REVIEW ARTICLE

Endothelial Progenitor Cells for the Vascularization of Engineered Tissues

Erica B. Peters, PhD

Self-assembled microvasculature from cocultures of endothelial cells (ECs) and stromal cells has significantly advanced efforts to vascularize engineered tissues by enhancing perfusion rates *in vivo* and producing investigative platforms for microvascular morphogenesis *in vitro*. However, to clinically translate prevascularized constructs, the issue of EC source must be resolved. Endothelial progenitor cells (EPCs) can be noninvasively supplied from the recipient through adult peripheral and umbilical cord blood, as well as derived from induced pluripotent stem cells, alleviating antigenicity issues. EPCs can also differentiate into all tissue endothelium, and have demonstrated potential for therapeutic vascularization. Yet, EPCs are not the standard EC choice to vascularize tissue constructs *in vitro*. Possible reasons include unresolved issues with EPC identity and characterization, as well as uncertainty in the selection of coculture, scaffold, and culture media combinations that promote EPC microvessel formation. This review addresses these issues through a summary of EPC vascular biology and the effects of tissue engineering design parameters upon EPC microvessel formation. Also included are perspectives to integrate EPCs with emerging technologies to produce functional, organotypic vascularized tissues.

Keywords: endothelial progenitor cells, vascularization, angiogenesis, vasculogenesis

Introduction

FOR THE PAST DECADE, the vascularization of engineered tissues to repair or replace failing organs has been a major focus in the regenerative medicine community.^{1–5} The vasculature consists of an organized, hierarchical network containing arteries, arterioles, capillaries, venules, and veins that ensure proper tissue function by delivering oxygen and nutrients while removing waste.^{6,7} Without perfusion from embedded microvasculature, the thickness of *in vitro* engineered tissue constructs is limited to the oxygen diffusional limit, which is $\sim 200\ \mu\text{m}$.^{8–10} In addition, host vessel ingrowth is limited to tens of microns per day, requiring several days to weeks to fully vascularize a several hundred micron-thick construct.^{11,12} The time required for vascular ingrowth can form oxygen gradients within the construct, producing hypoxic regions and causing necrosis in the center of the tissue, as well as nonuniform tissue integration and cell differentiation.^{2,3,13}

Incorporating cells that secrete angiocrine factors within the scaffold, such as endothelial cells (ECs) cocultured with mural cell precursors or fibroblasts, can induce *de novo* blood vessel development to create capillary networks and generate a prevascularized construct.^{14–17} These self-assembled capillary networks significantly reduce the time to anasto-

mose with host vasculature and enhance construct viability in comparison to implants of nonvascularized tissue constructs.^{14,18,19} Despite these significant advances, the source of ECs remains an issue.^{3,20,21} Isolation of tissue-specific ECs requires additional, invasive surgeries for the patient. As well, fully differentiated cells have limited expansion potential and may lack responsiveness to growth factors and cytokines, impeding integration with vascular and parenchymal cells, which allows recapitulation of the intricate vascular patterns that result from intimate cell associations.^{22–24}

The cell of greatest interest in vascularizing engineered tissues is the endothelial progenitor cell (EPC) because of its potential to differentiate into all capillary niches, such as the continuous capillaries in skeletal muscle, fenestrated capillaries in endocrine glands, and discontinuous capillaries in the liver.^{25,26} Furthermore, EPCs may be noninvasively isolated from recipients of tissue-engineering therapy through adult peripheral blood and umbilical cord blood, as well as derived from human-induced pluripotent stem cells (hiPSCs), avoiding immunogenicity concerns.^{27–30} EPCs form vascular structures *in vitro* and *in vivo*, possess permeability values similar to vessel-derived endothelium, and can outperform vascular-derived endothelium in forming vascular networks.^{15,31–36} In addition to their use in

therapeutic angiogenesis, vascularized engineered tissues provide *in vitro* platforms for personalized medicine.³⁷ For example, utilizing autologous EPCs to develop vasculature *in vitro* can identify optimal drug combinations, tailored to the patient's unique genetic background.

Still, the identity and differentiation mechanisms of EPCs are not well understood, likely due to a lack of standardization in their isolation and characterization.³⁸ Heterogeneity is also present in the vasculogenic potential of EPC subsets and in the ability of tissue-specific stromal and parenchymal cells to promote EPC microvessel formation.^{39–44} As a result, wide variation exists in the reported engraftment potential of EPCs to host vasculature.^{45–48} This review aids the tissue engineers in their efforts to utilize EPCs by clarifying EPC identity and characterization, reviewing the current understanding of EPC vascular morphogenesis and differentiation, and summarizing key design parameters to generate EPC microvessels *in vitro*. Also provided are perspectives to incorporate existing knowledge of EPCs with advances in materials science and genetic engineering to enable the next generation of tissue vascularization research.

Defining EPCs

The controversy surrounding EPC definition can be traced back to the 1917 observations of blood vessel development in the chick embryo by Florence Sabin.^{49,50} Sabin described angioblasts, which are cells derived through fibroblast growth factor (FGF-2 or bFGF) induction of the mesoderm, as giving rise to primitive erythroblasts enclosed by an EC border, known as blood islands, which formed primitive vascular networks that further developed into arteries and veins.^{49,51} The erythroblasts and ECs of the blood islands appeared to form from within the angioblasts, prompting Sabin to conclude that the angioblasts could produce blood plasma and red blood cells, as well as ECs.⁴⁹ This discovery led to the widely accepted hypothesis that hematopoietic stem cells and EPCs arose from a common precursor, the hemangioblast.^{52,53} Yet, subsequent observations utilizing fate mapping of murine embryonic cell populations *in situ* demonstrated that erythropoietic progenitors arose independently of ECs.⁵⁴ Similar findings from the developing vasculature within murine embryos are displacing the idea of a hemangioblast with the concept that while hematopoiesis and vasculogenesis occur almost simultaneously and in close proximity, the processes originate from distinct mesodermal precursors.^{25,50,55}

Nonetheless, the hypothesis that hematopoietic stem cells and EPCs arose from a common hemangioblast precursor led to the seminal work of Asahara in 1997 and Rafii in 1998, which proposed that angioblasts, the endothelial precursor cells, could be isolated from human peripheral blood and bone marrow utilizing markers for hematopoietic stem cells, CD34, and ECs, CD34, and Flk-1/KDR.^{56,57} Specifically, Asahara showed that adherent CD34 antibody-captured mononuclear cells (CD34⁺ MNCs) possessed hematopoietic lineage through CD45 expression (27.2% ± 2.2%), as well as endothelial lineage through the expression of CD31 (71.5% ± 7.1%), Flk-1 (35.8% ± 8.8%), Tie-2 (54.6% ± 14.2%), E-selectin (9.0% ± 3.0%), and >80% uptake of DiI-labeled acetylated low-density lipoprotein (Fig. 1A).

Furthermore, by demonstrating that CD34⁺ MNCs could assist in therapeutic angiogenesis on reinjection to ischemic tissue, Asahara catalyzed the idea of utilizing adult peripheral blood EPCs as an autologous cell therapy for patients afflicted with cardiovascular disease.^{56,58,59} Although the work of Asahara *et al.* was promising, it did not thoroughly confirm EPC identity of the examined CD34⁺ cell population.⁵⁶ As a result, studies attempting to replicate and compare Asahara's original findings to obtain a population of true EPCs, summarized in Table 1, have spanned nearly two decades of research. At present, a combination of surface antigen expression for EC markers and lack of expression for hematopoietic cell markers, as well as functional assays assessing proliferative and vasculogenic potential, is needed to define EPCs, summarized in Table 2. The rationale for these requirements is explained by a review and comparison of reported EPCs.

Bone marrow-derived EPCs

Beginning in 1998, Rafii and colleagues expanded on Asahara's work by demonstrating that vascular endothelial growth factor (VEGF or VEGF-A) is critical to developing EC colonies from CD34⁺ MNCs derived from peripheral blood, bone marrow, and cord blood based on von Willebrand factor (vWF) expression and EC monolayer formation (Fig. 1B).⁵⁷ Yet, CD34 cannot serve as a unique EPC marker because it is expressed on both hematopoietic progenitor cells and mature ECs, as well as fibroblasts and epithelial cells.⁶⁰ To overcome this lack of specificity, in 2000, Gehling *et al.* tried to isolate peripheral blood EPCs using antibodies for CD133/AC133, a selective marker for CD34⁺ hematopoietic stem cells that does not recognize mature, differentiated CD34⁺ ECs.^{61,62} Gehling *et al.* observed that 2 weeks of CD133⁺ MNC treatment with VEGF and stem cell growth factor produced EC-like colonies containing Weibel-Palade bodies and expressing 30–35% CD34, 16–32% CD31, 48–56% VE-cadherin, >99.5% KDR/Flk-1, Tie-2, ulex europaeus agglutinin-1 (UEA-1), and vWF.⁶¹

Around the same time of Gehling's work, however, Schmeisser *et al.* conducted a study that showed peripheral blood-derived monocytes, a potential progeny of CD133⁺ hematopoietic stem cells, could express vWF (45.2% ± 5%), VE-cadherin (12.4% ± 2%), endothelial constitutive nitric oxide synthase (ecNOS, 9.8% ± 3%), and form capillary-like network structures *in vitro* after 2 weeks of VEGF treatment.⁶³ The EC-like characteristics of monocytes continued to increase after 4 weeks of VEGF treatment, resulting in similar expression levels in EC markers vWF (94.2% ± 13%), VE-cadherin (89.7% ± 15%), ecNOS (58.8% ± 10%), as vascular EC controls, and decreased expression for hematopoietic markers CD45 (92.2% ± 6%) and CD14 (12.3% ± 4%).⁶³ Taken together with the knowledge that monocytes and macrophages can uptake acetylated low-density lipoprotein and express CD31, these findings suggest that instead of CD133⁺/CD34⁺ MNCs existing as hemangioblast progenitors as suggested by Asahara, Rafii, and Gehling, they could simply exist as monocytes, induced from hematopoietic progenitors to exhibit EC attributes on continuous VEGF treatment.^{25,56,57,60,61,63,64}

FIG. 1. Photomicrograph comparisons of reported human EPCs. (A) CD34⁺ putative EPCs derived from adult peripheral blood MNCs on fibronectin-coated substrates. Reprinted with permission from Asahara *et al.*⁵⁶ Reprinted with permission from AAAS. (B) Putative EPCs derived from adult bone marrow MNCs on fibronectin-coated substrates and treated with VEGF. 600× original magnification. Republished with permission from American Society of Hematology, Rafii *et al.*,⁵⁷ © 1998, permission conveyed through Copyright Clearance Center, Inc. (C) Brightfield image of CD34⁺ EPCs derived from hiPSCs through Wnt/β-catenin signaling. Reprinted from Lian *et al.*,⁷⁷ © 2014, with permission from Elsevier. (D) Phase-contrast image of NRP-1⁺CD31⁺ hiPSC-ECFCs. Scale bar: 50 μm. Reprinted with permission from Macmillan Publishers Ltd: Prasain *et al.*,⁷⁸ © 2014 (E) Hierarchy of EPCs as low proliferative potential-ECFCs in adult peripheral blood (*top panel*) and highly proliferative potential-ECFCs in umbilical cord blood (*bottom panel*). Scale bar: 100 μm. Republished with permission from American Society of Hematology, Ingram *et al.*,²⁸ © 2004, permission conveyed through Copyright Clearance Center, Inc. ECFCs, endothelial colony-forming cells; EPCs, endothelial progenitor cells; hiPSC, human-induced pluripotent stem cell; MNCs, mononuclear cells; VEGF, vascular endothelial growth factor. Color images available online at www.liebertpub.com/teb

Blood-derived EPCs

Lin and colleagues had previously shown that circulating mature, differentiated ECs were present in peripheral blood and possessed proliferative potential *in vitro*, leading to the hypothesis of an endothelium origin as likely as a hematopoietic origin for the CD34⁺ EPCs.^{56,57,65} EPC isolation by Lin *et al.* differed from Asahara and Rafii by utilizing adherence enrichment, rather than depletion, of MNCs, which were expanded on type I collagen-coated substrates (Table 1).⁶⁶ In 2004, Ingram *et al.* expanded on the work of Lin and colleagues by seeking a more functional property to characterize EPCs than cell surface antigens: clonogenic and proliferative potential assays.²⁸ These assays, used to describe hematopoietic progenitor cells, identified a hierarchy of EPCs classified by proliferation potential, highly proliferative potential-endothelial colony-forming cells (HPP-ECFCs) found in umbilical cord blood (hCB-ECFCs) and low proliferative potential-endothelial colony-forming cells (LPP-ECFCs) found in adult peripheral blood (hPB-ECFCs) (Fig. 1E). HPP-ECFCs could give rise to at least 10 million progeny from a single cell without compromising telomerase activity, which protects against senescence. Other character-

istics that distinguished cord blood EPCs from peripheral blood EPCs included 15-fold higher numbers of EC colonies per equivalent blood volume, faster appearance in culture and formation of capillary-like structures *in vitro* using a Matrigel™ assay, and expansion for 100 population doublings before demonstrating cellular senescence, in contrast to 20–30 population doublings for hPB-ECFCs.

Continued characterization of the EPC-derived ECFCs against EC colony forming unit ECs (CFU-ECs), which resemble the EPCs reported by Asahara, showed that ECFCs have significantly higher expression for EC-associated markers CD105, CD144, CD146, UEA-1, and lacked expression for the hematopoietic cell-associated markers CD14, CD45, and CD115.⁴⁷ Only ECFCs could form secondary EC colonies through a single-cell clonogenic assay, while CFU-ECs could form secondary hematopoietic colonies of myeloid lineage with methylcellulose assays. CFU-ECs and ECFCs were also shown to be clonally unrelated from experiments with polycythemia vera patients, who contain a mutated gene, *Janus Kinase 2 V617F*, in their hematopoietic stem cells.⁴⁷ All CFU-ECs contained this gene, in comparison to less than 4% of ECFCs. Only ECFCs could form capillaries within collagen and fibronectin gels,

TABLE 1. COMPARISON OF ENDOTHELIAL PROGENITOR CELLS DERIVED FROM HUMAN TISSUE

Source	Isolation/derivation technique	Expansion conditions	Characterization	Ref.
Blood	Adult PB-MNCs isolated with Ficoll density gradient centrifugation, plated on TCP for 1 h to remove differentiated adhesive cells, and treated with CD34 or Flk-1 antibody-coated magnetic beads.	Fibronectin-coated TCP substrate, M-199 media containing BBE and 20% v/v FBS.	>80% uptake of DiI acLDL. 27.2% $\pm$ 2.2% CD45 ⁺ , 32.8% $\pm$ 12.7% CD34 ⁺ , 71.5% $\pm$ 7.1% CD31 ⁺ , 35.8% $\pm$ 8.8% Flk-1 ⁺ /KDR ⁺ , 54.6% $\pm$ 14.2% Tie-2 ⁺ , 9.0% $\pm$ 3.0% E-selectin ⁺ . Nitric oxide release in response to treatment with acetylcholine and VEGF. <i>In vivo</i> incorporation of DiI-labeled CD34 ⁺ cells into the neovasculature of athymic ischemic hind limbs, 6 weeks after administration.	56
	G-CSF mobilized PB-MNCs isolated with Ficoll/Hypaque density gradient centrifugation and treated with AC133 antibody-conjugated magnetic beads.	Fibronectin-coated chamber slides, IMDM media containing 10% v/v FBS, 10% v/v horse serum, 10 ⁻⁶ M hydrocortisone, 100 ng/mL SCGF, 50 ng/mL VEGF. Nonadherent supernatant cells were removed during media changes and replated at a density of 2 $\times$ 10 ⁶ cells/mL.	30–35% CD34 ⁺ , 15–32% CD31 ⁺ , 48–56% VE-cadherin ⁺ , >99.5% KDR ⁺ /Flk-1 ⁺ , UEA-1 ⁺ , >99.5% vWF ⁺ , >99.5% Tie-2 ⁺ , >99.5%, 0% CD1a ⁺ , 0% CD14 ⁺ . Presence of Weibel-Palade bodies. AC133 ⁺ cells, conjoined with A549 cancer cells, incorporated into tumor neovessel 5 weeks after administration into SCID mice.	61
	Adult peripheral blood anticoagulated with heparin or buffered sodium citrate, diluted 1:2 with HBSS containing 0.5% v/v BSA, 1 mM EDTA and enriched for EPCs using PIH12 antibody-conjugated beads. PIH12 ⁺ MNCs were derived by Histopaque-1077 density gradient centrifugation using MCDB131 media that contained 1 μ g/mL hydrocortisone acetate, 0.5 mM dibutyl cAMP, 1.6 mM L-glutamine, 10 ng/mL VEGF, 0.004% v/v heparin, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 mg/mL amphotericin B.	Type I collagen-coated TCP, EBM TM -2 media containing 7% v/v FBS, EGM TM -2 bullet kit (hEGF, hydrocortisone, 30 mg/mL gentamicin, 15 μ g/mL amphotericin, VEGF, bFGF, human recombinant IGF-1, ascorbic acid, heparin). Expanded on fibronectin-coated TCP.	Uptake of DiI acLDL, positive immunostaining for vWF, presence of Weibel-Palade bodies. Cell surface antigen expression for VE-cadherin, Flk-1/KDR, vWF, CD36, and negative expression for CD14. Increased ICAM-1 expression and VCAM-1 and tissue factor expression after treatment with IL-1 and LPS. 17 $\pm$ 9-fold and 1023 $\pm$ 476-fold expansion of recipient and donor endothelial outgrowth cells, respectively, after 27 $\pm$ 4 days of culture.	66
	Umbilical cord blood or adult peripheral blood was anticoagulated with citrate phosphate dextrose and diluted 1:1 with HBSS. MNCs isolated with Histopaque-1077 density gradient centrifugation using EBM-2 media containing 10% v/v FBS, 2% v/v penicillin/streptomycin, and 0.25 μ g/mL amphotericin B.	MNCs plated on type I collagen-coated TCP. Nonadherent cells removed daily. Passaged cells expanded on collagen-coated TCP and maintained with EGM-2 media.	Single-cell assay clonogenic proliferative potential. 92.29% $\pm$ 1.32% CD31 ⁺ , 96.73% $\pm$ 1.79% CD105 ⁺ , 99.15% $\pm$ 0.85% CD144 ⁺ , 94.21% $\pm$ 3.71% CD146 ⁺ , 68.61% $\pm$ 11.26% KDR ⁺ /Flk-1 ⁺ , 97.09% $\pm$ 2.05% vWF ⁺ , 100% UEA-1 ⁺ , 1.20% $\pm$ 0.74% CD14 ⁺ , 0.37% $\pm$ 0.37% CD45 ⁺ , 0.28% $\pm$ 0.21% CD115 ⁺ . Expansion to at least 30 or 100 population doublings for adult peripheral and umbilical cord blood EPCs, respectively, by 60 days of culture. <i>In vitro</i> capillary-like network formation on Matrigel TM , uptake of DiI acLDL (99.75% $\pm$ 0.25%), increased VCAM-1 expression after TNF- α or IL-1 stimulation. Developed perfusable microvasculature when embedded in collagen/fibrin gels and implanted into SCID mice.	28,47

(continued)

TABLE 1. (CONTINUED)

TABLE 2. ENDOTHELIAL PROGENITOR CELL REQUIREMENTS

Clonal proliferative potential from a single cell to produce progeny restricted to endothelial lineage, as demonstrated by >90% expression of endothelial-associated markers CD31, CD105, CD144, CD146, and lack of expression for hematopoietic-associated markers CD14, CD45, CD115.
Expansion to at least 30 population doublings over 60 days of <i>ex vivo</i> culture.
Formation of lumenized capillary-like networks <i>in vitro</i> .
<i>De novo</i> generation of blood vessels that anastomose with host vasculature.

which do not recruit vessel ingrowth in murine species that anastomosed to host vasculature and were perfused.^{47,67} Hence, only ECFCs can be defined as true EPCs and recommended for use in the vascularization of engineered tissues.^{68,69} As well, based on the evidence that both LPP-ECFCs and HPP-ECFCs reside within mature vasculature, the most likely origin of CD34⁺ EPCs is the endothelium of all tissue vascular niches rather than a unique progenitor cell residing within the bone marrow.^{70,71}

Human-induced pluripotent stem cell-derived EPC

Although the EPC subset of ECFCs has potential for autologous angiogenic therapy, the peripheral blood of patients afflicted with preexisting disease, such as coronary artery disease, can contain reduced numbers of circulating EPCs with impaired angiogenic function.^{72–74} iPSC technology offers a potential solution by developing mesodermal precursor cells.^{75,76} In particular, Lian *et al.* showed that human fibroblasts, treated with nonintegrating episomal vectors containing *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-Myc*, and *KLF4* genes, could induce CD34⁺CD31⁺ EPCs through glycogen synthase kinase 3 (GSK3) inhibition and subsequent activation of Wnt/ β -catenin signaling (Table 1).⁷⁷ The hiPSC-EPCs expressed VE-cadherin, formed capillary-like network structures *in vitro*, demonstrated clonogenic ability, and exhibited physiological EC response to stimulation with inflammatory and permeability mediators (Fig. 1C).⁷⁷ However, hiPSC-EPCs generated only 20 population doublings over 60 days of culture, which is more characteristic of mature ECs rather than EPCs.⁶⁶

At the same time of Lian's work, Yoder and colleagues found that previously reported methods for deriving hiPSC-EPCs involving OP9 mouse embryonic stromal cell coculture, three-dimensional (3D) embryoid body formation, or continuous transforming growth factor beta (TGF- β) inhibition, yielded heterogeneous cell populations that did not maintain a stable EC phenotype after several weeks of culture.^{78–81} Instead, the group developed a single-step, serum-free, two-dimensional (2D) adherent cell culture, utilizing growth factors involved in EPC specification from the mesoderm—activin-A, BMP4, FGF-2, and VEGF₁₆₅—to generate a highly proliferative population of ECFCs from hiPSCs (Fig. 1D).⁷⁸ hiPSC-ECFCs were selected by fluorescence-activated cell sorting (FACS) for neuropilin-1 (NRP-1) and CD31 expression. Endothelial precursors express NRP-1 before CD34 and CD31.⁸² The NRP-1⁺CD31⁺ hiPSC-ECFCs displayed similar phenotype and proliferative potential as HPP-ECFCs, expanding to over 10¹² progeny by 90 days of culture, and maintaining homogeneous EC phenotype, as assessed through NRP-1, CD31, CD144 expression as well as lack of alpha-smooth muscle actin (α -SMA) expression.⁷⁸

Furthermore, the hiPSC-ECFCs possessed vasculogenic potential, evidenced by capillary network formation *in vitro* atop Matrigel, and functional microvessel formation *in vivo* within collagen gels that were subcutaneously implanted into severe combined immunodeficient (SCID) mice.⁷⁸ Addressing concerns of safety with hiPSCs, Prasain *et al.* found no teratoma formation of hiPSC-ECFCs after 6 months of implantation. The hiPSC-ECFCs showed therapeutic promise in oxygen-induced retinopathy within C57/BL6 wild-type mice, and in ischemic limbs within athymic mice.⁷⁸ Prasain *et al.* also showed that NRP-1 has a critical role in hiPSC-ECFC generation through VEGF₁₆₅-mediated KDR signaling. Intriguingly, NRP-1 activation in ECs, derived from the blood and vasculature of patients with peripheral artery disease, could increase proliferation and reduce apoptosis and senescence.⁷⁸ Taken together, these findings offer promising technologies to obtain therapeutic numbers of vasculogenic EPCs, autologously derived from patients with cardiovascular disease.

In summary, only ECFCs derived from adult peripheral blood and umbilical cord blood meet the criteria in Table 2 and are considered true EPCs.^{25,60,68} Ongoing EPC characterization efforts include advanced lineage tracing to assess whether ECFCs can generate the complete hierarchy of blood vessels, and gene expression analysis to determine whether ECFCs residing in the vascular niche of different organs possess similar angiogenic potential.^{25,68}

EPC Vascular Morphogenesis and Differentiation

In the developing embryo, angioblasts, the embryonic EPCs, are derived from the mesoderm through the soluble effector, Indian hedgehog (IHH) and FGF-2, which act together to increase Flk-1/KDR expression.^{83,84} Flk-1⁺ EPCs respond to autocrine and endoderm-derived VEGF-A by differentiating toward a more mature EC phenotype, evidenced by expression of VE-cadherin, PECAM-1, Tie-1, Tie-2, and Flt-1.^{84–87} Extraembryonic vasculogenesis occurs first with migrating EPCs entering the yolk sac in response to chemotactic gradients formed by VEGF and semaphorins.^{87–89} Using α v β 3 integrins, EPCs coalesce with hematopoietic cells and form blood islands that fuse together on a fibronectin-rich extracellular matrix, generating the primary capillary plexi, shown in Figure 2.^{90–92} These primitive vascular networks remodel to form the vitelline circulation, the blood vessels responsible for transferring nutrients from the yolk sac to the embryo.⁸⁷

In contrast, dispersed angioblasts/EPCs initiate vasculogenesis in the embryo proper, coalescing to directly form primary vascular networks without intermediary blood island formation.^{88,89} These networks fuse together and undergo bidirectional remodeling processes to generate the dorsal aorta and cardinal vein.^{87,93,94} ECs sprouting from

FIG. 2. Overview of vasculogenesis within the vertebrate embryo. Mesodermal germ layer stimulation with FGF-2 and IHH proteins produces angioblasts/EPCs that express Flk-1/KDR/VEGFR-2. Locally produced VEGF-A in the extraembryonic yolk sac and intraembryonic embryo proper creates chemokine gradients that activate and recruit EPCs. VEGF-A stimulates EPC expression of VE-cadherin, PECAM-1, Tie-1, Tie-2, and Flt-1. In extraembryonic vasculogenesis, EPCs form an endothelial border enclosing primitive erythrocytes, generating blood islands. Expression of arterial Nrp-1 or venous Nrp-2 is present on blood island ECs before capillary formation and blood perfusion. Blood islands coalesce to create primary vascular networks that develop into the vitelline circulatory system. In contrast, during intraembryonic vasculogenesis, EPCs coalesce directly creating a primary vascular network that fuses to form the dorsal aorta and cardinal vein. Angiogenesis of the dorsal aorta and cardinal vein produces intersegmental blood vessels that further remodel to form the embryonic circulatory system, assisted by Notch signaling, Semaphorin, Netrin, and Ephrin proteins, as well as Slit ligands. Arterial specification is induced by VEGF-A, which binds to Nrp-1 and VEGFR-2/Flk-1 on ECs, inducing Notch signaling that results in Ephrin-B2 expression. Retinoic acid regulates venous specification by activating COUP-TFII to interact with Nrp-2 and inhibit Notch signaling, promoting EphB4 expression. A subset of venous endothelium expressing Sox18 interacts with COUP-TFII to upregulate VEGFR-3 expression, which acts together with VEGF-C to initiate lymphangiogenesis. Not shown are mural cells, pericytes, and vascular SMCs that closely associate with the vascular network, maintain endothelial quiescence, and regulate blood flow. COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; EC, endothelial cell; FGF-2, fibroblast growth factor; SMC, smooth muscle cells. Color images available online at www.liebertpub.com/teb

these vessels are guided by positive and negative signals from VEGFR-2 and VEGFR-1, respectively, creating an intersegmental blood vessel that remodels to form the embryonic circulation system, with vascular patterns guided by Notch and VEGFR-2 signaling as well as Semaphorin, Netrin, Ephrin, and Slit ligands.^{95–97} Angiogenesis is initiated by proangiogenic factors VEGF-A and Ang-2 that activate ECs lining the interior of mature vessels to remove intercellular junctional contacts, secrete matrix metalloproteinases (MMPs), which break down basement membrane proteins, detach adjacent mural cells, and assume a migratory, proliferative phenotype with protruding filopodia.^{98,99} VEGF-induced vessel permeability during early angiogenesis releases plasma protein fibronectin, vitronectin, and fibrinogen that interact with interstitial collagens to create a

provisional matrix, which assists in angiogenic growth by sequestering VEGF-A to form a haptotactic gradient that directs EC migration.^{100–102} In addition to VEGF-A, vascular patterns are regulated by interactions of EC ligands plexin D1 and roundabout receptor (Robo) with semaphorin and Slit proteins, respectively.^{100,103,104} The sprouting vessels migrate along the chemoattractant gradient using integrins $\alpha\beta3$ and $\alpha\beta5$.¹⁰⁵

Vascular ECs that are exposed to the highest, local VEGF-A concentration differentiate to tip cells that lead the sprouting vessels.⁹⁸ Trailing the tip cells are stalk cells, which maintain connectivity with the parent vessel and proliferate to elongate the vascular sprout, as depicted in Figure 3A.^{98,100} Lateral inhibition from tip cells prevent stalk cell differentiation to tip cells.⁹⁸ Tip cell VEGFR-2

interaction with VEGF-A induces expression of the transmembrane Notch ligand, Delta-like 4 (DLL4), which interacts with Notch receptors expressed on neighboring stalk cells to initiate Notch signaling. Stalk cell notch intracellular domain is activated to transcriptionally increase VEGFR-1 expression and decrease VEGFR-2 and Nrp-1 expression.^{100,106} VEGFR-1 traps VEGF, preventing tip cell differentiation of stalk cells by acting as an antagonist of VEGF-mediated signaling.^{98,100,107}

Stalk cells develop lumen by first establishing apicobasal polarity through β 1 integrin interactions with the underlying basal matrix, enabling protease activated receptor 3 (PAR3)-mediated redistribution of junctional EC proteins, zonula occludens (ZO-1), VE-cadherin, claudin-5, from the apical surface to the sprouting EC cord periphery.^{98,108} Signaling of VEGFR-2 and Rho-associating coiled kinase (ROCK) then recruits actomyosin proteins, such as F-actin, to build lumen.^{98,109} Epidermal growth factor-like domain-7 (EGFL7), secreted into the extracellular matrix by neovessel ECs, has also been shown to regulate tubulogenesis.¹¹⁰ Fusion of intracellular vacuoles can also create lumen with the assistance of Rho family GTPases Cdc42 and Rac1.^{111,112} For example, EPC tubulogenesis *in vitro* was shown to occur through pinocytosis and fusion of intracellular vacuoles, regulated by MT1-MMP and Cdc42.^{17,113} Remodeling of the surrounding extracellular matrix occurs by release of additional MMPs and tissue inhibitor of metalloproteinases.¹⁰⁵ Angiogenesis ends when tip cells reach an adjacent vessel, initiating intercellular junctional contacts and anastomosis.⁹⁸ To further stabilize the nascent vasculature, tip cells secrete platelet-derived growth factor subunit B (PDGF-B), which binds to the surrounding extracellular matrix, creating a haptotactic gradient that recruits mural cells to PDGF-B via platelet-derived growth factor receptor beta (PDGFR- β).^{6,98,114,115} Once mural cells contact the vessel endothelium, ECs release sphingosine 1-phosphate, which recruits endogenous neural cadherin (N-cadherin)

within ECs to create adherens junctions between ECs and mural cells.¹¹⁶ TGF- β 1 is then released by vascular ECs to induce mural cell differentiation toward a vascular smooth muscle cell (SMC) phenotype, which can regulate vascular blood flow.^{6,117} Basement membrane proteins also promote vessel stabilization through EC interactions with the laminin- α 4 integrin, which promotes Notch signaling-induced EC quiescence.^{100,118}

Differentiation of newly formed vasculature toward the arterial, venous, and lymphatic lineages is genetically predetermined, as evidenced by expression of arterial Nrp-1 and venous Nrp-2 on blood island EPCs prior capillary plexus formation and onset of blood flow.^{119,120} Yet, hemodynamic forces, such as the high pressure, high shear stress, pulsatile flow in arteries and the low pressure, low shear stress flow in veins, are essential to regulating and maintaining venous or arterial fate.^{120–123} Arteriovenous specification begins with notochord-secreted sonic hedgehog ligand, which promotes VEGF-A secretion from somites.^{120,124–126} A VEGF gradient is established, starting at the dorsal artery and continuing toward the cardinal vein.^{125,127,128} For arterial differentiation, VEGF-A binds to heterodimers of Flk-1/VEGFR-2 and Nrp-1 to activate Notch signaling, which increases Ephrin-B2 expression and suppresses EphB4 expression.^{88,120,124,129–131} Venous specification is initiated by retinoic acid, which activates a nuclear orphan receptor, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII).^{120,125,132} COUP-TFII suppresses Notch signaling by interfering with Nrp-1 function, consequently increasing EphB4 expression.¹³²

Lymphatic vasculature differs from arterial and venous vasculature through lack of continuous basement membrane and loose intercellular junctions, and transport of interstitial tissue fluid instead of blood.^{122,133} Lymphatic ECs are produced by a subpopulation of cardinal vein cells that express lymphatic vessel hyaluronan-1 receptor-1 and induce Sox18 expression.^{125,134} Sox18 cooperates with COUP-

FIG. 3. Review of angiogenesis and endothelium specification. **(A)** Summary of angiogenesis, Reprinted with permission from Macmillan Publishers Ltd: Adams and Alitalo¹²² © 2007. **(a)** New vessels arise from preexisting vessels on exposure to proangiogenic (+) factors VEGF-A and ANG-2 that induce vascular ECs to remove intercellular and PC, secrete proteases that degrade the basement membrane and surrounding ECM, alter polarity, and migrate. The degree of exposure and vicinity to VEGF-A determines tip cell selection. VEGF-A and tip cell VEGFR-2 interactions promote transmembrane Notch ligand DLL4 expression, laterally inhibiting tip cell differentiation of adjacent stalk cells by promoting Notch signaling. **(b)** Enhanced vascular permeability releases plasma proteins, which form a provisional matrix that interacts with VEGF-A, Semaphorins, and SLIT proteins, creating an angiogenic gradient that directs migrating sprouts. Tip cells secrete PDGF-B to recruit mural cells. Stalk cells maintain parent vessel connectivity through intercellular tight junctions and deposition of basement membrane. **(c)** Encountering tip cells initiates the end of angiogenesis by reforming intercellular junctional contacts. Lumen formation occurs by re-establishing apicobasal polarity, redistributing junctional EC proteins and recruiting actomyosin. Fusion of intracellular vacuoles also creates lumen through Rho family GTPases Cdc42 and Rac1. **(d)** Neovessel ECs secrete EGFL7 into the surrounding ECM to assist in tubulogenesis. Stabilization of mural cell contacts promotes vessel maturation by initiating quiescence. TGF- β 1 is released by ECs to further mural cell differentiation toward a vascular SMC phenotype. **(B)** Organotypic endothelium types, modified and reprinted with permission from Macmillan Publishers Ltd.: Hoffman and Calabrese¹⁶⁷ © 2014 and with permission of Springer, Pries and Kuebler.¹⁶⁸ Nonfenestrated continuous endothelium possesses tight and adherens junctions that regulate permeability. Wnt signaling, *Wnt7a*, *Wnt7b*, *Wnt3a* genes, β -catenin, and astrocyte SSECKS control BBB differentiation. Fenestrated endothelium in the EG contains diaphragms regulated through EG-VEGF, VEGF-A, and type II membrane glycoprotein PV-1. Podocyte-secreted VEGF and the Rho/Rac signaling pathway specify the kidney glomeruli. Discontinuous endothelium contains fenestrae 100–200 nm in size and lack a continuous basement membrane. BBB, blood–brain barrier; DLL4, Delta-like 4; ECM, extracellular matrix; EG, endocrine-gland; EGFL7, epidermal growth factor-like domain-7; PC, pericyte contacts; PDGF-B, platelet-derived growth factor subunit B; PV-1, plasmalemmal vesicle-associated protein-1; SSECKS, Src-suppressed C kinase; TGF- β 1, transforming growth factor beta 1. Color images available online at www.liebertpub.com/teb

TFII to stimulate prospero-related homeobox-1 (Prox1) transcription factor expression.^{120,135,136} Prox1 upregulates VEGFR-3 expression, which acts together with its coreceptor Nrp-2 to respond to VEGF-C.^{122,137–140} VEGF-C induces lymphatic ECs to create lymph sacs that have sprouting, migratory lymphatic ECs which form the lymphatic plexus that further remodels to form lymphatic capillaries and collection vessels and ducts.^{120,122,141,142} Still, mature vascular ECs are not permanently confined to arterial, venous, and lymphatic fate. Postnatal reprogram-

ming ECs to an alternate macrovascular fate can occur when hemodynamic forces and levels of COUP-TFII and Prox1 expression are altered, consequently affecting Notch signaling and subsequent Ephrin-B2 and EphB4 expression.^{121,125,132,143–145}

The entire macrovascular and microvascular hierarchy must undergo further differentiation to accommodate the unique function and metabolic demands of each organ.¹²⁵ Organ-specific specialization of capillary plexus EPCs is a consequence of local factors secreted by tissue cells.^{122,146}

Organ	Brain, Muscle, Heart, Lung, Skin	Endocrine glands, Kidney glomeruli, Pancreas, Gastrointestinal tract	Liver, Bone marrow, Spleen
Molecular Specification	BBB: Wnt signaling, Wnt7a, Wnt7b, Wnt3a, β -catenin, SSeCKS	Endocrine glands: VEGF-A, EG-VEGF, PV-1 Kidney glomeruli: VEGF-A, Rho/Rac signaling	

Currently, three known forms of endothelium structure exist: nonfenestrated continuous, fenestrated continuous, and fenestrated discontinuous, as illustrated in Figure 3B.^{125,147} Fenestrae are porous openings in the vascular wall that enable the swift exchange between tissue constituents and circulating blood.^{125,148} Fenestrae range in size from 60 to 200 nm and may contain diaphragms, spoke wheel-like subdivisions that filter blood based on solute size.^{125,148,149} Nonfenestrated, continuous endothelium is found in the brain, muscle, heart, lung, and skin.¹²⁵ These organs provide critical motor, respiratory, and protective functions that require endothelium with strong barrier properties. Accordingly, nonfenestrated continuous capillaries are characterized by tight junctions and adherens junctions as well as caveolae and vesiculo-vacuolar organelles that regulate permeability.^{125,150–152}

At present, the understanding of endothelium specification toward nonfenestrated continuous phenotype is limited to studies involving the blood-brain barrier (BBB). The BBB tightly controls transendothelial passage of biomolecules and cells by tight junctional occludin and claudin proteins, and selective membrane transporter systems that comprised glucose transporter type 1 (Glut-1) and ATP binding cassette transporter proteins.¹⁵³ Together with canonical Wnt signaling, *Wnt7a* and *Wnt7b* genes promote Glut-1 expression.^{146,154–156} Claudin-3 tight junction expression in the BBB is regulated by the *Wnt3a* gene as well as EC-derived β -catenin protein, which induces claudin-3 expression while simultaneously reducing the expression of fenestra-promoting type II membrane glycoprotein plasmalemmal vesicle-associated protein-1 (PV-1).^{146,156} Brain-specific pericytes, astrocytes, and neurons provide local cues that assist in EC differentiation by regulating junctional protein expression and transport vesicles.^{125,157} As an example, to maintain BBB integrity, astrocytes produce Src-suppressed C kinase (SSeCKS), stimulating Ang-1 production while also suppressing VEGF, a permeability enhancer.¹⁵⁸

Kidney glomeruli, pancreas, intestine, and endocrine glands all contain fenestrated endothelium.¹⁴⁶ Endothelium differentiation to diaphragm-containing fenestrae found on endocrine glands is regulated by PV-1 and induced by VEGF-A and endocrine gland-VEGF (EG-VEGF).^{122,159–161} Although the fenestra of the kidney glomerular endothelium is also induced by VEGF, diaphragm expression is lost after development.^{125,161,162} Podocytes provide the local source of VEGF to induce EC differentiation.¹⁶³ The podocyte VEGF activates the Rho/Rac signaling pathway to reorganize the EC actin cytoskeleton, and form fenestrae.^{164–166} The current understanding of endothelium specification to fenestrated discontinuous phenotype is limited to anatomical descriptions.^{167,168} This endothelium type is found in the liver and bone marrow and characterized by a lack of continuous basement membrane and the presence of clathrin-coated pits and vesicles that regulate endocytosis.¹²⁵ The fenestrae are large, ranging 100–200 nm in diameter, and lack diaphragms, facilitating the release of niche cells and metabolites.¹⁴⁹

Tissue Engineering Vascular Niches with EPCs

Establishing design parameters for generating EPC-vascularized, tissue niches *in vitro* is a daunting task because cells, scaffold, and culture media must be optimized

to provide the distinct biochemical and biophysical signals of each niche. Fortunately, several *in vitro* model systems for investigating microvessel formation utilizing vascular-derived ECs have been established.^{16,169–171} These systems mimic *in vivo* vasculogenic and angiogenic processes by incorporating ECs with stromal cells, such as mural cells and fibroblasts, within scaffolds that mimic the provisional matrix, supplemented with media containing VEGF-A and bFGF. These initial design parameters have been tailored to tissue engineer microvessels from EPCs.^{34,172} The EPC-derived microvessels described in this section are restricted to EPC subtypes that possess true EPC character, currently only hCB-ECFCs or hPB-ECFCs.^{25,60,68,69} Classifying the EPC progeny as ECFCs is due to the loss of expression for the progenitor-associated markers CD34 and CD133 during expansion, and is often interpreted as EPC differentiation toward a mature endothelial phenotype.¹⁷² Yet, given that neither CD34 nor CD133 has been shown to regulate EPC vasculogenic function or differentiation toward organotypic endothelium, to describe expanded EPCs and differentiated ECs is misleading. Therefore, the terms EPC and ECFCs have been used synonymously.

Beginning in 2004, Bischoff's group demonstrated safe expansion of hCB-ECFCs to therapeutically relevant numbers by 3 weeks *in vitro*, assessed through continued expression of CD31, VE-cadherin, vWF, E-selectin, and KDR.¹⁷² The group also showed that mural cells were required for *in situ* hCB-ECFC microvessel formation within a polyglycolic acid-poly-L-lactic acid (PGA-PLLA) bioresorbable scaffold.¹⁷² Without direct mural cell coculture, hCB-ECFCs and hPB-ECFCs fail to generate microvascular networks *in vitro* and *in vivo*.^{33–35,44,172}

In 2008, Jain's group examined whether the *de novo* vasculature generated from hCB-ECFCs or hPB-ECFCs, in coculture with 10T1/2 murine mesenchymal progenitor cells (MPCs) within fibronectin/collagen gels, remained stable after several weeks of implantation into SCID mice.¹⁷³ hCB-ECFCs were capable of generating functional blood vessels that were durable for at least 4 months, as demonstrated by intravital microscopy of green fluorescent protein (GFP)-labeled hCB-ECFC microvessels perfused with rhodamine-conjugated dextran.¹⁷³ Intravital microscopy permeability measurements against 67 kDa Texas Red-labeled bovine serum albumin showed that hCB-ECFCs possessed permeability values similar to normal brain capillaries, $0.73 \pm 0.21 \times 10^{-7}$ cm/s.^{173,174} In addition, there were no significant differences between hCB-ECFC and host microvessels in red blood cell velocity or the ability to recruit leukocytes on treatment with interleukin-1 β (IL-1 β).¹⁷³ In contrast, hPB-ECFC and 10T1/2 cocultures produced unstable microvessels that regressed after 3 weeks of implantation *in vivo*.¹⁷³

Melero-Martin *et al.* expanded on the work of Jain's group by replacing the 10T1/2 cells with clinically viable mesenchymal stem/progenitor cells, derived from human adult bone marrow (bm-MPCs) or umbilical cord blood (cb-MPCs).³⁵ Both cb-MPCs and bm-MPCs could support hCB-ECFC and hPB-ECFC vasculogenesis in Matrigel constructs implanted within athymic mice for at least 1 week *in vivo*, differentiating toward α -SMA⁺, pericyte-like cells that covered EPC neovessels.³⁵ As well, hCB-ECFC microvessels resulting from bm-MPC cocultures were functional

for at least 4 weeks *in vivo* based on erythrocyte perfusion.³⁵ Yet, for a tissue-engineered system to be considered as a therapeutic device, all design parameters must be clinically acceptable, prohibiting systems utilizing Matrigel, a xenogeneic-derived scaffold.

To date, a small number of studies have successfully tissue engineered vascular niches utilizing bona fide EPCs, hCB-ECFCs, or hPB-ECFCs, with translatable coculture cells and scaffolds. These include the adipogenic, vasa vasorum, dermal, osteogenic, and pulmonary tissue niches, summarized in Table 3. As well, a few groups have attempted to mimic the embryonic niche by incorporating both endothelial and MPCs within a synthetic scaffold.^{17,175} The advantage of this approach is the potential to generate the vascular niche of any organ, provided the appropriate differentiation cues are present within the scaffold and culture media. Few studies have attempted to understand the singular effects of coculture cell type, scaffold mechanical properties, or media composition on *in vitro* EPC network formation. Still, there are common themes across vascular niche design that are critical to generating stable, functional EPC microvessels *in vitro*.

Coculture choice

In addition to mural cells, coculture with fibroblasts, stromal, parenchymal, and mesenchymal stem cells (MSCs) or MPCs, is critical to developing EPC microvessels *in vitro* and *in vivo*.^{17,34,176–178} These coculture cells serve as a source of angiocrine factors that function beyond stabilizing neovessel formation to initiating and supporting vasculogenic and angiogenic processes. For example, *in vitro* capillary formation from EC elongation and intercellular adherens junctions requires mural cell VEGF and EC-associated VEGFR-2 signaling, and cannot be simulated with exogenous VEGF or mural cell-conditioned media.¹⁶ As well, *in vitro* capillary lumen formation from EC and fibroblast cocultures depends on the combined expression of fibroblast-associated extracellular matrix proteins collagen I, insulin growth factor-binding protein 7, procollagen C endopeptidase enhancer 1, transforming growth factor- β -induced protein ig-h3, and secreted protein acidic and rich in cysteine (SPARC).¹⁷⁹ Coculture cells also provide immunogenic shielding properties, as recently reported by Souidi *et al.*, which showed that MSCs downregulated human leukocyte antigen-antigen D related (HLA-DR) expression on hCB-ECFCs *in vitro* and *in vivo*, reducing EPC-mediated allogenic T cell proliferation.¹⁸⁰

Parenchymal cells are often the initial coculture cell choice for engineering vascularized tissues, grounded on the assumption that mature niche cells can provide differentiation cues to EPCs, resulting in the desired endothelium phenotype. Wu *et al.* mimicked the vasa vasorum niche by coculture of hCB-ECFCs with human saphenous vein-derived SMCs within PGA-PLLA constructs, yielding lumenized microvessels after 6 days of *ex vivo* culture.¹⁷² In addition, hCB-ECFC and human aortic SMC coculture, with minimal supplemental growth factors, gave rise to neovessels that contained properties of physiological microvessels, such as quiescence and lumen formation.³² When encapsulated in a polyethylene glycol (PEG) hydrogel system, the hCB-ECFC microvessels expressed intercellular

EC junctional proteins VE-cadherin and connexin 32, endothelial nitric oxide synthase (eNOS), perivascular localization of α -SMA⁺/PDGFR- β ⁺ cells, and basement membrane formation with collagen IV and laminin, shown in Figure 4A–C.³³ Fuchs *et al.* determined that human osteoblasts could support microvessel formation of hPB-ECFCs, generating lumenized microvessels with intercellular tight junctions after 1 week of *in vitro* culture.¹⁵

Even so, mural and parenchymal cells have major drawbacks limiting their application in regenerative medicine, including invasive isolation procedures and limited expansion potential. Tissue stromal cells, such as dermal fibroblasts and adipose stromal cells, require less invasive isolation, have expansion potential, and can support EPC microvessel formation.^{176–178,181} For instance, Athanassopoulos *et al.* demonstrated hCB-ECFC and dermal fibroblast cocultures could vascularize clinical-grade, dermal-substitute scaffolds.¹⁸¹ George's group also found that fibroblasts, derived from human lung tissue, supported hCB-ECFC microvessel formation within fibrin gels *in situ* and differentiate toward an α -SMA⁺ myofibroblast, pericyte-like phenotype that encircled the EPC vessels.¹⁷⁶ Furthermore, the prevascularized fibrin constructs anastomosed with host vasculature and were perfused within 1 day of implantation.¹⁷⁶ Offering promise for reconstructive surgery application of EPCs, Traktuev *et al.* revealed that coculture with liposuction-derived adipocytes and stromal cells within collagen/fibronectin gels could support hCB-ECFC microvessel formation, express α -SMA⁺, exhibit perivascular localization, and protect against hCB-ECFC apoptosis within SCID mice.¹⁷⁸

All the same, stromal cells possess limited differentiation potential toward niche parenchymal cells and, in the case of lung fibroblasts, are not easily accessible. Using multipotent MSCs from adipose, bone marrow, umbilical cord blood, and amniotic fluid can overcome this barrier. During vasculogenesis and angiogenesis, MSCs/MPCs undergo mural cell differentiation on recruitment to neovasculture by means of EC-secreted TGF- β 1 and gap junctions.^{122,182,183} Chen *et al.* demonstrated that bone marrow-derived MSCs (BM-MSCs), cocultured with hCB-ECFCs within gelatin methacrylate hydrogels, could support vasculogenesis and differentiate toward mural lineage cells expressing α -SMA and smooth muscle-myosin heavy chain (SM-MHC) within 1 week of culture *in vitro* (Fig. 4D, E).¹⁷ Athanassopoulos *et al.* also demonstrated BM-MSC support of hCB-ECFC microvessel formation *in vitro*, with no significant differences in total tubule length, number of tubules, or number of junctions in comparison to dermal fibroblast cocultures after 2 weeks of culture.¹⁸¹ Encouraging pediatric applications with EPCs, MSCs, and MPCs derived from amniotic fluid and umbilical cord blood have been shown to support hCB-ECFC network formation *in vitro* (Fig. 4F, G).^{43,184}

Adipose-derived stem cells, another promising autologous coculture cell choice, contain multilineage differentiation potential as well as support EC network formation.^{185,186} Adipose-derived stem cells can be produced following clinical-grade procedures and used safely in patients.¹⁸⁷ Mural cells can also be directly transdifferentiated from blood-derived ECFCs through myocardin (*MYOCD*) expression, exhibiting SMC phenotype and function through smooth muscle aortic alpha-actin (*ACTA2*) and myosin heavy chain 11 (*MYH11*) expression, contractility, and calcium signaling

TABLE 3. TISSUE-ENGINEERED MODELS OF ENDOTHELIAL PROGENITOR CELL MICROVESSEL FORMATION

Vascular niche	Cells ^a	Culture media	Biomaterial	Key findings	Ref.
Adipose	EPCs: Umbilical CB-MNCs obtained through Histopaque 1077 gradient centrifugation and cultured on type I collagen-coated TCP. Coculture cell: hASCs and mature adipocytes isolated from liposuction samples treated with type I collagenase. Adipocytes separated from hASCs with centrifugation.	EPCs: EBM-2 media supplemented with EGM-2 aliquots and 10% v/v FBS. hASCs: EGM-2 MV media. Coculture and adipocyte triculture: same as EPC expansion media.	Type I collagen (1.5 mg/mL) and fibronectin (100 ng/mL) solution mixed with 1.5 mg/mL sodium bicarbonate, 25 mM HEPES, 30% v/v EPC expansion media, an additional 10% v/v FBS, and 2×10^6 cells/mL of 4:1 EPCs to hASCs. Similar gel composition for triculture with adipocytes except EPC and hASC number reduced to 1×10^6 cells/mL and combined with 129 μ L packed cell volume of adipocytes.	hASCs can differentiate to α -SMA ⁺ mural cells that support <i>in vivo</i> EPC microvessel formation within collagen/fibronectin gels with 147 ± 22.1 vessels/mm ² by day 6. hASC and EPC cocultures can vascularize adipocytes within collagen/fibronectin gels with 86.2 ± 2.9 vessels/mm ² by 2 weeks <i>in vivo</i> . hASCs decrease EPC apoptosis <i>in vivo</i> PDGF-BB secreted by EPCs upregulates hASC VEGF production by several fold. EPCs, when cocultured with BM-MSCs or hDFs, can penetrate dermal scaffolds and form lumenized microvessels with intercellular tight junctions. BM-MSCs can support EPC vasculogenesis as well as hDFs.	178
Dermal	EPCs: CB-MNCs obtained by Ficoll-Histopaque density gradient centrifugation and plated on type I collagen-coated TCP. Endothelial colonies isolated with polystyrene clonal rings and expanded on collagen-coated TCP. Coculture cell: hDFs or BM-MSCs.	EPC: EBM-2 containing EGM-2 aliquots, 10% v/v FCS. hDFs: DMEM with high glucose and sodium pyruvate, supplemented with L-glucose, penicillin, and streptomycin, 10% v/v FCS. BM-MSCs: MSCGM containing penicillin, streptomycin, 10% v/v FBS. Coculture: EPC expansion media except FCS reduced to 8% v/v.	EPCs were mixed with hDFs or BM-MSCs at a 1:5 ratio, 4.2×10^3 cells/mm ² , and seeded atop Integra [®] or MatriDerm [®] scaffolds.		181
	EPCs: umbilical cord blood-derived ECFCs [®] . Coculture cell: fibroblasts derived from neonatal foreskin (hDFs).	EPCs: EBM-2 media containing EGM-2 aliquots and ECFC [®] serum supplement. hDFs: FGM TM -2 media. Coculture: EBM-2 media containing EGM-2 aliquots.	N-isopropylacrylamide grafted on tissue culture polystyrene and polymerized to PNIPAM with electron beam irradiation. A sandwich coculture of hDFs and EPCs preparing each layer separately. hDF monolayers formed from 13.5×10^4 cells/cm ² , EPCs cultured at 2×10^4 cells/cm ² for 12 h. Layers harvested by incubation at 20°C for 15–30 min followed by 20-min incubation at 37°C to promote adhesion. A fibrin-coated plunger assisted assembly of stacked sheets.	Cell-sheet-based tissue engineering technology can promote EPC microvessel formation, when placed in sandwich coculture with hDFs <i>in vitro</i> . Preformed EPC networks can anastomose with host vasculature on implantation <i>in vivo</i> with $\sim 315 \pm 90$ vessels/mm ² after 1 week.	177

(continued)

TABLE 3. (CONTINUED)

<i>Vascular niche</i>	<i>Cells^a</i>	<i>Culture media</i>	<i>Biomaterial</i>	<i>Key findings</i>	<i>Ref.</i>
Developing embryo	EPCs: CB-MNCs isolated with Histopaque 1077 density gradient centrifugation and seeded atop TCP precoated with 1% gelatin. Outgrowth cells purified with anti-CD31-coated magnetic beads. EPCs expanded on fibronectin-coated TCP. Coculture cell: BM-MSCs, isolated from bone marrow MNCs with 1% gelatin-coated TCP, expanded on uncoated TCP.	EPC: primary EPCs cultured in EBM-2 containing EGM-2 aliquots, 1× GPS, 20% v/v FBS, 15% v/v autologous plasma. Expanded in identical media, without plasma and hydrocortisone. BM-MSC: expanded with MSCGM containing 10% v/v FBS, 1× GPS, 10 ng/mL bFGF. Coculture: same as EPC expansion media except FBS reduced to 2% v/v.	GelMA polymeric precursors formed by reacting 10% w/w porcine skin gelatin with 1%, 5%, or 10% v/v methacrylic anhydride. EPCs and BM-MSCs at a 1:1 ratio were pelleted via centrifugation and mixed with 5 w/v% GelMA at 2×10^6 cells/mL before photopolymerization with 0.5 w/v% Irgacure® 2959 under UV light.	GelMA hydrogels support microvessel formation from EPC and BM-MSC cocultures <i>in vitro</i> as well as anastomosis with host vasculature <i>in vivo</i> with 58.34 ± 32.70 vessels/mm ² 1 week after implantation. EPCs form lumen via fusion of intracellular vesicles. Degree of methacrylation affects EPC microvessel morphology and MSC mural cell differentiation.	17
Osteogenic	EPCs: PB-MNCs obtained by Ficoll gradient centrifugation and expanded on collagen-coated TCP. Coculture cell: pOBs isolated from minced bone fragments that were digested with collagenase.	EPCs: cultured in EBM-2 containing EGM-2 aliquots, 5% v/v FCS, 1% penicillin/streptomycin. pOBs: expanded in 1:1 DMEM/Hams F12 blend with 10% v/v FCS and 1% penicillin/streptomycin. Coculture: EPC expansion media supplemented with 10 mM β -glycerolphosphate, 10^{-7} M dexamethasone, 0.2 mM sodium ascorbate.	Glass coverslips coated with fibronectin, EPCs plated atop pOB monolayer at a 1:1.5 ratio, a total of 5×10^5 cells. 3D cell aggregates formed by 1:1 EPC to pOB seeding, a total of 4×10^6 cells, in rotating culture.	EPCs cocultured with pOBs form lumen-containing microvessels that exhibit intercellular tight junctions, by 1 week <i>in vitro</i> . Osteogenic differentiation supplements do not affect EPC microvessel formation or EC phenotype and maintain pOB phenotype.	15
Pulmonary	EPCs: CB-MNCs derived by lymphocyte separation medium and seeded atop 1% gelatin-coated TCP. EPCs selected from MNC outgrowth cells with anti-CD31-coated magnetic beads. Coculture cell: NHLDFs.	EPCs expanded in 1:1 blend of M199 and EBM-2 media containing EGM-2 aliquots, 20% v/v FBS, 1% v/v ECGS. NHLDFs expanded in FGM-2 media containing 2% v/v FBS. Coculture: EBM-2 media containing EGM-2 aliquots and 2% v/v FBS.	Fibrinogen, 10 mg/mL, mixed with EPCs and NHLDFs before gelation from the addition of 2 U/mL thrombin, at ratios ranging from 5:1 to 1:2. EPC density was kept constant at 1×10^6 cells/mL and NHLDF number varied from 2×10^5 to 2×10^6 cells/mL.	First study to demonstrate 3D constructs prevascularized by EPCs can anastomose with host vasculature within 1 day of implantation. Higher density of NHLDF cocultured with EPCs within fibrin gels enhances rate of microvessel formation <i>in vitro</i> as well as anastomosis rate <i>in vivo</i> . NHLDFs support lumenized microvessel formation by EPCs and differentiate toward pericyte-like cells that encircle neovessels.	176

(continued)

TABLE 3. (CONTINUED)

<i>Vascular niche</i>	<i>Cells^a</i>	<i>Culture media</i>	<i>Biomaterial</i>	<i>Key findings</i>	<i>Ref.</i>
Vasa vasorum	EPCs: CB-MNCs derived from Ficoll-Hypaque density gradient sedimentation, seeded on 1% gelatin-coated TCP for 2 days. Collected nonadherent cells treated with anti-CD34-conjugated magnetic beads. CD34 ⁺ MNCs treated with anti-CD133-conjugated magnetic beads. Expanded on 1% gelatin-coated TCP. Coculture cell: saphenous vSMCs.	EPCs: isolated in EBM-2 containing EGM-2 aliquots, without hydrocortisone, 1× GPS, 20% v/v FBS, 15% human plasma. Expanded in identical media without 15% human plasma. vSMCs: expanded in DMEM containing 10% v/v FBS, 250 µg/mL amphoteracin, 1× GPS. Coculture: identical to EPC expansion media.	Nonwoven mesh textile composed of PGA-PLLA fibers, 65 mg/mL. EPCs and vSMCs seeded at a 1:1 ratio for a total of 2×10 ⁶ cells/cm ² .	First demonstration of <i>de novo</i> EPC microvessel formation within a tissue-engineered scaffold <i>in vitro</i> . EPCs are capable of stable expansion for at least 3 weeks <i>in vitro</i> by protein expression of EC markers CD31, VE-cadherin, vWF, E-selectin, and KDR gene expression. vSMCs promote spontaneous <i>in vitro</i> self-assembly of EPCs to lumenized capillary structures within PGA-PLLA scaffolds at a density of 76.6±35 vessels/mm ² . Synthetic PEG hydrogels containing adhesive and protease-sensitive peptides support EPC microvessel formation from AoSMC coculture with 80.4±32.8 vessels/mm ² , by 2 weeks <i>in vitro</i> . EPC microvessels represent physiological microvasculature <i>in vitro</i> by demonstrating quiescence, lumen formation, expression of VE-cadherin, connexin 32, eNOS, perivascular localization of α-SMA ⁺ /PDGFR-β ⁺ cells, laminin, and collagen IV basement formation.	172
	EPCs: CB-MNCs isolated by Histopaque 1077 density gradient centrifugation. Expanded on type I collagen-coated TCP. Clonal isolation rings used to isolate EPC colonies. Coculture cell: AoSMCs.	EPCs: EBM-2 containing EGM-2 aliquots, 9% v/v FBS, 0.9% v/v PSA. AoSMCs: SmBM TM media containing SmGM-2 TM aliquots (insulin, bFGF, EGF), 4.7% v/v FBS, 0.9% v/v PSA. Coculture: EBM-2 media containing 9% v/v FBS, 0.9% v/v PSA.	PEG conjugated to either RGDS (PEG-RGDS) or MMP-2/9 sensitive peptide GGGPQGIWGGK (PEG-PQ) were synthesized by reacting with acrylate-PEG-succinimidyl valerate at 2:1 or 1:2.1 molar ratios, respectively. 1:1 EPC and AoSMC pellets were mixed with 5% w/v PEG-RGDS and 20% w/v PEG-PQ at 30×10 ⁶ cells/mL and crosslinked under white light with a photoinitiator solution of triethanolamine, eosin Y, and 1-vinyl-2 pyrrolidinone.		33

^aAll cells are derived from human sources.

3D, three-dimensional; AoSMC, aortic smooth muscle cells; BM-MSCs, human bone marrow-derived mesenchymal stem cells; CB-MNCs, cord blood MNCs; ECGS, endothelial cell growth supplement; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal calf serum; FGM-2, fibroblast growth medium; GelMA, gelatin methacrylate; GPS, glutamine-penicillin-streptomycin; hASC, human adipose stromal cells; hDFs, human dermal fibroblasts; MMP, matrix metalloproteinases; MSCGM, mesenchymal stem cell growth media; NHLDFs, normal lung-derived fibroblasts; PDGFR-β, platelet-derived growth factor receptor beta; PEG, polyethylene glycol; PGA-PLLA, polyglycolic acid-poly-L-lactic acid; PNIPAM, poly(*N*-isopropylacrylamide); pOBs, primary osteoblasts; PSA, penicillin-streptomycin-amphotericin; vSMCs, vascular smooth muscle cells.

FIG. 4. Representative images of microvessels that were tissue engineered from hCB-ECFC cocultures *in vitro*. (A–C) GFP-transduced hCB-ECFC microvessels resulting from 1:1 coculture with vascular SMCs within a cell adhesive and proteolytically degradable polyethylene glycol hydrogel. (A) Confocal x-y plane projection of EPC network formation. Scale bar: 250 μm. (B) EPC microvessels expressed VE-cadherin adherens junctions (red) and connexin 32 gap junctions (light blue). Arrow depicts intercellular connexin 32 gap junctions. Nuclei are counterstained with DAPI. (C) EPC microvessels expressed eNOS. Scale bar: 100 μm. Reprinted with permission from Springer, Peters *et al.*³³ (D, E) Images of hCB-ECFC network formation from 1:1 coculture with BM-MSCs within gelatin methacrylate hydrogels. (D) Red fluorescent protein-labeled EPC networks (DsRed-ECFCs) after 6 days of coculture with BM-MSCs (not shown). Scale bar: 250 μm. (E) BM-MSCs, expressing α-SMA, localized to EPC microvessels in a pericyte-like manner after 1 week of coculture. Right inset shows higher magnification and cross-sectional images. Reprinted with permission from Chen *et al.*¹⁷ (F) GFP-labeled hCB-ECFC networks resulting from 2 weeks of coculture on collagen I-coated TCP with 1:4 spindle-shaped amniotic fluid-derived MSCs (not shown). Reprinted with permission from Roubelakis *et al.*¹⁸⁴ (G) hCB-ECFC networks, labeled with tdTomato fluorescent protein, after 18 days of coculture on uncoated TCP with 1:4 GFP-labeled cord blood-derived mesenchymal progenitor cells. Scale bar: 250 μm. Reprinted with permission from Springer, Peters *et al.*⁴³ α-SMA, alpha-smooth muscle actin; BM-MSCs, bone marrow-derived mesenchymal stem cells; DAPI, 4,6-diamidino-2-phenylindole; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; hCB-ECFC, endothelial colony-forming cells, derived from human umbilical cord blood EPCs; TCP, tissue culture plastic. Color images available online at www.liebertpub.com/teb

activity.¹⁸⁸ This finding offers promise for utilizing blood as a single tissue source to isolate both mural and ECs.

Still, differences exist among mural, stromal, and MSC/MPC ability to support EPC network formation, which may be masked from the presence of angiogenic growth factors commonly used in the culture medium. Supporting this hypothesis, we found that when culture media contained no exogenous angiocrine factors beyond those present in serum, only vascular SMCs and cb-MPCs, in contrast to brain pericytes and BM-MSCs, could support hCB-ECFC network formation.^{43,44} Moreover, without the addition of exogenous VEGF, FGF-2, EGF, hydrocortisone, insulin growth factor-1, ascorbic acid, and heparin, hCB-ECFC networks formed in coculture with BM-MSCs regressed and underwent cell loss by 2 weeks of culture, possibly due to trace amounts of CD45⁺ cells with phagocytic potential.⁴⁴ As well, *ex vivo* expanded BM-MSCs secrete less than half of the angiocrine factors produced by amniotic fluid-MSCs and dermal fibroblasts, which includes molecules that regulate tubulogenesis, FGF-2, MMP-9, and endothelium differentiation, EG-VEGF, VEGF-C.¹⁸⁴

In addition to coculture choice, parameters of coculture ratios, seeding densities, and coculture spatial arrangement can also influence EPC network formation.^{32,33,176,181,189} The optimal coculture design parameters depend on whether the cells are grown atop a 2D biomaterial or within a 3D scaffold. In the context of cell-sheet tissue engineering technologies, 2D systems with mixed coculture spatial arrangements yield more connected, homogeneous networks than lamellar systems.³² Alternatively, a sandwiched coculture system containing a layer of hCB-ECFCs between fibroblast monolayers can also produce interconnected, lumenized microvessels.¹⁷⁷ Uniformly distributed cells enable greater sprouting and interconnected networks than spheroid cell aggregates in 3D systems.^{17,33,176,189} As well, the ideal coculture ratio to promote hCB-ECFC microvessel formation is 1:1 for 3D systems and near 1:4 for 2D systems.^{17,32,33,176,181,184} Increasing densities of supportive stromal cells can enhance the rate of microvessel formation *in vitro* and anastomosis rate *in vivo* in 3D systems, but this effect was not observed in 2D systems.^{176,181}

Scaffold

Fibrin gels have been approved for patient use, possess excellent cytocompatibility, and have shown success as a tissue engineering scaffold.¹⁹⁰ Fibrin gelation occurs by recapitulating the blood coagulation cascade using thrombin, fibrin, and calcium chloride. Calcium activates thrombin to cleave fibrinogen, generating fibrin.¹⁹¹ As well, fibrinolytic inhibitors aprotinin and tranexamic acid can adjust fibrin degradative properties.¹⁹¹ The mechanical properties of fibrin gels can be tuned by varying thrombin concentrations.¹⁹² Although collagen and fibrin gels can support EPC microvessel formation *in vitro* and *in vivo*, they possess batch variability and allow limited control over degradation rate and mechanical properties.^{17,193,194}

On the contrary, while having tunable mechanical properties, semicrystalline polymers, such as PGA-PLLA, have limited ability to incorporate bioactive cues to imitate tissue niches.^{172,195} A potential solution proposed by Okano's group is cell-sheet-based tissue engineering technology,

which has the advantage over 3D scaffolds in mimicking microenvironments of niche cells by conserving their extracellular matrix and intercellular connections formed during *ex vivo* culture.¹⁹⁶ As well, cell-sheet tissue engineering offers a lower risk of toxicity to cells that can occur from polymer degradation by-products.^{195,196} Cell sheets are produced from tissue culture plastic covalently grafted with poly(*N*-isopropylacrylamide) (PNIPAM) polymer chains that swell at temperatures below its lower critical solution temperature of 32°C, due to increased aqueous solubility, repelling the cell monolayer from the culture dish as a continuous cell sheet.¹⁹⁶ Sasagawa *et al.* demonstrated that EPCs, sandwiched between human dermal fibroblast layers, formed network structures by 3 days *in vitro* that contained partially formed lumens.¹⁷⁷ These preformed networks connected with host vasculature and were perfused with blood by 1 week after subcutaneous transplantation.¹⁷⁷ Yet, the cell-sheet engineering approach is limited by poor control over microarchitecture and mechanical strength.

Chemically modifying extracellular matrix proteins to create hydrogels can improve the mechanical properties of naturally derived gels while maintaining their biofunctional properties. Gerecht's group has explored hyaluronic hydrogel design factors of matrix viscoelasticity and adhesion peptide density to support EPC capillary formation and uncover underlying mechanisms of vascular morphogenesis.^{113,197} Hyaluronic acid is a glycosaminoglycan present during embryonic development. hCB-ECFCs express hyaluronic acid receptors and can interact with hyaluronic acid hydrogels through surface membrane CD44.¹⁹⁷ Gerecht found that crosslinking thiol-modified hyaluronic acid-gelatin hydrogels with a chemical crosslinker, PEG diacrylate, allowed greater control over physical properties and microarchitecture.

Specifically, soft hydrogels with a Young's modulus of 10 Pa supported hCB-ECFC vascular formation, from monoculture, to a significantly greater extent than 73 Pa hydrogels, as assessed by tube length and tube area.¹⁹⁷ In contrast, hCB-ECFCs remained rounded without observable vascular morphogenesis in 640 Pa hydrogels. The extensive network formation in the 10 Pa hydrogel was correlated with 200- μ m average diameter microchannels that were not observed in the 73 or 640 Pa hydrogels.¹⁹⁷ Similarly, Khademhosseini's group chemically functionalized gelatin with methacrylate groups to produce a photocrosslinkable hydrogel that could support hCB-ECFC microvessel formation in coculture with BM-MSCs *in vitro* and within athymic mice *in vivo*.¹⁷ They found that the degree of methacrylation affected microvessel morphology and MSC mural differentiation, with softer, 1 M methacrylate gels (2.0 ± 0.18 kPa) stimulating BM-MSC expression of α -SMA and SM-MHC, increasing microvessel density and lumen diameter *in vivo* in contrast to stiffer, 10 M methacrylate gels (4.5 ± 0.33 kPa).¹⁷

Despite these advantageous, chemically modified biological gels do not allow precise control over biochemical and biophysical cues to recapitulate each tissue niche. The extracellular matrix composition is unique to each tissue niche, with varying amounts of constituent proteins, such as collagen, elastin, and fibrin, which activate separate cell signaling pathways to create the distinct cell phenotypes of each tissue.¹⁹⁸ A promising candidate for tailored scaffold microenvironments in vascular niche tissue engineering is

PEG hydrogel. PEG hydrogels are considered a “blank slate” that resists protein adsorption, can incorporate specific cell-adhesive and protease-sensitive peptides in addition to angiogenic factors.^{199,200} We found that PEG hydrogels, (18 ± 5 kPa) containing fibronectin-derived RGDS adhesive peptide and MMP-2/9 sensitive peptides, provided an adequate microenvironment to induce vasculogenesis of hCB-ECFCs within SMC coculture, with minimal supplemental growth factors.³³ Importantly, this system used a mild photocrosslinking process, suitable for encapsulation of progenitor and stem cells.^{33,201}

Culture media

Currently, almost all tissue-engineered models of EPC microvessel formation utilize culture media enhanced with angiogenic growth factors and animal-derived serum (Table 3). This is problematic as continuous supply of angiogenic factors may not occur on implantation of the construct *in vivo*, leading to vessel regression. Although a few systems can support EPC microvessel formation without additional angiogenic factors, animal-derived serum is still required, which is immunogenic and clinically unacceptable.^{33,43} Fortunately, xenogeneic-free methods exist for EPC expansion that replaces animal-derived serum with human platelet lysates, which contain VEGF, PDGF-AB, and PDGF-BB, and preserves EPC vasculogenic potential *in vivo*.^{202,203} Palecek's group has also developed a translatable culture media to support hiPSC-EPCs, consisting of basal media and ascorbic acid.²⁰⁴

In summary, when combined with appropriate coculture cells in natural or synthetic biomaterials, EPCs from hCB-ECFCs can form microvascular structures *in vitro* and *in vivo* that mimic vascular tissue niches. Conversely, hPB-ECFC-derived microvessels lack stability and durability *in vitro* and *in vivo*.^{173,181} Cell engineering strategies, notably iPSC technology, have the potential to improve the expansion and vasculogenic potential of hPB-ECFCs, significantly broadening EPC therapeutic applications.

In Vitro Differentiation of EPCs to Organotypic Endothelium

The intimate associations between microvascular ECs and parenchymal cells enable exchange of soluble and membrane-bound angiocrine signals that provide essential induction signals for organogenesis.²⁰⁵ For example, during liver development, hepatocyte invasion is significantly impaired in Flk-1/VEGFR-2-negative embryos.^{205,206} In addition, without the presence of microvascular ECs during pancreatic tissue development, insulin production is stopped and pancreatic cell differentiation is inhibited.^{205,207} In the adult, vascular ECs serve as professional niche cell regulators of tissue function, producing angiocrine factors that orchestrate stem cell self-renewal and fibrosis-free organ regeneration.^{205,208,209} Vascular ECs also convey cues between the intravascular to extravascular space that control tissue metabolic activity and homeostasis.²⁰⁵

Expanded hCB-ECFCs possess an intermediate arteriovenous phenotype based on similar levels of gene expression for EphB4, Nrp-2, Notch4, and Ephrin-B2.²¹⁰ Intriguingly, hPB-ECFCs contain populations of both blood vascular and lymphatic ECs, based on expression of VEGFR-1 and

VEGFR-3/Prox1, respectively, suggesting potential for use of blood-derived ECFCs in blood vascular and lymphatic tissue engineering.²¹¹ Yet, the molecular basis for EPC specialization to arteriovenous and organ-specific vasculature is not well known. Methods to induce tissue-specific endothelium differentiation *in vitro* include coculture with parenchymal cells, directed differentiation, and direct reprogramming.²¹²

For example, Boyer-Di Ponio *et al.* found that early passage hCB-ECFCs, indirectly cocultured with rat-derived astrocytes, differentiate toward cerebral microvascular cells that possess characteristics of the BBB based on decreased permeability and increased expression of tight and adherens junction proteins.²¹⁰ Specifically, after 2 weeks of coculture, hCB-ECFCs, plated atop polyester cell culture inserts, demonstrated a significant decrease in permeability against 457 Da fluorescent small molecules, at 2.05×10^{-5} cm/s, in comparison to hCB-ECFC monocultures. Furthermore, hCB-ECFCs increased gene and protein expression of Glut-1 and occludin, and more homogeneous expression of VE-cadherin, ZO-1, claudin-3, and claudin-5 than hCB-ECFC monocultures.²¹⁰ Moreover, Boyer-Di Ponio *et al.* used directed differentiation of hCB-ECFCs to an arterial phenotype by treatment with 50 ng/mL of VEGF, which promoted Notch signaling and increased gene expression of arterial associated markers Ephrin-B2, *DLL4*, *Notch 3*, *HES1*, *HEY1*, and *HEY2*.²¹⁰ Palecek's group utilized directed codifferentiation of hiPSCs through activation of Wnt/ β -catenin signaling to produce neural progenitor cells and cerebral microvascular ECs.²¹³ The cerebral ECs displayed permeability of 0.37×10^{-5} cm/s against 180 Da glucose molecules and continuous cell-cell contact expression of ZO-1, occludin, and claudin-5.²¹³

Examining gene transcriptional profiles for organotypic endothelium may identify molecular candidates to induce EC specification. Providing data for this approach, Nolan *et al.* performed a systematic investigation to determine the unique molecular signatures of vascular endothelium from murine liver, bone marrow, heart, brain, lung, spleen, kidney, muscle, and testis.²⁶ They showed that each tissue expresses distinct clusters of angiocrine factors, with the ETS transcription factor family being likely responsible for regulating organotypic endothelium differentiation.^{26,214} Specifically, *SFP11* was correlated with differentiation toward fenestrated, discontinuous endothelium due to its high expression in the bone marrow and liver vasculature.²⁶

Future Perspectives

Although significant progress has been made toward utilizing EPCs for vascularizing engineered tissues, there is no evidence of EPC integration with tissue niche parenchymal and stromal cells to produce organotypic endothelium *in vitro*. Contributing to this lack of progress is the common approach to generate vascularized tissues *in vitro*, which is mixing EPCs or ECs alongside tissue stromal and progenitor cells, utilizing previously optimized growth conditions for the tissue of interest. The fallacy in this concept is the underlying assumption that the growth conditions for avascular tissue development will also promote vasculogenesis and that the angiogenic conditions will not interfere with parenchymal differentiation of tissue progenitor cells.

Organ development is an elaborate feedback control system in which temporally and spatially secreted factors by tissue niche cells provide positive and negative regulation.²⁰⁵ The use of angiogenic initiators, such as VEGF, can redirect parenchymal progenitor differentiation toward vascular lineages.²¹⁵ As a consequence, novel techniques are required to guide orthogonal differentiation of vascular and parenchymal progenitor cells *in situ*. The use of reductionist, *in vitro* models utilizing synthetic hydrogels and chemically defined media can provide a translatable platform to probe the intricate relationship among endothelial, stromal, and parenchymal cells. For instance, Lutolf and colleagues demonstrated VEGF could be covalently bound with protease-sensitive peptides within synthetic PEG hydrogels and released by cell-secreted proteases, enabling studies of vascular morphogenesis within a chemically defined microenvironment.²¹⁶

In addition to angiocrine factors, angiogenic transcription factors, such as the transcription factor hypoxia-inducible factor 1, could also be identified in reductionist, synthetic hydrogel models of vascular morphogenesis. Activators for the master angiogenic regulators of each tissue niche could then be incorporated within the scaffold to indirectly stimulate the production of angiocrine factors in mural and parenchymal cells surrounding the vascular cells.^{37,205} This approach would enable complex angiocrine profile production at physiological concentrations, confined to localized regions, thus eliminating the complexity of releasing multiple angiocrine factors within the scaffold in a temporally and spatially controlled manner.^{217–220} As well, clustered regularly interspaced short palindromic repeat-mediated gene activation may be utilized to regulate the angiogenic transcription factors.²²¹

Also, deriving EPCs and tissue-specific progenitors from mesodermal precursors would closely mimic the process in organ development and eliminate the need for multiple cell isolations. Gerecht, Palecek, and colleagues have provided proof-of-principle for this approach, generating EPCs or organotypic ECs and parenchymal progenitors from hiPSCs.^{175,213,222} In addition, inducing EPCs to form macrovasculature within the prevascularized construct could create a vascular axis within the construct, enabling microsurgical connection with the host vasculature and instantly perfusing the scaffold with blood.^{46,223} Assisting efforts to advance EPC vascularization research is omnidirectional, direct-contact bioprinting that can recapitulate the distinct 3D hierarchical vascular networks for each tissue, as well as mimic the local matrix microenvironment by finely controlled deposition of biomolecules.^{224–226} Thus, combining reductionist *in vitro* models with recent advances in polymer science, genetic engineering, and 3D bioprinting can provide the sophisticated systems needed to develop the next generation of tissue-engineered models that can further EPC vascularization research.

Acknowledgments

E.B.P. was supported by the NIH T32 National Institutional Research Service Award T32 HL07670, and gratefully acknowledges Dr. George Truskey for review of this article. This review is dedicated to the living memory of Gregory Duke Brown.

Disclosure Statement

No competing financial interests exist.

References

1. Nerem, R.M. Tissue engineering: the hope, the hype, and the future. *Tissue Eng* **12**, 1143, 2006.
2. Rouwkema, J., Rivron, N.C., and van Blitterswijk, C.A. Vascularization in tissue engineering. *Trends Biotechnol* **26**, 434, 2008.
3. Lovett, M., Lee, K., Edwards, A., and Kaplan, D.L. Vascularization strategies for tissue engineering. *Tissue Eng Part B Rev* **15**, 353, 2009.
4. Novosel, E.C., Kleinhans, C., and Kluger, P.J. Vascularization is the key challenge in tissue engineering. *Adv Drug Deliv Rev* **63**, 300, 2011.
5. Rouwkema, J., and Khademhosseini, A. Vascularization and angiogenesis in tissue engineering: beyond creating static networks. *Trends Biotechnol* **34**, 733, 2016.
6. Jain, R.K. Molecular regulation of vessel maturation. *Nat Med* **9**, 685, 2003.
7. Carmeliet, P., and Jain, R.K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298, 2011.
8. Zarem, H.A. The microcirculatory events within full-thickness skin allografts (homografts) in mice. *Surgery* **66**, 392, 1969.
9. Carmeliet, P., and Jain, R.K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249, 2000.
10. Rücker, M., *et al.* Vascularization and biocompatibility of scaffolds consisting of different calcium phosphate compounds. *J Biomed Mater Res Part A* **86**, 1002, 2008.
11. Clark, E.R., and Clark, E.L. Microscopic observations on the growth of blood capillaries in the living mammal. *Am J Anat* **64**, 251, 1939.
12. Laschke, M.W., and Menger, M.D. Vascularization in tissue engineering: angiogenesis versus inosculation. *Eur Surg Res* **48**, 85, 2012.
13. Malda, J., *et al.* Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling. *Biotechnol Bioeng* **86**, 9, 2004.
14. Levenberg, S., *et al.* Engineering vascularized skeletal muscle tissue. *Nat Biotechnol* **23**, 879, 2005.
15. Fuchs, S., Hofmann, A., and Kirkpatrick, C.J. Microvessel-like structures from outgrowth endothelial cells from human peripheral blood in 2-dimensional and 3-dimensional co-cultures with osteoblastic lineage cells. *Tissue Eng* **13**, 2577, 2007.
16. Evensen, L., *et al.* Mural cell associated VEGF is required for organotypic vessel formation. *PLoS One* **4**, e5798, 2009.
17. Chen, Y.C., *et al.* Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. *Adv Funct Mater* **22**, 2027, 2012.
18. Tremblay, P.L., Hudon, V., Berthod, F., Germain, L., and Auger, F.A. Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. *Am J Transplant* **5**, 1002, 2005.
19. Shepherd, B.R., *et al.* Vascularization and engraftment of a human skin substitute using circulating progenitor cell-derived endothelial cells. *FASEB J* **20**, 1739, 2006.
20. Jain, R.K., Au, P., Tam, J., Duda, D.G., and Fukumura, D. Engineering vascularized tissue. *Nat Biotechnol* **23**, 821, 2005.

21. Baldwin, J., *et al.* In vitro pre-vascularisation of tissue-engineered constructs A co-culture perspective. *Vasc Cell* **6**, 1, 2014.
22. Gong, Z., and Niklason, L.E. Blood vessels engineered from human cells. *Trends Cardiovasc Med* **16**, 153, 2006.
23. Jackson, S.A., Schiesser, J., Stanley, E.G., and Elefanty, A.G. Differentiating embryonic stem cells pass through 'temporal windows' that mark responsiveness to exogenous and paracrine mesendoderm inducing signals. *PLoS One* **5**, e10706, 2010.
24. Ikami, K., *et al.* Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development* **142**, 1582, 2015.
25. Yoder, M.C. Human endothelial progenitor cells. *Cold Spring Harb Perspect Med* **2**, a006692, 2012.
26. Nolan, D.J.D.J., *et al.* Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. *Dev Cell* **26**, 204, 2013.
27. Boyer, M., *et al.* Isolation of endothelial cells and their progenitor cells from human peripheral blood. *J Vasc Surg* **31**, 181, 2000.
28. Ingram, D.A., *et al.* Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **104**, 2752, 2004.
29. Mead, L.E., Prater, D., Yoder, M.C., and Ingram, D.A. Isolation and characterization of endothelial progenitor cells from human blood. *Curr Protoc Stem Cell Biol* 2008; DOI:10.1002/9780470151808.sc02c01 s6
30. Samuel, R., *et al.* Generation of functionally competent and durable engineered blood vessels from human induced pluripotent stem cells. *Proc Natl Acad Sci U S A* **110**, 12774, 2013.
31. Rouwkema, J., Westerweel, P.E., de Boer, J., Verhaar, M.C., and van Blitterswijk, C.A. The use of endothelial progenitor cells for prevascularized bone tissue engineering. *Tissue Eng Part A* **15**, 2015, 2009.
32. Peters, E.B., Christoforou, N., Leong, K.W., and Truskey, G.A. Comparison of mixed and lamellar coculture spatial arrangements for tissue engineering capillary networks in vitro. *Tissue Eng Part A* **19**, 697, 2013.
33. Peters, E.B., Christoforou, N., Leong, K.W., Truskey, G.A., and West, J.L. Poly(ethylene glycol) hydrogel scaffolds containing cell-adhesive and protease-sensitive peptides support microvessel formation by endothelial progenitor cells. *Cell Mol Bioeng* **9**, 38, 2016.
34. Melero-Martin, J.M., *et al.* In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* **109**, 4761, 2007.
35. Melero-Martin, J.M., *et al.* Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res* **103**, 194, 2008.
36. Cheung, T.M., Ganatra, M.P., Peters, E.B., and Truskey, G.A. Effect of cellular senescence on the albumin permeability of blood-derived endothelial cells. *Am J Physiol Heart Circ Physiol* **303**, H1374, 2012.
37. Lam, G.C., and Sefton, M.V. Harnessing gene and drug delivery for vascularizing engineered tissue platforms. *Drug Discov Today* **21**, 1532, 2016.
38. Richardson, M.R., and Yoder, M.C. Endothelial progenitor cells: quo Vadis? *J Mol Cell Cardiol* **50**, 266, 2011.
39. Zampetaki, A., Kirton, J.P., and Xu, Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res* **78**, 413, 2008.
40. Rouwkema, J., De Boer, J., and Van Blitterswijk, C.A. Endothelial cells assemble into a 3-dimensional pre-vascular network in a bone tissue engineering construct. *Tissue Eng* **12**, 2685, 2006.
41. Ford, M.C., *et al.* A macroporous hydrogel for the co-culture of neural progenitor and endothelial cells to form functional vascular networks in vivo. *Proc Natl Acad Sci U S A* **103**, 2512, 2006.
42. Menge, T., *et al.* Human mesenchymal stem cells inhibit endothelial proliferation and angiogenesis via cell-cell contact through modulation of the VE-cadherin/ β -catenin signaling pathway. *Stem Cells Dev* **22**, 120626080437008, 2012.
43. Peters, E.B., Liu, B., Christoforou, N., West, J.L., and Truskey, G.A. Umbilical cord blood-derived mononuclear cells exhibit pericyte-like phenotype and support network formation of endothelial progenitor cells in vitro. *Ann Biomed Eng* **43**, 2552, 2015.
44. Peters, E.B., Christoforou, N., Moore, E.M., West, J.L., and Truskey, G.A. CD45+ Cells present within mesenchymal stem cell populations affect network formation of blood-derived endothelial outgrowth cells. *Biores Open Access* **4**, 75, 2015.
45. Ziegelhoeffer, T., *et al.* Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res* **94**, 230, 2004.
46. Schwarz, T.M., *et al.* Vascular incorporation of endothelial colony-forming cells is essential for functional recovery of murine ischemic tissue following cell therapy. *Arterioscler Thromb Vasc Biol* **32**, e13, 2012.
47. Yoder, M.C., *et al.* Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* **109**, 1801, 2007.
48. Krenning, G., *et al.* CD34+ cells augment endothelial cell differentiation of CD14+ endothelial progenitor cells in vitro. *J Cell Mol Med* **13**, 2521, 2009.
49. Sabin, F.R. Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. *Anat Rec* **13**, 199, 1917.
50. Ferkowicz, M.J., and Yoder, M.C. Blood island formation: longstanding observations and modern interpretations. *Exp Hematol* **33**, 1041, 2005.
51. Cox, C.M., and Poole, T.J. Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. *Dev Dyn* **218**, 371, 2000.
52. Risau, W., and Flamme, I. Vasculogenesis. *Annu Rev Cell Dev Biol* **11**, 73, 1995.
53. Choi, K. The hemangioblast: a common progenitor of hematopoietic and endothelial cells. *J Hematother Stem Cell Res* **11**, 91, 2002.
54. Kinder, S.J., *et al.* The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development* **126**, 4691, 1999.
55. Ferkowicz, M.J., *et al.* CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130**, 4393, 2003.
56. Asahara, T., *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964, 1997.
57. Shi, Q., *et al.* Evidence for circulating bone marrow-derived endothelial cells. *Blood* **92**, 362, 1998.

58. Assmus, B., *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* **106**, 3009, 2002.
59. Erbs, S., *et al.* Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. *Circ Res* **97**, 756, 2005.
60. Hirschi, K.K., Ingram, D.A., and Yoder, M.C. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 1584, 2008.
61. Gehling, U.M., *et al.* In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* **95**, 3106, 2000.
62. Yin, A.H., *et al.* AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90**, 5002, 1997.
63. Schmeisser, A., *et al.* Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel® under angiogenic conditions. *Cardiovasc Res* **49**, 671, 2001.
64. Tontonoz, P., Nagy, L., Alvarez, J.G., Thomazy, V.A., and Evans, R.M. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **93**, 241, 1998.
65. Solovey, A., *et al.* Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med* **337**, 1584, 1997.
66. Lin, Y., Weisdorf, D.J., Solovey, A., and Hebbel, R.P. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* **105**, 71, 2000.
67. Schechner, J.S., *et al.* In vivo formation of complex microvessels lined by human endothelial cells in an immunodeficient mouse. *Proc Natl Acad Sci U S A* **97**, 9191, 2000.
68. Patel, J., Donovan, P., and Khosrotehrani, K. Concise review: functional definition of endothelial progenitor cells: a molecular perspective. *Stem Cells Transl Med* **5**, 1302, 2016.
69. Basile, D.P., and Yoder, M.C. Circulating and tissue resident endothelial progenitor cells. *J Cell Physiol* **229**, 10, 2014.
70. Ingram, D.A., *et al.* Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. *Blood* **105**, 2783, 2005.
71. Yoder, M.C. Is endothelium the origin of endothelial progenitor cells? *Arterioscler Thromb Vasc Biol* **30**, 1094, 2010.
72. Choi, J.-H., *et al.* Decreased number and impaired angiogenic function of endothelial progenitor cells in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol* **24**, 1246, 2004.
73. Fadini, G.P., *et al.* Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* **45**, 1449, 2005.
74. Hristov, M., *et al.* Reduced numbers of circulating endothelial progenitor cells in patients with coronary artery disease associated with long-term statin treatment. *Atherosclerosis* **192**, 413, 2007.
75. Lian, X., *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions. *Nat Protoc* **8**, 162, 2013.
76. Yu, J., *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324**, 797, 2009.
77. Lian, X., *et al.* Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. *Stem Cell Reports* **3**, 804, 2014.
78. Prasain, N., *et al.* Differentiation of human pluripotent stem cells to cells similar to cord-blood endothelial colony-forming cells. *Nat Biotechnol* **32**, 1151, 2014.
79. Choi, K., *et al.* Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells* **27**, 559, 2009.
80. James, D., *et al.* Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. *Nat Biotechnol* **28**, 161, 2010.
81. Goldman, O., *et al.* A boost of BMP4 accelerates the commitment of human embryonic stem cells to the endothelial lineage. *Stem Cells* **27**, 1750, 2009.
82. Cimato, T., *et al.* Neuropilin-1 identifies endothelial precursors in human and murine embryonic stem cells before CD34 expression. *Circulation* **119**, 2170, 2009.
83. Dyer, M.A., Farrington, S.M., Mohn, D., Munday, J.R., and Baron, M.H. Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neuroectodermal cell fate in the mouse embryo. *Development* **128**, 1717, 2001.
84. Goldie, L.C., Nix, M.K., and Hirschi, K.K. Embryonic vasculogenesis and hematopoietic specification. *Organogenesis* **4**, 257, 2008.
85. Seghezzi, G., *et al.* Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol* **141**, 1659, 1998.
86. Gory-Fauré, S., *et al.* Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093, 1999.
87. Ferguson, J.E., Kelley, R.W., and Patterson, C. Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler Thromb Vasc Biol* **25**, 2246, 2005.
88. Epstein, J.A., Aghajanian, H., and Singh, M.K. Semaphorin signaling in cardiovascular development. *Cell Metab* **21**, 163, 2015.
89. Drake, C.J., and Fleming, P.A. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* **95**, 1671, 2000.
90. Drake, C.J., Cheresch, D.A., and Little, C.D. An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. *J Cell Sci* **108**, 2655, 1995.
91. George, E.L., Georges-Labouesse, E.N., Patel-King, R.S., Rayburn, H., and Hynes, R.O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079, 1993.
92. Schmidt, A., Brixius, K., and Bloch, W. Endothelial precursor cell migration during vasculogenesis. *Circ Res* **101**, 125, 2007.
93. Drake, C.J., and Little, C.D. VEGF and vascular fusion: implications for normal and pathological vessels. *J Histochem Cytochem* **47**, 1351, 1999.
94. Dzierzak, E. Ontogenic emergence of definitive hematopoietic stem cells. *Curr Opin Hematol* **10**, 229, 2003.
95. Ellertsdóttir, E., *et al.* Vascular morphogenesis in the zebrafish embryo. *Dev Biol* **341**, 56, 2010.

96. Gore, A.V., Monzo, K., Cha, Y.R., Pan, W., and Weinstein, B.M. Vascular development in the zebrafish. *Cold Spring Harb Perspect Med* **2**, a006684, 2012.
97. Larrivée, B., Freitas, C., Suchting, S., Brunet, I., and Eichmann, A. Guidance of vascular development: lessons from the nervous system. *Circ Res* **104**, 428, 2009.
98. Herbert, S.P., and Stainier, D.Y.R. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* **12**, 551, 2011.
99. Maisonpierre, P.C., *et al.* Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55, 1997.
100. Welte, J., *et al.* Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest* **123**, 3190, 2013.
101. Senger, D.R. Molecular framework for angiogenesis: a complex web of interactions between extravasated plasma proteins and endothelial cell proteins induced by angiogenic cytokines. *Am J Pathol* **149**, 1, 1996.
102. Senger, D.R., and Davis, G.E. Angiogenesis. *Cold Spring Harb Perspect Biol* **3**, 1, 2011.
103. Kim, J., Oh, W.J., Gaiano, N., Yoshida, Y., and Gu, C. Semaphorin 3E-plexin-d1 signaling regulates VEGF function in developmental angiogenesis via a feedback mechanism. *Genes Dev* **25**, 1399, 2011.
104. Jones, C.A., *et al.* Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nat Med* **14**, 448, 2008.
105. Belvisi, L., *et al.* Biological and molecular properties of a new α v β 3/ α v β 5 integrin antagonist. *Mol Cancer Ther* **4**, 1670, 2005.
106. Krueger, J., *et al.* Flt1 acts as a negative regulator of tip cell formation and branching morphogenesis in the zebrafish embryo. *Development* **138**, 2111, 2011.
107. Hiratsuka, S., *et al.* Membrane fixation of vascular endothelial growth factor receptor 1 ligand-binding domain is important for vasculogenesis and angiogenesis in mice. *Mol Cell Biol* **25**, 346, 2005.
108. Zovein, A.C., *et al.* Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. *Dev Cell* **18**, 39, 2010.
109. Strilić, B., *et al.* The molecular basis of vascular lumen formation in the developing mouse aorta. *Dev Cell* **17**, 505, 2009.
110. Parker, L.H., *et al.* The endothelial-cell-derived secreted factor Egr1 regulates vascular tube formation. *Nature* **428**, 754, 2004.
111. Kamei, M., *et al.* Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* **442**, 453, 2006.
112. Davis, G.E., and Bayless, K.J. An integrin and Rho GTPase-dependent pinocytic vacuole mechanism controls capillary lumen formation in collagen and fibrin matrices. *Microcirculation* **10**, 27, 2003.
113. Hanjaya-Putra, D., *et al.* Vascular endothelial growth factor and substrate mechanics regulate in vitro tubulogenesis of endothelial progenitor cells. *J Cell Mol Med* **14**, 2436, 2010.
114. Lindahl, P. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277**, 242, 1997.
115. Lindblom, P., *et al.* Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes Dev* **17**, 1835, 2003.
116. Paik, J.H., *et al.* Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. *Genes Dev* **18**, 2392, 2004.
117. Chen, S., and Lechleider, R.J. Transforming growth factor-beta-induced differentiation of smooth muscle from a neural crest stem cell line. *Circ Res* **94**, 1195, 2004.
118. Stenzel, D., *et al.* Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. *EMBO Rep* **12**, 1135, 2011.
119. Herzog, Y., Guttman-Raviv, N., and Neufeld, G. Segregation of arterial and venous markers in subpopulations of blood islands before vessel formation. *Dev Dyn* **232**, 1047, 2005.
120. Marcelo, K.L., Goldie, L.C., and Hirschi, K.K. Regulation of endothelial cell differentiation and specification. *Circ Res* **112**, 1272, 2013.
121. le Noble, F., *et al.* Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development* **131**, 361, 2004.
122. Adams, R.H., and Alitalo, K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* **8**, 464, 2007.
123. Kwei, S., *et al.* Early adaptive responses of the vascular wall during venous arterialization in mice. *Am J Pathol* **164**, 81, 2004.
124. Lawson, N.D., Vogel, A.M., and Weinstein, B.M. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell* **3**, 127, 2002.
125. Atkins, G.B., Jain, M.K., and Hamik, A. Endothelial differentiation: molecular mechanisms of specification and heterogeneity. *Arterioscler Thromb Vasc Biol* **31**, 1476, 2011.
126. Vokes, S.A., *et al.* Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development* **131**, 4371, 2004.
127. Swift, M.R., and Weinstein, B.M. Arterial-venous specification during development. *Circ Res* **104**, 576, 2009.
128. Lanner, F., Sohl, M., and Farnebo, F. Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. *Arterioscler Thromb Vasc Biol* **27**, 487, 2007.
129. Lee, P., *et al.* Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. *Proc Natl Acad Sci U S A* **99**, 10470, 2002.
130. Lawson, N.D., *et al.* Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675, 2001.
131. Gu, C., *et al.* Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* **5**, 45, 2003.
132. You, L.-R., *et al.* Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**, 98, 2005.
133. Cueni, L.N., and Detmar, M. New insights into the molecular control of the lymphatic vascular system and its role in disease. *J Invest Dermatol* **126**, 2167, 2006.
134. Gale, N.W., *et al.* Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. *Mol Cell Biol* **27**, 595, 2007.
135. Francois, M., *et al.* Sox18 induces development of the lymphatic vasculature in mice. *Nature* **456**, 643, 2008.

136. Srinivasan, R.S., *et al.* The nuclear hormone receptor Coup-TFII is required for the initiation and early maintenance of Prox1 expression in lymphatic endothelial cells. *Genes Dev* **24**, 696, 2010.
137. Wigle, J.T., *et al.* An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* **21**, 1505, 2002.
138. Hong, Y.K., *et al.* Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* **225**, 351, 2002.
139. Yuan, L., *et al.* Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129**, 4797, 2002.
140. Kärpänen, T., *et al.* Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. *FASEB J* **20**, 1462, 2006.
141. Karkkainen, M.J., *et al.* Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* **5**, 74, 2004.
142. Karpanen, T., *et al.* Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *Am J Pathol* **169**, 708, 2006.
143. Lee, S., Choi, I., and Hong, Y. K. Heterogeneity and plasticity of lymphatic endothelial cells. *Semin Thromb Hemost* **36**, 352, 2010.
144. Johnson, N.C., *et al.* Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev* **22**, 3282, 2008.
145. Kang, J., *et al.* An exquisite cross-control mechanism among endothelial cell fate regulators directs the plasticity and heterogeneity of lymphatic endothelial cells. *Blood* **116**, 140, 2010.
146. Rocha, S.F., and Adams, R.H. Molecular differentiation and specialization of vascular beds. *Angiogenesis* **12**, 139, 2009.
147. Tse, D., and Stan, R. V. Morphological heterogeneity of endothelium. *Semin Thromb Hemost* **36**, 236, 2010.
148. Simionescu, M., Simionescu, N., and Palade, G.E. Morphometric data on the endothelium of blood capillaries. *J Cell Biol* **60**, 128, 1974.
149. Braet, F., and Wisse, E. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp Hepatol* **1**, 1, 2002.
150. Dejana, E. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol* **5**, 261, 2004.
151. Bendayan, M. Morphological and cytochemical aspects of capillary permeability. *Microsc Res Tech* **57**, 327, 2002.
152. Dvorak, A.M., and Feng, D. The vesiculo-vacuolar organelle (VVO): a new endothelial cell permeability organelle. *J Histochem Cytochem* **49**, 419, 2001.
153. Paolinelli, R., Corada, M., Orsenigo, F., and Dejana, E. The molecular basis of the blood brain barrier differentiation and maintenance. Is it still a mystery? *Pharmacol Res* **63**, 165, 2011.
154. Daneman, R., *et al.* Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proc Natl Acad Sci U S A* **106**, 641, 2009.
155. Stenman, J.M., *et al.* Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* **322**, 1247, 2008.
156. Liebner, S., *et al.* Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J Cell Biol* **183**, 409, 2008.
157. Daneman, R., Zhou, L., Kebede, A.A., and Barres, B.A. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* **468**, 562, 2010.
158. Lee, S.W., *et al.* SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. *Nat Med* **9**, 900, 2003.
159. LeCouter, J., *et al.* Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* **412**, 877, 2001.
160. Ioannidou, S., *et al.* An in vitro assay reveals a role for the diaphragm protein PV-1 in endothelial fenestra morphogenesis. *Proc Natl Acad Sci U S A* **103**, 16770, 2006.
161. Esser, S., *et al.* Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol* **140**, 947, 1998.
162. Ichimura, K., Stan, R.V., Kurihara, H., and Sakai, T. Glomerular endothelial cells form diaphragms during development and pathologic conditions. *J Am Soc Nephrol* **19**, 1463, 2008.
163. Eremina, V., Baelde, H.J., and Quaggin, S.E. Role of the VEGF-A signaling pathway in the glomerulus: evidence for crosstalk between components of the glomerular filtration barrier. *Nephron Physiol* **106**, 32, 2007.
164. Satchell, S.C., and Braet, F. Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier. *Am J Physiol Renal Physiol* **296**, F947, 2009.
165. Yokomori, H., *et al.* Rho modulates hepatic sinusoidal endothelial fenestrae via regulation of the actin cytoskeleton in rat endothelial cells. *Lab Invest* **84**, 857, 2004.
166. Eriksson, A., *et al.* Small GTP-binding protein Rac is an essential mediator of vascular endothelial growth factor-induced endothelial fenestrations and vascular permeability. *Circulation* **107**, 1532, 2003.
167. Hoffman, G.S., and Calabrese, L.H. Vasculitis: determinants of disease patterns. *Nat Rev Rheumatol* **10**, 454, 2014.
168. Pries, A.R., and Kuebler, W. M. Normal endothelium. *Handb Exp Pharmacol* **176**, 1, 2006.
169. Ingber, D.E., and Folkman, J. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J Cell Biol* **109**, 317, 1989.
170. Finkenzeller, G., Torio-Padron, N., Momeni, A., Mehlforn, A.T., and Stark, G.B. In vitro angiogenesis properties of endothelial progenitor cells: a promising tool for vascularization of ex vivo engineered tissues. *Tissue Eng* **13**, 1413, 2007.
171. Chen, X., *et al.* Prevascularization of a fibrin-based tissue construct accelerates the formation of functional anastomosis with host vasculature. *Tissue Eng Part A* **15**, 1363, 2009.
172. Wu, X., *et al.* Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am J Physiol Heart Circ Physiol* **287**, H480, 2004.
173. Au, P., *et al.* Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. *Blood* **111**, 1302, 2008.
174. Yuan, F., Leunig, M., Berk, D.A., and Jain, R.K. Microvascular permeability of albumin, vascular surface area, and vascular volume measured in human adenocarcinoma

- LS174T using dorsal chamber in SCID mice. *Microvasc Res* **45**, 269, 1993.
175. Kusuma, S., *et al.* Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. *Proc Natl Acad Sci U S A* **110**, 12601, 2013.
 176. Chen, X.F., *et al.* 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.
 177. Sasagawa, T., Shimizu, T., Yamato, M., and Okano, T. Endothelial colony-forming cells for preparing pre-vascular three-dimensional cell-dense tissues using cell-sheet engineering. *J. Tissue Eng Regen Med* **10**, 739, 2016.
 178. Traktuev, D.O., *et al.* Robust functional vascular network formation in vivo by cooperation of adipose progenitor and endothelial cells. *Circ Res* **104**, 1410, 2009.
 179. Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, P.D., and Hughes, C.C.W. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell* **22**, 3791, 2011.
 180. Souidi, N., *et al.* Stromal cells act as guardians for endothelial progenitors by reducing their immunogenicity after co-transplantation. *Stem Cells* **35**, 1233, 2017.
 181. Athanassopoulos, A., *et al.* Microvessel networks preformed in artificial clinical grade dermal substitutes in vitro using cells from haematopoietic tissues. *Burns* **38**, 691, 2012.
 182. Hirschi, K.K., Burt, J.M., Hirschi, K.D., and Dai, C. Gap junction communication mediates transforming growth factor-beta activation and endothelial-induced mural cell differentiation. *Circ Res* **93**, 429, 2003.
 183. Fang, J.S., Dai, C., Kurjiaka, D.T., Burt, J.M., and Hirschi, K.K. Connexin45 regulates endothelial-induced mesenchymal cell differentiation toward a mural cell phenotype. *Arterioscler Thromb Vasc Biol* **33**, 362, 2013.
 184. Roubelakis, M.G., Tsaknakis, G., Pappa, K.I., Anagnou, N.P., and Watt, S.M. Spindle shaped human mesenchymal stem/stromal cells from amniotic fluid promote neovascularization. *PLoS One* **8**, e54747, 2013.
 185. Zuk, P.A., *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279, 2002.
 186. Rohringer, S., *et al.* Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. *Angiogenesis* **17**, 921, 2014.
 187. Vériter, S., *et al.* Human adipose-derived mesenchymal stem cells in cell therapy: safety and feasibility in different 'hospital exemption' clinical applications. *PLoS One* **10**, 2015.
 188. Ji, H.Y., *et al.* Transdifferentiation of human endothelial progenitors into smooth muscle cells. *Biomaterials* **85**, 180, 2016.
 189. Finkenzeller, G., Graner, S., Kirkpatrick, C.J., Fuchs, S., and Stark, G.B. Impaired in vivo vasculogenic potential of endothelial progenitor cells in comparison to human umbilical vein endothelial cells in a spheroid-based implantation model. *Cell Prolif* **42**, 498, 2009.
 190. Li, Y., Meng, H., Liu, Y., and Lee, B.P. Fibrin gel as an injectable biodegradable scaffold and cell carrier for tissue engineering. *ScientificWorldJournal* **2015**, 685690, 2015.
 191. Cholewinski, E., Dietrich, M., Flanagan, T.C., Schmitz-Rode, T., and Jockenhoevel, S. Tranexamic acid—an alternative to aprotinin in fibrin-based cardiovascular tissue engineering. *Tissue Eng Part A* **15**, 3645, 2009.
 192. Rowe, S.L., Lee, S., and Stegemann, J.P. Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels. *Acta Biomater* **3**, 59, 2007.
 193. Helary, C., *et al.* Concentrated collagen hydrogels as dermal substitutes. *Biomaterials* **31**, 481, 2010.
 194. Allen, P., Melero-Martin, J., and Bischoff, J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. *J Tissue Eng Regen Med* **5**, e74, 2011.
 195. Asti, A., and Gioglio, L. Natural and synthetic biodegradable polymers: different scaffolds for cell expansion and tissue formation. *Int J Artif Organs* **37**, 187, 2014.
 196. Akiyama, Y., Kikuchi, A., Yamato, M., and Okano, T. Ultrathin poly(N-isopropylacrylamide) grafted layer on polystyrene surfaces for cell adhesion/detachment control. *Langmuir* **20**, 5506, 2004.
 197. Yee, D., Hanjaya-Putra, D., Bose, V., Luong, E., and Gerecht, S. Hyaluronic acid hydrogels support cord-like structures from endothelial colony-forming cells. *Tissue Eng Part A* **17**, 1351, 2011.
 198. Minuth, W.W., Strehl, R., and Schumacher, K. Concepts of tissue creation: diversity of development. In: Minuth, W.W., Strehl, R., and Schumacher, K., eds. *Tissue engineering: essentials for daily laboratory work*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co., 2005, pp. 142–143.
 199. Hahn, M.S., *et al.* Photolithographic patterning of polyethylene glycol hydrogels. *Biomaterials* **27**, 2519, 2006.
 200. Moon, J.J., *et al.* Biomimetic hydrogels with pro-angiogenic properties. *Biomaterials* **31**, 3840, 2010.
 201. Bahney, C.S., *et al.* Visible light photoinitiation of mesenchymal stem cell-laden bioresponsive hydrogels. *Eur Cells Mater* **22**, 43, 2011.
 202. Reinisch, A., *et al.* Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood* **113**, 6716, 2009.
 203. Dutenhoefer, F., *et al.* 3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells: evidence of prevascularisation within 7 days. *Eur Cells Mater* **26**, 49, 2013.
 204. Bao, X., *et al.* Chemically-defined albumin-free differentiation of human pluripotent stem cells to endothelial progenitor cells. *Stem Cell Res* **15**, 122, 2015.
 205. Rafii, S., Butler, J.M., and Ding, B.S. Angiocrine functions of organ-specific endothelial cells. *Nature* **529**, 316, 2016.
 206. Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K.S. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559, 2001.
 207. Lammert, E., Cleaver, O., and Melton, D. Induction of pancreatic differentiation by signals from blood vessels. *Science* **294**, 564, 2001.
 208. Shen, Q., *et al.* Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* **304**, 1338, 2004.
 209. Ding, B.S., *et al.* Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature* **505**, 97, 2014.
 210. Boyer-Di Ponio, J., *et al.* Instruction of circulating endothelial progenitors in vitro towards specialized blood-brain

- barrier and arterial phenotypes. PLoS One **9**, e84179, 2014.
211. DiMaio, T.A., Wentz, B.L., and Lagunoff, M. Isolation and characterization of circulating lymphatic endothelial colony forming cells. *Exp Cell Res* **340**, 159, 2016.
 212. Wilson, H.K., Canfield, S.G., Shusta, E.V., and Palecek, S.P. Concise review: tissue-specific microvascular endothelial cells derived from human Pluripotent stem cells. *Stem Cells* **32**, 3037, 2014.
 213. Lippmann, E.S., *et al.* Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nat Biotechnol* **30**, 783, 2012.
 214. Meadows, S.M., Myers, C.T., and Krieg, P.A. Regulation of endothelial cell development by ETS transcription factors. *Semin Cell Dev Biol* **22**, 976, 2011.
 215. Wang, N., *et al.* Vascular endothelial growth factor stimulates endothelial differentiation from mesenchymal stem cells via Rho/myocardin-related transcription factor-A signaling pathway. *Int J Biochem Cell Biol* **45**, 1, 2013.
 216. Zisch, A.H., *et al.* Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. *FASEB J* **17**, 2260, 2003.
 217. Pola, R., *et al.* The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med* **7**, 706, 2001.
 218. Deckers, M.M., *et al.* Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* **143**, 1545, 2002.
 219. Déry, M.A.C., Michaud, M.D., and Richard, D.E. Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. *Int J Biochem Cell Biol* **37**, 535, 2005.
 220. Rivron, N.C., *et al.* Sonic Hedgehog-activated engineered blood vessels enhance bone tissue formation. *Proc Natl Acad Sci U S A* **109**, 4413, 2012.
 221. González, F. CRISPR/Cas9 genome editing in human pluripotent stem cells: harnessing human genetics in a dish. *Dev Dyn* **245**, 788, 2016.
 222. Macklin, B.L., and Gerecht, S. Bridging the gap: induced pluripotent stem cell derived endothelial cells for 3D vascular assembly. *Curr Opin Chem Eng* **15**, 102, 2017.
 223. Ferrara, N., and Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* **438**, 967, 2005.
 224. Wu, W., Deconinck, A., and Lewis, J.A. Omnidirectional printing of 3D microvascular networks. *Adv Mater* **23**, 2011.
 225. Kolesky, D.B., *et al.* 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv Mater* **26**, 3124, 2014.
 226. Kolesky, D.B., Homan, K.A., Skylar-Scott, M.A., and Lewis, J.A. Three-dimensional bioprinting of thick vascularized tissues. *Proc Natl Acad Sci U S A* **113**, 3179, 2016.

Address correspondence to:

Erica B. Peters, PhD

Department of Chemical and Biological Engineering

University of Colorado

Boulder, CO 80303

E-mail: erbr8553@colorado.edu

Received: March 15, 2017

Accepted: May 24, 2017

Online Publication Date: July 3, 2017