulus was significantly greater in 1:4 EC:fibroblast toroids than 1:1 EC:fibroblast, 1:7 EC:fibroblast, and fibroblast-only toroids (Fig. 6B).

Matrix-promoting factors affect vascular network patterning and tissue biomechanical properties

Having demonstrated that altering the ratio of cell types resulted in changes to primary vascular network patterning, ECM presentation, and biomechanical tissue properties, we

next investigated whether we could enhance tissue strength and rapid fabrication without disrupting network patterning. We investigated whether factors known to affect neovascularization *in vivo* could enhance tissue strength and rapid fabrication without disrupting network patterning. To this end we generated toroids under nonadherent conditions using high-density 1:4 EC:fibroblast cell suspension in which media was supplemented with either VEGF,^{11,12} TGF β 1,¹³ or RA.¹⁴ The time course for these experiments was 3 days and each culture received fresh media and supplemental factors daily. As shown in Figure 7, addition of VEGF (300 ng/mL) resulted in an increase in the size (thickness) of the prevascularized tissues (compare Fig. 7B to 7A). VEGF-mediated changes in tissue size and vascular patterning were most readily observed when the toroids were allowed to relax through cutting. Comparing the boxed regions of the cut toroid images, it appears that VEGF treatment (Fig. 7B) resulted in a qualitative reduction in avascular space within 1:4 EC:fibroblast toroids.

In contrast to VEGF, treatment with RA or TGF β 1 did not noticeably affect vascular network morphologies compared to control (Fig. 8). Biomechanical analysis of toroids generated from RA- and TGF β 1-treated cultures revealed that treatment with 1 μ g/mL RA significantly decreased the

FIG. 3. Confocal image projections of whole-mount toroids illustrating how changing the ratio of HAMECs:fibroblasts in initial high-density cell suspension changes the primary vascular network morphologies in the resulting tissues. Images on left are CD31⁺ primary networks (green). Images on right are merged CD31 (green), vimentin (red), phalloidin (cyan), Hoechst (blue). (A) 1:1 HAMEC:NHDF toroid; (B) 1:4 HAMEC:NHDF toroid. Scale bar 50 μ m; all images 20 $\times$. NHDF, normal human dermal fibroblast.

FIG. 4. Confocal image projections of whole-mount 1:4 HUVEC:fibroblast-derived rod tissue illustrating presence of fibronectin (A) and laminin (B) ECM proteins on day 3 of culture. Nuclei are labeled with Hoechst (blue). Scale bar 100 μ m; all images 20 $\times$. ECM, extracellular matrix.

displacement at failure as compared to control conditions (Fig. 9). As shown in Figure 9, toroids treated with 5 ng/mL TGF β 1 had a significantly greater average ultimate tensile strength as compared to RA-treated toroids. To evaluate if TGF β 1 effects were dose-dependent, toroids were treated with either 5 or 25 ng/mL TGF β 1 over the course of 3 days in culture. Addition of 5 ng/mL, but not 25 ng/mL TGF β 1

was found to significantly increase average ultimate tensile strength (10.48 ± 0.11 vs. 7.71 ± 0.94 kPa) and average Young's modulus (26.28 ± 0.59 vs. 14.90 ± 1.13 kPa) compared to control, indicative of increased tissue strength (Fig. 10).

In addition to evaluating the effects of factors known to affect neovascularization *in vivo*, we also investigated

FIG. 5. Confocal image projections of whole-mount toroids illustrating how changing the ratio of HAMECs:fibroblasts in initial high-density cell suspension changes the presentation of type I collagen. (A) type I collagen; (B) type I collagen (red), Hoechst (blue). Scale bar 50 μ m; all images 40 $\times$.

FIG. 6. Graphs depicting average ultimate tensile strengths and displacements at failure (**A**) and average Young's modulus (**B**) from biomechanical analysis of high-density cell suspension-derived toroids. 1:4 HAMEC:fibroblast toroids are stronger than 1:1 and 1:7 HAMEC:fibroblast-derived toroids in terms of significantly greater ultimate tensile strength and displacement at failure (**A**), and 1:4 HAMEC:fibroblast toroids have a larger average Young's modulus than fibroblast-only, 1:7 HAMEC:fibroblast, and 1:1 HAMEC:fibroblast toroids (**B**). * $p < 0.05$, one-way ANOVA, Bonferroni post test. Error bars represent standard deviations. ANOVA, analysis of variance.

whether the addition of exogenous transglutaminase 2 (a cross-linking agent known to affect type I collagen) would increase toroid strength. On day 3 of culture, toroids generated from 1:4 EC:fibroblast cell suspension were treated for 6 h with 50 $\mu\text{g}/\text{mL}$ transglutaminase 2. Following treat-

ment, the toroids were immediately subjected to biomechanical analysis. As Figure 10 indicates, treatment with transglutaminase 2 did not significantly alter average Young's modulus (Fig. 10B), displacement at failure (Fig. 10A), nor ultimate tensile strength (Fig. 10A) as compared

FIG. 7. Confocal image projections of CD31-labeled whole-mount toroid tissues illustrating how the addition of exogenous VEGF (**B**) increases tissue size. (**A**) 1:4 HAMEC:NHDF; (**B**) 1:4 HAMEC:NHDF + 300 ng/mL VEGF. White boxed regions illustrate qualitative reduction in avascular space upon treatment with VEGF. Scale bar 50 μm ; all images 40 $\times$. VEGF, vascular endothelial growth factor.

FIG. 8. Confocal image projections of CD31-labeled whole-mount toroids illustrating how the addition of exogenous retinoic acid (RA) and transforming growth factor beta 1 (TGFβ1) affect primary vascular network patterning. (A) 20×; (B) 40×. Scale bars 50 μm.

to untreated toroids. No significant change to vascular network morphology was noted (data not shown).

Discussion

Primary vascular network formation requires specific ratio of cell types

The work reported in this study investigated the feasibility of bioengineering prevascularized human tissues un-

der scaffold-free conditions. The minimum requirements for the generation of primary vascular networks are ECs and synthetic cells that together produce an ECM. Fibroblasts are an appropriate synthetic cell for *in vitro* network formation as they are known to enhance EC viability *in vitro* and are an abundant cell source. The study of Germain *et al.*¹⁵ evaluating network formation in cocultures of fibroblasts and ECs seeded into collagen-based matrices suggests that ECs require physical and/or biochemical interaction with fibroblasts to participate in appropriate vascular network assembly.

The generation of prevascularized tissues under nonadherent, scaffold-free conditions has two major components. The first and not directly related to primary vascular network formation is the behavior of cells in nonadherent culture as it pertains to the formation of the gross shape of a tissue. In Czajka *et al.*⁷ we report on tissue morphogenesis in scaffold-free, nonadherent culture and discuss the organization of the tissue cortical cytoskeleton. In the current study we observed that changing the relative ratio of cell types under these same scaffold-free, nonadherent conditions alters the kinetics of tissue generation, with fibroblast-only tissues showing the fastest lateral condensation and EC-only showing little and, therefore, slow condensation (data not shown). That tissues composed of different ratios of cell types exhibit different kinetics reflects differences among cell types' abilities to establish and contract the tissue cortical cytoskeleton in a coordinated manner.

Primary vascular network formation in scaffold-free, nonadherent cultures is governed by an interrelated set of rules related to the cell type(s) and numbers used for tissue fabrication. Immunofluorescence analysis of cell suspension-derived rods and toroids revealed that a 1:4 ratio of ECs to fibroblasts was conducive to the development of capillary-like vascular networks throughout the tissues. This finding was empirical. Suspensions containing ratios of 1:1 ECs: fibroblasts cultured under nonadherent conditions formed

FIG. 9. Graph depicting average ultimate tensile strengths and displacements at failure from biomechanical analysis of high-density cell suspension-derived toroids. RA-treated toroids illustrated significantly decreased average displacement at failure compared to control 1:4 HAMEC:fibroblast toroids (#) and significantly lower average ultimate tensile strength as compared to TGFβ1-treated toroids (*). $p < 0.05$, one-way ANOVA, Bonferroni post test. Error bars represent standard deviations.

FIG. 10. Graphs depicting average ultimate tensile strengths and displacements at failure (**A**) and average Young's modulus (**B**) from biomechanical analysis of high-density cell suspension-derived toroids. Treatment with 5 ng/mL TGFβ1 significantly increased average ultimate tensile strength compared to untreated control toroids (**A**), and significantly greater average Young's modulus compared to control and transglutaminase-treated toroids (**B**). * $p < 0.05$, one-way ANOVA, Bonferroni post test. Error bars represent standard deviations.

tissues that lacked avascular space (too many ECs), whereas ratios of 1:7 ECs:fibroblasts formed EC clusters, but failed to form primary vascular networks (not enough ECs, data not shown). That changes to vascular patterning resulted from a relatively small change (i.e., changing the ratio of cell types from 1:4 to 1:7 without changing the total cell density) is in accord with findings demonstrating that vascular network formation is a tightly conserved process.^{8,9,12,16} Differential network patterning resulting from different ratios of cell types was also supported by previous studies investigating the relationship between the EC mitogen VEGF and embryonic vasculogenesis.^{10–12,17}

While observing that CD31⁺ networks spanned the entirety of the 1:4 EC:fibroblast-derived tissues, it was surprising to notice distinct directional organization specific to regions within the tissues. Specifically for rod constructs, networks organized perpendicular to the direction of lateral condensation within the central region of the construct and parallel to the tissue-media border at the periphery of the construct (Fig. 2). The organization of networks running parallel to the tissue-media border was noted in toroidal and sheet-like tissues as well. That the pattern runs parallel to the surface of the tissue, regardless of tissue shape, confirms that high-density cell suspension-based *in vitro* tissue morphogenesis under nonadherent conditions is influenced by surface tension, which is in keeping with our previous insight into the formation of the tissue cortical cytoskeleton.⁷

Directional alignment of endothelial networks within tissues derived *in vitro* has not been explored in detail. Previous uses of ECs to generate tissues under nonadherent conditions have used entirely ECs, EC coating on spheroids of another cell type, or a 1:1 ratio with another cell type (fibroblasts, smooth muscle cells).^{18–21} Our observation that altering the ratio of cell types in the initial cell suspension altered the sorting behavior, whereas also resulting in the formation of *in vivo*-like vascular network patterns, was a novel finding. Stated another way: without changing the strength of cell-cell cadherin junctions or total cell density, we were able to influence EC patterning within a scaffold-

free, self-assembled tissue construct simply by altering the ratio of cell types. Importantly, the results presented in this study describe the formation of generic prevascular networks. ECs are known to have unique characteristics depending upon their tissue of origin²²; however, that we obtained similar results using two types of ECs (HUVCEs and HAMECs) illustrates that the tissue of origin and size of vessels the cells were obtained from do not dictate their ability to function as pseudo-angioblasts during vascular network formation within our culture system.

Perhaps the most striking and surprising aspect of this work was the rapid time frame during which cells generated a supportive ECM. By culturing the cells at high density and failing to provide exogenous matrix we hypothesized that these conditions would necessitate and/or facilitate the synthesis of ECM. It was, therefore, gratifying to see the presence of laminin, type I collagen, and fibronectin after 3 days of culture (Figs. 4 and 5). Laminin and type I collagen appeared to be expressed rather ubiquitously throughout the tissues, whereas fibronectin appeared to be enriched at the tissue-media border zone (Fig. 4). Laminin is noted to be restricted to the basement membrane of adult blood vessels,²³ however, the global ECM expression pattern we noted in these studies is consistent with pan-laminin expression observed in embryoid bodies,²⁴ suggesting EC:fibroblast cell suspension cultured under nonadherent conditions elaborates embryonic or provisional rather than adult ECM. All three of these ECM proteins have been shown to be present in the vascularized regions *in vivo*.^{25–28}

In addition to affecting vascular patterning (Fig. 3), tissue cortical cytoskeleton organization (Fig. 3), and presentation of ECM components (Figs. 4 and 5), altering the ratio of cell types affected the biomechanical properties of the resultant tissues. This suggests that the cross-talk between cell types during tissue formation is influenced by the relative number of cell types and by the resulting matrix production. It is noteworthy that the 1:4 EC:fibroblast constructs were optimal for vascular network formation and exhibited the greatest average ultimate tensile strength (Fig. 5A) and

Young's modulus (Fig. 5B). Perhaps these findings reflect the interrelation between structure and function. In support of this, we showed that the fibroblast-only tissues showed a statistically smaller average Young's modulus (Fig. 5B). Based on our data it would appear that the cross-talk between ECs and fibroblasts modulates the synthetic capacity of the fibroblasts to alter matrix production and resultant tissue material properties. Regardless of the underlying mechanisms, our findings suggest that vascularized tissues generated from 1:4 ECs:fibroblasts have the most favorable properties for future use as tissue implants.

The medical utility of prevascularized tissue constructs fabricated using a scaffold-free approach would benefit from an enhancement of tissue strength, allowing constructs to be sutured into a variety of anatomical sites. Based on the rapid elucidation of matrix and having evidence for the production of type I collagen in our constructs, we hypothesized that the introduction of exogenous type I collagen might produce a stronger tissue. However, tissues that formed from high-density cell suspensions with the addition of exogenous collagen (1 mg/mL) showed altered vascular network patterning and delayed tissue maturation (data not shown). This observation suggested that cell-cell contact and step-wise elucidation of a complex ECM was essential for proper vascular network formation, and reinforced our decision to pursue a scaffold-free method.

An alternative approach to enhance the tissue strength of prevascularized tissue constructs is to introduce factors into the culture system that are known to affect vascular network formation and/or ECM elaboration. To this end we evaluated the ability of exogenous VEGF, TGF β 1, and RA to alter tissue strength. As a known EC proliferative agent it was not surprising that VEGF-treatment increased gross tissue size (Fig. 7B), nor that VEGF altered the resulting vascular patterning. That day 3 constructs exhibited evidence of fusion upon incubation with VEGF (data not shown) suggests that *in vitro* primary vascular network formation closely mimics the steps of *in vivo* vasculogenesis.¹¹ It should be noted that VEGF-mediated increase in EC numbers could have also affected ECM synthesis and composition, as altering the EC:fibroblast ratio could alter cellular cross-talk and thus synthetic capacity of the fibroblasts leading to a change in tissue size.

That treatment with RA did not seem to affect vascular patterning is in keeping with *in vivo* data illustrating that the formation of primary vascular plexuses is not altered in RA-treated embryos, but subsequent remodeling of plexuses into larger-caliber vessels is impeded.⁹ Our finding that RA-treatment led to a significantly less strong (as measured through UTS, Fig. 9) tissue than TGF β 1-treatment suggests that, while an appreciable difference in network patterning was not observed as a result of RA-treatment, the overall integrity of the tissue was compromised. This finding was unexpected as RA has been shown to induce fibroblast proliferation and synthesis of type I collagen.¹⁴

The maintenance of normal vascular patterns in TGF β 1-treated tissues was also unexpected, as TGF β 1 is known to affect fibroblasts' ability to synthesize type I collagen and is also a known fibroblast mitogen.¹³ Evidence of increased average ultimate tensile strength and Young's modulus upon treatment with the 5 ng/mL dose of TGF β 1 (Fig. 10) sug-

gests that TGF β 1-treatment is a means to increase tissue strength without sacrificing vascular network patterning. That increasing the dose of TGF β 1 did not correspond to a significant increase in tissue strength suggests that TGF β 1-induced changes to tissue biomechanical properties underlying increased tissue strength are neither linear nor dose-dependent. This observation suggests that TGF β 1 treatment initiates a complex change to the cellular and/or ECM components of forming tissues, and further investigation into the mechanism by which TGF β 1 affects tissue material properties is merited.

Given the fact that our cultures elaborated type I collagen, we sought an alternative approach to capitalize on the inherent biomechanical properties of type I collagen rather than adding exogenous collagen, which produced unfavorable results and went against our scaffold-free approach. Following our paradigm that organization of an ECM permissive for primary vascular network formation is a complex step-by-step process, we allowed the ECM to be elaborated before treatment of prevascularized tissues with transglutaminase 2. Analysis of treated prevascularized toroidal tissue constructs did not show a significant effect on average Young's modulus (Fig. 10B). This was surprising as transglutaminases are known to initiate collagen cross-linking and, by association, increase tissue strength: transglutaminase-treated collagen shows enhanced resistance to proteolytic degradation and increased rigidity.²⁹ These changes in biomaterial properties would be expected to show an associated decrease in elasticity (i.e., increased Young's modulus, increased ultimate tensile strength, and decreased displacement at failure). Transglutaminase-treated toroids did, however, show trends toward increased average displacement at failure and increased average ultimate tensile strength (Fig. 10A), of which only increased average ultimate tensile strength is consistent with increased tissue strength. There are multiple potential explanations as to why treatment with transglutaminase did not increase tissue strength in this experiment including: dose, duration of treatment, time at which transglutaminase was introduced into the culture, and the culture media itself (i.e., the availability of calcium, a cofactor that regulates transglutaminase activity).³⁰ Given the biomaterial properties of type I collagen, we believe further investigation into enhancing cross-linking is warranted.

In conclusion, we have provided data showing that isolated adult ECs are capable of forming primary vascular networks within self-assembled tissue constructs when cocultured with fibroblasts in the absence of exogenous matrix. Coculture of ECs and fibroblasts at a 1:4 ratio synthesize ECM permissive to primary vascular network formation concomitant with *in vitro* primary vascular network formation, resulting in tissues capable of physical manipulation and biomechanical analysis. Overall, the use of high-density cell suspensions containing endothelial and fibroblast cells seems a promising approach to address the need for self-assembled, prevascularized tissues.

Acknowledgments

The authors thank Dr. Michael J. Yost for his review of this article. This work was supported by NIH R01

HL080168 (C.J.D.), NSF EPSCoR RII EPS-0903795 (C.J.D.), and NIH predoctoral fellowship T32 HL007260 (supporting C.A.C., awarded to Dr. Donald R. Menick, MUSC). This study used the Morphology, Imaging, and Instrumentation Core, which is supported by NIH-NIGMS P30 GM103342 to South Carolina COBRE for Developmentally Based Cardiovascular Diseases.

Disclosure Statement

No competing financial interests exist.

References

1. Ferriera, L.S., Gerecht, S., Shieh, H.F., Watson, N., Ruppnick, M.A., Dallabrida, S.M., Vunjak-Novakovic, G., and Langer, R. Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle-like cells and form vascular networks *in vivo*. *Circ Res* **101**, 286, 2007.
2. Melero-Martin, J.M., De Obaldia, M.E., Kang, S.Y., Zhan, A.Z., Yuan, L., Oettgen, P., and Bischoff, J. Engineering robust and functional vascular networks *in vivo* with human adult and cord blood-derived progenitor cells. *Circ Res* **103**, 194, 2008.
3. Chen, X., Aledia, A.S., Popson, S.A., Him, L., Hughes, C.C.W., and George, S.C. Rapid anastomosis of endothelial progenitor cell-derived vessels with host vasculature is promoted by a high density of cotransplanted fibroblasts. *Tissue Eng Part A* **16**, 585, 2010.
4. Kang, K.T., Allen, P., and Bischoff, J. Bioengineered human vascular networks transplanted into secondary mice reconnect with the host vasculature and re-establish perfusion. *Blood* **118**, 6718, 2011.
5. Traktuev, D.O., Prater, D.N., Merfeld-Clauss, S., Saneevaiah, A.R., Saadatizadeh, M.R., Murphy, M., Johnstone, B.H., Ingram, D.A., and March, K.L. Robust functional vascular network formation *in vivo* by cooperation of adipose progenitor and endothelial cells. *Circ Res* **104**, 1410, 2009.
6. Griffith, C.K., Miller, C., Sainson, R.C., Calvert, J.W., Jeon, N.L., Hughes, C.C., and George, S.C. Diffusion limits of an *in vitro* thick prevascularized tissue. *Tissue Eng* **11**, 257, 2005.
7. Czajka, C.A., Mehez, A.N., Trusk, T.C., Yost, M.J., and Drake, C.J. Scaffold-free tissue engineering: organization of the tissue cortical cytoskeleton and its effects on tissue shape. *Ann Biomed Eng* **42**, 1049, 2014.
8. LaRue, A.C., Mironov, V.A., Argraves, W.S., Czirok, A., Fleming, P.A., and Drake, C.J. Patterning of embryonic blood vessels. *Dev Dyn* **228**, 21, 2003.
9. LaRue, A.C., Argraves, W.S., Zile, M.H., and Drake, C.J. Critical role for retinol in the generation/differentiation of angioblasts required for embryonic blood vessel formation. *Dev Dyn* **230**, 666, 2004.
10. Giles, P.B., Candy, C.L., Fleming, P.A., Owens, R.W., Argraves, W.S., and Drake, C.J. VEGF directs newly gastrulated mesoderm to the endothelial lineage. *Dev Biol* **279**, 169, 2005.
11. Drake, C.J., and Little, C.D. Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization. *Proc Natl Acad Sci U S A* **92**, 7657, 1995.
12. Drake, C.J., LaRue, A., Ferrara, N., and Little, C.D. VEGF regulates cell behavior during vasculogenesis. *Dev Biol* **224**, 178, 2000.
13. Tan, A.B., Kress, S., Castro, L., Sheppard, A., and Raghunath, M. Cellular re- and de-programming by micro-environmental memory: why short TGF- β 1 pulses can have long effects. *Fibrogenesis Tissue Repair* **6**, 12, 2013.
14. Varani, J., Mitra, R.S., Gibbs, D., Phan, S.H., Dixit, V.M., Mitra, R., Jr., Wang, T., Siebert, K.J., Nickoloff, B.J., and Voorhees, J.J. All-trans retinoic acid stimulates growth and extracellular matrix production in growth-inhibited cultured human skin fibroblasts. *J Invest Dermatol* **94**, 717, 1990.
15. Germain, L., Remy-Zolghardi, M., and Auger, F. Tissue engineering of the vascular system: from capillaries to larger blood vessels. *Med Biol Eng Comput* **38**, 232, 2000.
16. Iruela-Arispe, M.L., and Davis, G.E. Cellular and molecular mechanism of vascular lumen formation. *Dev Cell* **16**, 222, 2009.
17. Drake, C.J., and Little, C.D. VEGF and vascular fusion: implications for normal and pathological vessels. *J Histochem Cytochem* **47**, 1351, 1999.
18. Foty, R.A., Pflieger, C.M., Forgacs, G., and Steinberg, M.S. Surface tensions of embryonic tissues predict their mutual envelopment behavior. *Development* **122**, 1611, 1996.
19. Kelm, J.M., Djonov, V., Ittner, L.M., Fluri, D., Born, W., Hoerstrup, S.P., and Fussenegger, M. Design of custom-shaped vascularized tissues using microtissue spheroids as minimal building units. *Tissue Eng* **12**, 2151, 2006.
20. Kelm, J.M., Lorber, V., Snedeker, J.G., Schmidt, D., Brogini-Tenzer, A., Weissstanner, M., Odermatt, B., Mol, A., Zund, G., and Hoerstrup, S.P. A novel concept for scaffold-free vessel tissue engineering: self-assembly of microtissue building blocks. *J Biotechnol* **148**, 46, 2010.
21. Dean, D.M., Napolitano, A.P., Youssef, J., and Morgan, J.R. Rods, tori, and honeycombs: the directional self-assembly of microtissues with prescribed microscale geometries. *FASEB J* **21**, 4005, 2007.
22. Thorin, E., and Shreeve, S.M. Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol Ther* **78**, 155, 1998.
23. Hallmann, R., Horn, N., Selg, M., Wendler, O., Pausch, F., and Sorokin, L.M. Expression and function of laminins in the embryonic and mature vasculature. *Physiol Rev* **85**, 979, 2005.
24. Jakobsen, L., Domogatskaya, A., Tryggvason, K., Edgar, D., and Claesson-Welsh, L. Laminin deposition is dispensable for vasculogenesis but regulates blood vessel diameter independent of flow. *FASEB J* **22**, 1530, 2005.
25. Schoen, F.J., and Mitchell, R.N. Tissues, the extracellular matrix, and cell-biomaterial interactions. In: Ratner, B.D., Schoen, F.J., and Lemons, J.E., eds. *Biomaterials Science: An Introduction to Materials and Medicine*. San Diego, CA: Elsevier Academic Press, 2004, pp. 260–281.
26. Masuda, H., Hosokawa, N., and Nagata, K. Expression and localization of collagen-binding stress protein Hsp47 in mouse embryo development: comparison with types I and II collagen. *Cell Stress Chaperones* **3**, 256, 1998.
27. Nagata, K. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol* **16**, 379, 1998.

28. Cross, V.L., Zheng, Y., Won Choi, N., Verbridge, S.S., Sutermeister, B.A., Bonassar, L.J., Fischback, C., and Stroock, A.D. Dense type I collagen matrices that support cellular remodeling and microfabrication for studies of tumor angiogenesis and vasculogenesis *in vitro*. *Biomaterials* **31**, 8596, 2010.
29. Lorand, L., and Graham, R.M. Transglutaminases: cross-linking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* **4**, 140, 2003.
30. Griffin, M., Casadio, R., and Bergamini, C.M. Transglutaminases: nature's biological glues. *Biochem J* **368**, 377, 2002.

Address correspondence to:

Christopher J. Drake, PhD

Department of Regenerative Medicine and Cell Biology

Medical University of South Carolina

171 Ashley Ave., BSB 626, MSC 508

Charleston, SC 29425

E-mail: drakec@musc.edu

Received: April 1, 2014

Accepted: July 23, 2014

Online Publication Date: October 9, 2014