




Cellular Based Strategies for Microvascular Engineering

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Abstract

Vascularization is a major hurdle in complex tissue and organ engineering. Tissues greater than 200 μm in diameter cannot rely on simple diffusion to obtain nutrients and remove waste. Therefore, an integrated vascular network is required for clinical translation of engineered tissues. Microvessels have been described as $<150 \mu\text{m}$ in diameter, but clinically they are defined as $<1 \text{ mm}$. With new advances in super microsurgery, vessels less than 1 mm can be anastomosed to the recipient circulation. However, this technical advancement still relies on the creation of a stable engineered microcirculation that is amenable to surgical manipulation and is readily perfusable. Microvascular engineering lays on the crossroads of microfabrication, microfluidics, and tissue engineering strategies that utilize various cellular constituents. Early research focused on vascularization by co-culture and cellular interactions, with the addition of angiogenic growth factors to promote vascular growth. Since then, multiple strategies have been utilized taking advantage of innovations in additive manufacturing, biomaterials, and cell biology. However, the anatomy and dynamics of native blood vessels has not been consistently replicated. Inconsistent results can be partially attributed to cell sourcing which remains an enigma for microvascular engineering. Variations of endothelial cells, endothelial progenitor cells, and stem cells have all been used for microvascular network fabrication along with various mural cells. As each source offers advantages and disadvantages, there continues to be a lack of consensus. Furthermore, discord may be attributed to incomplete understanding about cell isolation and characterization without considering the microvascular architecture of the desired tissue/organ.

Keywords Endothelial progenitor cells · EPCs · Adipose tissue · ADSCs · CD34⁺ · Microvessel fragments · Angiogenesis · Vasculogenesis · Vascularization · iPSCs · Adult stem cells · MSCs

Introduction

One of the aims of tissue engineering is to create replacement parts for damaged tissue and organs. This achievement would

have profound clinical implications, especially in the disciplines of reconstructive surgery, transplant medicine and vascular surgery. Over 110,000 patients are currently waiting for an organ transplant and thousands of others could benefit from a vascularized composite allograft [1]. However, clinical translation of engineered tissue has been limited by the insufficient diffusion of oxygen and nutrients [2] into the graft. At diffusion distances greater than 150–200 μm , tissue constructs without a perfusable vascular network undergo rapid cell death and necrosis [3]. Therefore, in order to achieve successful implantation, the engineered replacement mandates rapid perfusion and integration with the recipient vasculature [4]. This typically requires a surgical anastomosis between the vascularized construct and recipient vasculature. Parameters such as size of the vascular connection and perfusion rate can affect patency [5]. Therefore, an engineered construct that contains a vascular tree from the capillary level ($\sim 5 \mu\text{m}$ diameter) to one that permits surgical anastomosis ($\sim 1 \text{ mm}$ diameter) would be welcomed. Advances in microvascular engineering would also be embraced by any medical or surgical

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field in which microvascular dysfunction is present, such as diabetic retinopathy [6], microvascular myocardial angina [7], and critical limb ischemia. For example, it is estimated that 1% of Americans over 50 years will develop critical limb ischemia [8]. While surgical advances have reduced the incidence of patients that ultimately require amputation, they are not feasible in all situations. This is particularly true when the blood flow limiting pathology occurs in the microvascular segment, as typically seen in diabetic patients [9].

Current approaches for vascularization rely on a variety of cell sources and approaches [10], with endothelial cells (EC) being a foundation. However, as ECs are significant in the rejection of allografts [11], it is imperative that cells used for microvascular engineering are compatible with the planned recipient. Therefore, cell sourcing must account for immunogenicity, in addition to other factors such as ease of isolation, reliable characterization, and vasculogenic/angiogenic potential. This has led to the investigation of mature endothelial cells, endothelial progenitor cells (EPCs), and other stem cell sources for the formation of microvascular networks in tissue engineered constructs and the treatment of critical ischemia.

Microvascular Development, Angiogenesis, and Revascularization

Embryologically, vascular development occurs through the processes of vasculogenesis and angiogenesis (Fig. 1) [12]. Vasculogenesis is the process of vessel formation in the early stage of development during which *de novo* blood vessels are formed from endothelial progenitor cells [13]. The lateral and posterior mesoderm in the gastrulating embryo express vascular endothelial growth factor receptor-2 (VEGFR2), the receptor for VEGF, which is the most crucial signal for embryonic vascular formation [14]. This receptor tyrosine kinase is encoded by the KDR (kinase insert domain receptor) gene, which is also known as fetal liver kinase-1 (FLK-1). The VEGFR2 expressing cells cluster to form hemangioblastic aggregates [15]. The peripheral cells (angioblasts) flatten

and differentiate into ECs, while the central cells form hematopoietic precursors. These progenitor cells migrate in response to endoderm derived signals, such as FGF (fibroblast growth factor) and BMP4 (bone morphogenic protein), to the sites of vascularization [16]. Following this, the ECs rapidly anastomose to form capillaries/primary vascular plexus and serve as a scaffold for the circulation. After the onset of heart-beat and blood flow, the capillary plexus is rapidly remodeled into a functional circulatory system [14]. Angiogenesis begins on embryonic day 8.5 in the mouse yolk sac [17, 18] and occurs through a coordinated series of events where vascular sprouts grow from specialized endothelial “tip cells” in response to angiogenic stimuli [19]. Initially, local destruction of the basement membrane occurs, followed by sprouting, proliferation, and alignment of endothelial cells into a tube. Angiogenesis is responsible for vascular remodeling through both sprouting and intussusceptive mechanisms with VEGF maintaining a critical role. Once the definitive vascular network is established, ECs remain quiescent until physiological or pathological events occur that require neovascularization. Neovascularization is achieved by both vasculogenesis (EPCs) and angiogenesis (adult ECs) [20] through gene expression, transcriptional factors, and microRNA (miRNA) regulation. Growth factors, cytokines and proteins play a major role in endothelial differentiation (Table 1; Figure 2) with VEGF and its receptors (VEGFR1 and VEGFR2) indispensable for blood vessel development and remodeling [46]. However, numerous signals/proteins are involved including Notch, Ephrin, Sonic hedgehog, and angiopoietin (Ang)-Tie. Notch signaling regulates both cellular proliferation and development, and in association with delta-like 4 (Dll4) modulates sprouting angiogenesis [47] via tip cells [48]. Ang-Tie is an EC specific signaling system that is necessary in both embryonic vascular development and regulation of postnatal angiogenesis [49]. Transcription factors regulate these critical gene expressions to modulate angiogenesis (Table 2). The ETS (E26 transformation-specific) transcription factor family plays a significant role in angiogenesis by targeting genes such as von Willebrand factor (vWF), Endoglin, Ang-Tie,

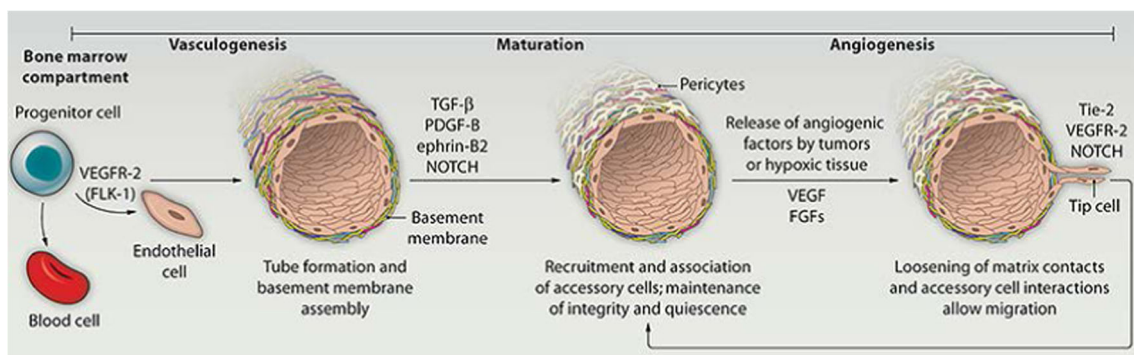


Fig. 1 Vasculogenesis and angiogenesis: Two distinct mechanisms of blood vessel formation. Vasculogenesis gives rise to the primitive vascular plexus during embryogenesis. Stimulated by tumors and hypoxic conditions, angiogenesis remodels and expands the vascular network [12]

Table 1 Proteins described in the literature that play important roles in angiogenesis, sprouting, and vascular development. The coordinated action of these proteins are essential in the regulation of both vasculogenesis and angiogenesis. #For example, during the early stages

of sprouting angiogenesis the secretion of VEGF from tissues results in KDR activation in nearby vessels. The endothelial cell exposed to the highest concentration of VEGF becomes a “tip cell” followed by the expression of DLL4 and Notch

Proteins	References	Role
VEGF	[19, 21]	Angiogenesis, Sprouting, Vascular Development
VEGFR1, VEGFR2	[22–24]	Angiogenesis, Sprouting
Tie1, Tie2	[25–27]	Angiogenesis, Sprouting, Vascular Development
ID3	[28]	Angiogenesis, Sprouting
eNOS	[29, 30]	Angiogenesis, Sprouting,
AKT1	[31–33]	Angiogenesis, Sprouting, Vascular Development
CD31	[34, 35]	Angiogenesis
VE-Cadherin	[36, 37]	Angiogenesis, Sprouting
Notch, DLL4	[38, 39]	Inhibits angiogenesis
KDR/Flt1	[40, 41]	Angiogenesis, Vascular Development, Sprouting
ANGPT1	[33, 42, 43]	Angiogenesis, Sprouting
ANGPT2	[44, 45]	Vascular Development, Sprouting

VEGFR2, eNOS (endothelial nitric oxide synthase), and VE-cadherin [72]. For example, the ETS transcription factor Erg is required for endothelial tube formation through its association with VE-cadherin [36].

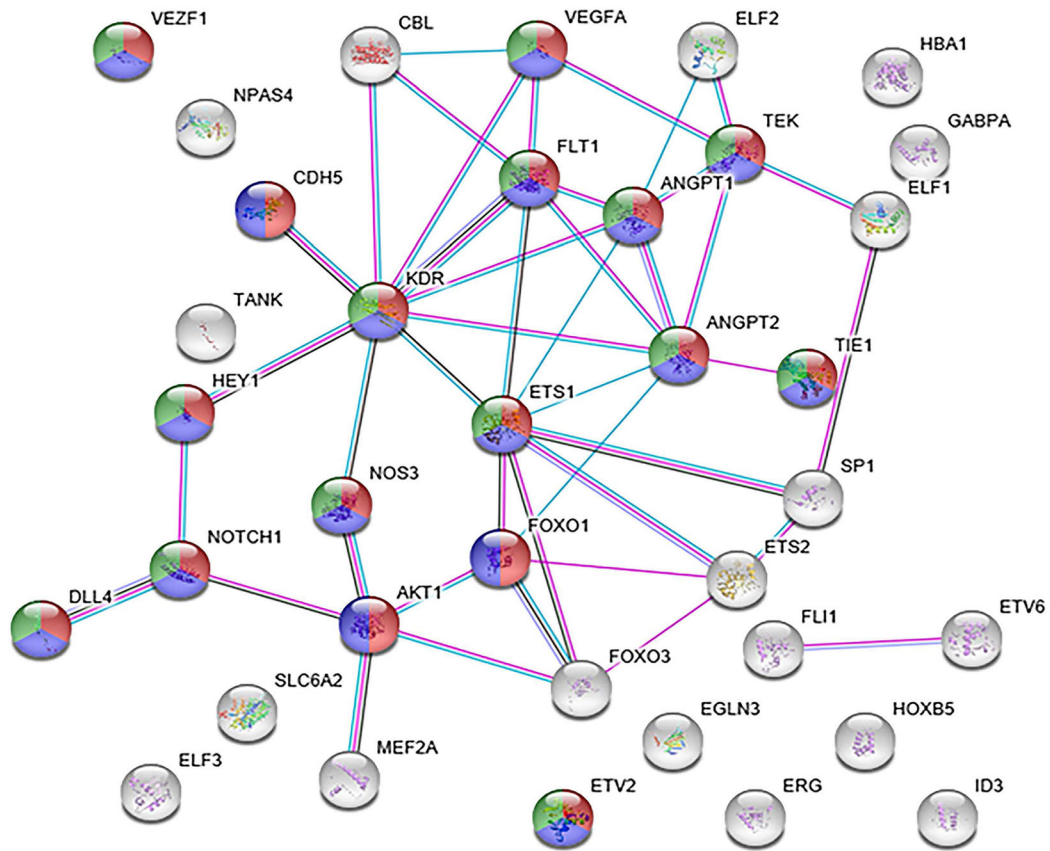
Gene expression is also controlled by miRNAs, which are small non-coding RNA molecules containing approximately 17–25 nucleotides that negatively regulate gene expression either by reducing translational efficiency or transcript degradation. A single miRNA has the ability to regulate hundreds of genes and affect the downstream transcriptome and proteome. Therefore, they have a profound ability to affect multiple biological processes, such as development, differentiation and growth. Recent studies have highlighted their involvement in vascular development (Table 3), with many studies focusing on their involvement in the VEGF signaling pathway. MicroRNAs affect vascular development by playing substantial modulatory roles, both at the ligand level and further downstream through intracellular signaling pathways, such as phosphoinositide-3-kinase (PI3K) and MAPK/ERK [87]. Their importance in embryonic angiogenesis has further been verified through Dicer-knockout mice which die mid-gestation [88]. However, miRNA involvement has also been suggested in post-natal vascular pathologies, including critical limb ischemia. Spinetti *et al.* have suggested that miRNA-15a and miRNA-16 impair circulating proangiogenic cell function and are increased in patients with critical limb ischemia [89]. Additionally, the authors further demonstrated that *ex vivo* inhibition of these miRNAs improved post-ischemia blood flow and muscular arteriole density in a murine model of critical limb ischemia [89]. Therefore, it is not surprising that miRNA modulation has emerged as a technology to facilitate vascular engineering [90], both *in vivo* and *ex vivo*. However, as the vascular system is diverse in size, architecture, and

functionality, multiple engineering approaches may need to be considered in a coordinated fashion Table 4.

Histology and Anatomy

The vasculature can be apportioned between the microvasculature and the macrovasculature. The microvasculature cannot be seen with the naked eye and is typically defined anatomically as having a luminal diameter <150 μm and functionally as regulating the total peripheral resistance in the vascular tree [102]. This includes arterioles, capillaries, and venules. Arterioles originate from arteries to arborize into capillaries which coalesce into venules that drain into veins. All constituents of the vascular tree are lined by endothelial cells (tunica intima) with varying degrees of stromal cells. Arterioles (100 to 300 μm diameter) have muscular walls (tunica media) that consist of a few layers of smooth muscle cells surrounded by a collagen layer (tunica adventitia). Capillaries (5 to 10 μm diameter) only have a single EC layer without any other distinct layers. This allows efficient material exchange between the blood and interstitium. Venules (7 μm to 1 mm diameter) contain a middle layer of muscle/elastic tissue and an outer layer of connective tissue. The ECs in capillaries and venules are enveloped by pericytes which are located in the basement membrane. Pericytes regulate microvascular morphogenesis and homeostasis while exhibiting some mesenchymal stem/progenitor cell functionality [103]. However, these cellular behaviors and interactions are also profoundly affected by the surrounding extracellular matrix (ECM).

The ECM is a structural support system that provides a matrix for cell adhesion and serves as a storage depot for growth factors [104]. Therefore, it is not surprising that it



Network Stats

number of nodes: 36
 number of edges: 43
 average node degree: 2.39
 avg. local clustering coefficient: 0.365

expected number of edges: 3
 PPI enrichment p-value: < 1.0e-16
your network has significantly more interactions than expected

Functional enrichment in your network

Biological Process (GO)

Pathway ID	Pathway description	count in gene set	FDR
GO:0001568	blood vessel development	17	1.69e-15
GO:0001944	vasculature development	17	1.91e-15
GO:0072358	cardiovascular system development	19	5e-15
GO:0072359	circulatory system development	19	5e-15
GO:0048514	blood vessel morphogenesis	14	1.63e-12

Nodes:

Network nodes represent proteins

splice isoforms or post-translational modifications are collapsed, i.e. each node represents all the proteins produced by a single, protein-coding gene locus

Node Color

- colored nodes: query proteins and first shell of interactors
- white nodes: second shell of interactors

Node Content

- empty nodes: Protein of unknown 3D structure
- filled nodes: some 3D structure is known or predicted

Edges:

Edges represent protein-protein associations

Associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other.

Known Interactions

- from curated databases
- experimentally determined

Predicted Interactions

- gene neighborhood
- gene fusions
- gene co-occurrence

Others

- textmining
- co-expression
- protein homology

Fig. 2 STRING analysis of functional protein association networks in vasculogenesis: Key proteins and transcription factors that are involved in vasculogenesis were identified by a literature search. Protein-protein interactions were evaluated using STRING analysis. The interactions between proteins and transcriptional factors within major Biological Processes (GO) such as vasculature development (blue color), blood vessel development (red color) and morphogenesis (green color) are highlighted

plays a large role in coordinating the angiogenic process through pro- and anti-angiogenic factor regulation, matrix-degrading proteases, and cell-ECM interactions [105]. The ECM is synthesized by fibroblasts and includes structural proteins (e.g. collagen), adhesive glycoproteins (e.g. fibronectin, laminin), glycosaminoglycans (e.g. hyaluronan), and proteoglycans (e.g. heparin sulfate) [104, 105]. It can broadly be subdivided into the interstitial matrix and the extracellular basement membrane, with the former consisting of collagen, fibronectin, elastin, and proteoglycans and the latter consisting of collagen IV, laminin, entactin, and heparin sulfate which binds to VEGF [106]. These ECM molecules/fragments can function in either a pro-angiogenic (e.g. collagen I, fibronectin, laminin-1) or anti-angiogenic fashion (e.g. arresten, endostatin) [106]. Angiogenesis occurs in ischemic tissues when oxygen sensing mechanisms detect a level of hypoxia that requires new blood vessel formation to satisfy metabolic demands. The most common form occurs in stages through a sprouting mechanism (Fig. 3) as follows: enzymatic degradation of the capillary basement membrane, endothelial cell proliferation, directed migration, tubulogenesis, vessel fusion; and pericyte [107], smooth muscle, and fibroblast stabilization [108]. This coordinated activity is orchestrated by the mechanical forces in the ECM along with cell-cell and cell-matrix interactions, bound proteins, and soluble growth factors. For example, $\alpha V\beta 3$ integrin present on ECs allows for binding to ECM vitronectin which augments the EC

Table 3 Key miRNAs described in the literature involved in angiogenesis, sprouting and tube formation. Improved understanding of vascular development has allowed for the identification of miRNAs involved in RNA silencing and post-transcriptional gene regulation

miRNAs	References	Role
miR-1224	[73]	Angiogenesis
miR-93	[74]	Tube formation
miR-505	[75]	Angiogenesis
miR-329	[76]	Inhibits angiogenesis
miR-126	[77, 78]	Vascular integrity and angiogenesis
miR-130a	[79]	Angiogenesis
miR-210	[80]	Tube formation
miR-296	[81]	Angiogenesis
miR-221, miR-222	[82–84]	Inhibits tube formation
miR-17-92	[81]	Angiogenesis
miR-27b	[85]	Angiogenesis
Let-7b	[86]	Angiogenesis

responsiveness to soluble factors, such as VEGF [104]. This intricate interplay allows for varied angiogenic responses depending on the initiating factors and anatomic site.

Variability in the microcirculatory bed across different tissues and organs is secondary to differences in cellular composition leading to adaptable physiology. For example, the microcirculation of the skin is distinct from that seen in the pancreatic islets as the skin microvasculature contains a large number of post-capillary venules that aid in preventing unwanted external elements from entering the body and thermoregulation [109]. The endocrine pancreatic islets are arranged around a central capillary that allows blood to first pass through centrally located β -cells and initiate an insulin response to circulating glucose prior to encountering α -cells and δ -cells that are responsible for appropriate counter-

Table 2 Key transcription factors described in the literature that play important roles in angiogenesis, sprouting and vascular development. The discovery of genes important in vasculogenesis and angiogenesis has enabled the identification of transcriptional mechanisms required for their expression

Transcription Factors	References	Role
HIF-1 α	[50]	Angiogenesis
SP-1	[50]	Angiogenesis
ETS family: ETV2, TEL, ETS1, ETS2, FLI1, ERG, ELF1, NET, NRF2/GABP, ELF2/NERF2, ELF3/ESE1	[36, 51–63]	Endothelial Differentiation Angiogenesis, Sprouting
NF- κ B	[64]	Angiogenesis
SCL/tal-1	[65]	Vascular Development
FOXO1	[66]	Angiogenesis
FOXO3a	[66]	Angiogenesis
Vezf1	[67]	Endothelial Differentiation
MEF2	[68]	Vascular Development, Sprouting
HESR1	[69]	Endothelial Tube Formation
NPAS4	[70]	Sprouting
HoxB5	[71]	Sprouting

Table 4 Representative studies of human derived cellular starting materials for microvascular engineering applications/investigations. Adult ECs along with multipotent and pluripotent stem cells have been cultured in various scaffolds and conditions to demonstrate *in vitro* and *in vivo* possibilities

Cell Type	Cell Source	Phenotype	Scaffold	<i>In vitro/in vivo</i> outcome	Ref
ECs	Umbilical Cord	CD31, VE, vWF	Matrigel	Angiogenesis-promoting effect of thrombin <i>in vivo</i> and the induction by thrombin of the angiogenic phenotype of endothelial cells <i>in vitro</i> in the absence of other cell types such as smooth muscle cells, pericytes, and inflammatory cells.	[91]
			Fibrin	HUVECs/fibroblast loaded fibrin or collagen-fibrin composite microbeads, authors demonstrated that endothelial networks formed with anastomosis between microbeads.	[92]
	Dermal	CD31	PLLA Matrigel	Differentiated into functional microvessels within 7 to 10 days in SCID mice. Became invested by perivascular smooth muscle α -actin-expressing mouse cells 21 days after implantation.	[93]
ESCs	Peripheral Blood	PECAM-1	Matrigel	After six passages, cells displayed CD34 and FLK-1 markers. After a week of animal implantation an ESC-EC coated scaffold integrated with the host vasculature and had evidence of perfusion.	[94]
			PLLA PLGA	Addition of embryonic fibroblasts increased the levels of vascular endothelial growth factor expression in the construct and prevascularization improved vascularization, blood perfusion and survival of the constructs after implantation.	[95]
iPSCs	Fibroblasts	Reprogramming using Oct3/4, Sox2, Klf4, and c-Myc	Matrigel	iPSC derived ECs demonstrated CD31, VE-Cadherin, and endothelial nitric oxide synthase (eNOS) similar to HUVECs but with increased CD34 and CD133 expression.	[96]
			Fibrin	iPSC-ECs formed mature capillary-like vessels, however, they revealed a five-fold reduction in capillary network creation when compared to HUVECs.	[97]
			PEG	PEG hydrogels functioned as a synthetic ECM and allowed for stable capillary lumens over a two-week period.	[98]
EPCs	PB	CD34	Collagen Fibronectin	Network formation and cord-like structures <i>in vitro</i> .	[99]
	BM	CD133, CD34, VEGFR2, VE-cadherin, E-selectin, and vWF	Fibronectin	Enhanced angiogenesis when co-cultured in the presence of mesenchymal stem cells.	[100]
	Adipose	CD31, CD34, VEGFR2	Matrigel	Adipose derived EPCs (adEPCs) formed capillary-like structures in Matrigel and enhanced the capability of HUVECs to form capillary-like tubes.	[101]

regulatory hormone secretion [110]. These subtle differences in microvascular anatomy and cell subtype are paramount in the regulation of blood flow to diverse tissues and organs. Therefore, specific cellular starting materials will be required to generate the desired microvascular bed for the engineered replacement tissue.

Cell Source and Type

ECs and mural cells (pericytes and smooth muscle cells) are the mainstay of engineering strategies, as they play essential developmental roles of the vascular tree [111]. However, varied cell sources have been utilized with each having innate advantages and disadvantages in microvascular network assembly.

Endothelial Cells

ECs typically express von Willebrand factor, vascular endothelial (VE)-cadherin, and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) [112]. They are instrumental in diapedesis, vascular tone, and smooth muscle cell proliferation [113]. As they are ubiquitous to the entire vascular tree, they have been investigated extensively for *in vitro* vascular engineering. It has long been appreciated that vascular graft failure is mediated by platelet initiated thrombosis and mitigated by an intact EC monolayer [114]. However, grafts less than 6 mm are still unreliable and not routinely used in clinical practice [115]. This limitation is problematic for the development of engineered and immediately perfusable tissues as they require the integration of vessels ranging from 5 μ m to 1 mm to permit both physiologic functionality and the ability for surgical anastomosis. However, there have been substantial

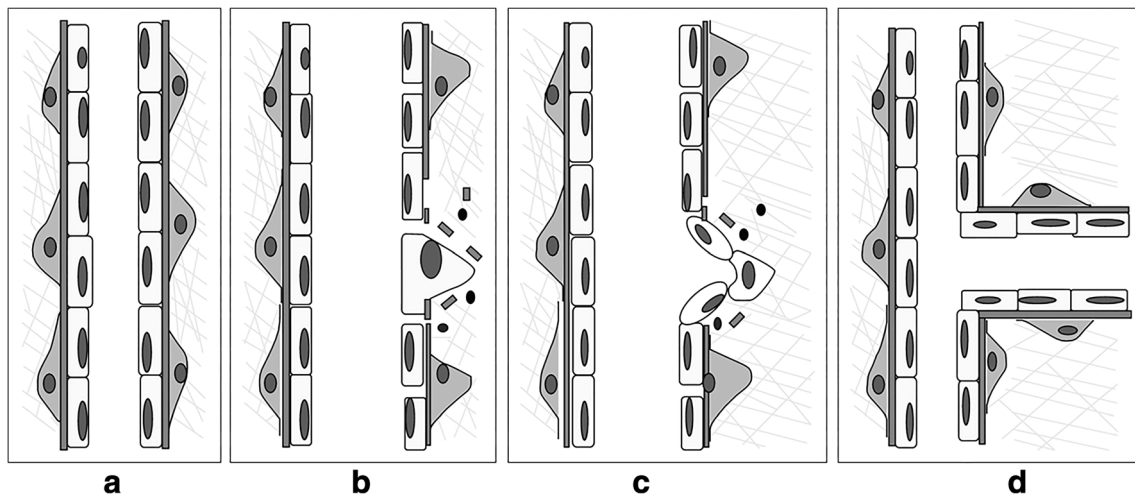


Fig. 3 Sprouting angiogenesis: After stimulation with angiogenic factors of a quiescent vessel (a), basement membrane degradation, pericyte detachment, and loosening of endothelial cell junctions occur (b). Endothelial cells begin to proliferate, migrate, and take part in

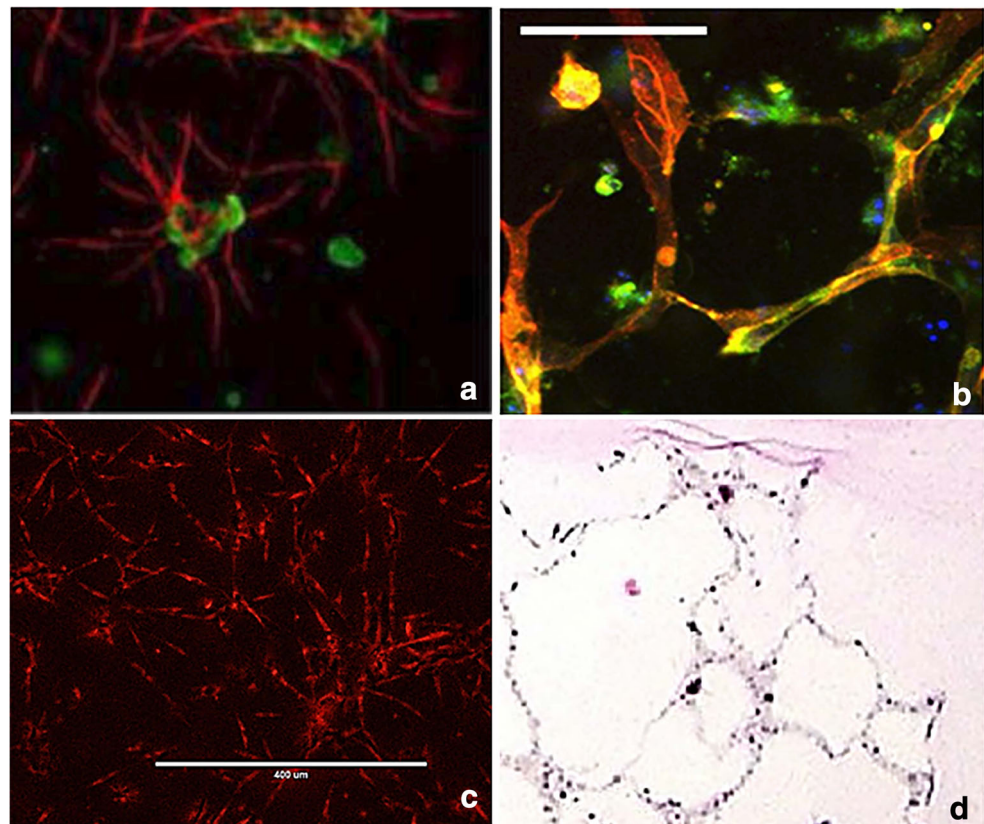
forming an immature capillary structure and depositing a new basement membrane (c). Finally, pericytes are recruited thereby providing stabilization of the new vessel (d) [106]

advances in *in vitro* microvascular engineering, especially with the generation of prevascularized tissues. Both macrovascular and microvascular endothelial cells have been utilized for this purpose. Differences do exist between them and are further amplified depending on the tissue source. As such, macrovascular cells include those originating from the pulmonary artery, coronaries, aorta, saphenous vein, umbilical cord, and dermis. Likewise, microvascular cells from virtually all these locations are available as well. Human umbilical vein endothelial cells (HUVECs) are the most widely used type of macrovascular cell for the purpose of forming a neovasculature. HUVECs have been demonstrated to undergo self-assembly into capillary structures in a variety of studies, especially when used in the presence of a basement-membrane matrix, such as Matrigel [91]. Although Matrigel is useful for microvascular engineering and study, its disadvantages are its sarcoma cell origin and lot-to-lot variability [116]. These factors may limit its potential for clinical translation and other scaffolds have been established using HUVECs. Nakatsu *et al.* described a fibrin gel based bead assay where Cytodex (Sigma-Aldrich, St. Louis, MO) beads were coated with ECs prior to being embedded in a fibrin gel that was subsequently coated with fibroblasts [117]. The fibroblasts provide the soluble factors that allow for angiogenesis. This model has been replicated with various EC and feeder cell sources and has been used extensively in the study of microvascular engineering. Rioja *et al.*, described a modular approach to microvascular engineering where HUVECs/fibroblast loaded fibrin or collagen-fibrin composite microbeads were embedded in a fibrin hydrogel (Fig. 4a) [92]. The authors demonstrated that endothelial networks formed and anastomosis between microbeads was established. However, in order to recapitulate the desired native microvascular anatomy, a

patterning strategy may need to be employed that can optimize incorporation and physiologic function. Baranski *et al.*, utilized a micropatterning technique to organize HUVECs into cords which following implantation acted as a guide for capillary formation [119]. When implanted with hepatocytes, prevascularized constructs with cords integrated and survived better than constructs that had randomly organized microvascular networks [119]. However, even with this approach it takes 5 to 14 days for mature capillaries to develop, which is still suboptimal to prevent necrosis of the innermost cell mass. Angiogenic integration of a prevascularized construct following implantation still requires progression through several stages as follows: enzymatic degradation of the capillary basement membrane, endothelial cell proliferation, directed migration, tubulogenesis, vessel fusion, and pericyte stabilization [107]. Specifically, the initial step of angiogenesis is controlled ECM degradation by matrix metalloproteinases (MMPs) that are secreted by ECs and other cells. As angiogenesis occurs in the microvasculature, differences in the expression of MMPs has been demonstrated between microvascular and macrovascular ECs, with substantially more MMP9 being secreted by microvascular cells [120]. Therefore, it may be worthwhile to revascularize grafts with microvascular cells to hasten angiogenic integration. It is also known that microvascular cells are critical to the formation of new blood vessels through sprouting mechanisms and may not require exogenous pro-angiogenic growth factors when used in specific co-cultures [121]. This may have significant implications for engraftment and rapid perfusion of prevascularized tissues upon implantation. Dermal microvascular endothelial cells (DMVECs) represent a type of microvascular cell that have been used extensively [93]. However, there have been concerns that mature ECs lack significant regenerative potential

Fig. 4 Various cell sources for microvascular engineering:

Microvascular bioengineering from various cell types: **a** HUVECs labeled with UEA-1 (red) were loaded onto microbeads (green) and demonstrated sprouting at 14 days [92], **b** iPSC→ECs were cultured for 3 days on PEG hydrogels and stained for CD31 (green), VE-Cadherin (red) and DAPI (blue) [98], **c** human adipose derived EPCs (CD34⁺/CD31⁻) loaded with CellTracker Red (Thermo Fisher, Eugene, OR) cultured on a 3D collagen gel demonstrating tube formation at 24 hours (our laboratory unpublished work) and **d** cross section of microvascular fragments seeded onto a porous scaffold and stained with hematoxylin and eosin [118]



[122], autologous sources may be problematic to obtain in ample quantities, and that phenotype expression is tissue dependent [123, 124]. These concerns have prompted the examination of other cell sources for microvascular engineering.

Embryonic Stem Cells

Embryonic stem cells (ESCs) were first reported in the mouse in 1981 followed by identification in humans in 1998 [125, 126, 127]. ESCs are located in the inner cell mass of the embryo [126] and are true pluripotent stem cells. As such, they can proliferate indefinitely and differentiate into any cell type while imparting low immunogenicity. These characteristics makes them especially appropriate for tissue engineering strategies, including microvascular replacement [128]. Levenberg *et al.*, described the differentiation of human ESCs into endothelial cells in 2002 [94]. Following the formation of embryoid bodies (EBs), cells expressing PECAM-1 were isolated and sorted. After six passages, cells displayed CD34 and FLK-1 markers similar to that seen in HUVECs. Cords with lumens developed following seeding onto Matrigel. Furthermore, after a week of animal implantation an ESC→EC coated scaffold integrated with the host vasculature and had evidence of perfusion [94]. In a follow-up study the same group utilized ESC derived ECs to engineer vascularized skeletal muscle [95]. This was accomplished by co-culturing human ESC→ECs with mouse skeletal myoblast

cells and mouse embryonic fibroblasts on a highly porous, biodegradable polymer scaffold. It was shown that the addition of fibroblasts increased the expression of VEGF and promoted microvessel stability following a month-long *in vitro* culture. Animal implantation demonstrated vigorous anastomosis with the host vasculature which was similar to that seen with HUVECs. Most interestingly, the vessels were completely integrated with the differentiating skeletal muscles fibers [95]. While the therapeutic advantages of ESCs are sizable, it is unfortunate that their downsides may be greater. The mechanisms that regulate ESC pluripotency and differentiation are still poorly understood with the contribution of various transcription factors, cofactors, chromatin regulators, and noncoding RNAs still imprecise [129]. Additionally, as ESCs are derived from the inner cell mass of a pre-implantation embryo, there are significant ethical concerns with cell retrieval. These considerations significantly hinder the potential for clinical translation. As such, other stem cell and progenitor cell sources are continuously being explored.

Induced Pluripotent Stem Cells

Advances in cellular reprogramming has led to the detection of a noteworthy alternative cell source for therapeutic vascularization, induced pluripotent stem cells (iPSCs). iPSCs have the capacity for pluripotency and self-renewal, much like ESCs [130]. When initially described by Yamanaka in 2006, they

were generated from mouse embryonic or adult fibroblasts (somatic cells) through the introduction of four transcription factors; OCT 3/4, SOX2, KLF4, and c-MYC via integrative retroviral delivery [130, 131]. The subsequent year, production using human fibroblasts was described using the same transcription factors [132], while a separate group used OCT4, NANOG, SOX2, and LIN28 [133] for reprogramming. These early efforts were hampered by low reprogramming efficiency, viral integration, and severe genomic modifications [134]. However, since then programming efficiency has been improved and the use of DNA-free vectors [135], non-integrating vectors, or vector excision has mitigated the incorporation of viral transgenes [136], improving safety. As such, the use of iPSCs has expanded across multiple disciplines, including microvascular engineering. Ikuno *et al.*, utilized human iPSC lines for differentiation into endothelial cells. The authors utilized a 2D monolayer serum-free culture system and ordered differentiation using stage-specific stimulation with VEGF and cAMP while removing non-responder cells early in the process [96]. iPSC derived ECs demonstrated CD31, VE-Cadherin, and endothelial nitric oxide synthase (eNOS) similar to HUVECs but with increased CD34 and CD133 expression. CD34 and CD133 are markers of both ECs and multipotent progenitor cells; therefore, it is not surprising that they were expressed on iPSC derived ECs. Additionally, these cells exhibited tube forming ability and morphology similar to the gold-standard HUVECs [96] when cultured on Matrigel. However, their propensity for capillary morphogenesis has also been evaluated in fibrin-based scaffolds. Bezenah *et al.*, employed a coated bead approach to assess the ability of iPSC→ECs to form microvascular networks in fibrin 3D culture in the presence of fibroblasts [97]. However, the authors discovered that although iPSC→ECs formed mature capillary-like vessels they also revealed a five-fold reduction in capillary network creation when compared to HUVECs. The authors concluded that differences in EC phenotypes must be better understood, especially when considering iPSCs for clinical use [97]. Zanutelli *et al.*, encapsulated human iPSCs derived ECs in peptide-functionalized polyethylene glycol (PEG) hydrogels to engineer a microvascular network *in vitro* using either well-culture or a microfluidic device (Fig. 4b) [98]. The PEG hydrogels functioned as a synthetic ECM and allowed for stable capillary lumens over a two-week period. The *in vitro* differentiation of iPSCs to ECs has been described using a variety of selective medias [137] and platforms. For example, Kurokawa *et al.*, have shown microvascular development in an organ-on-a-chip platform using iPSC→ECs and polydimethylsiloxane [138].

Adult Stem Cells

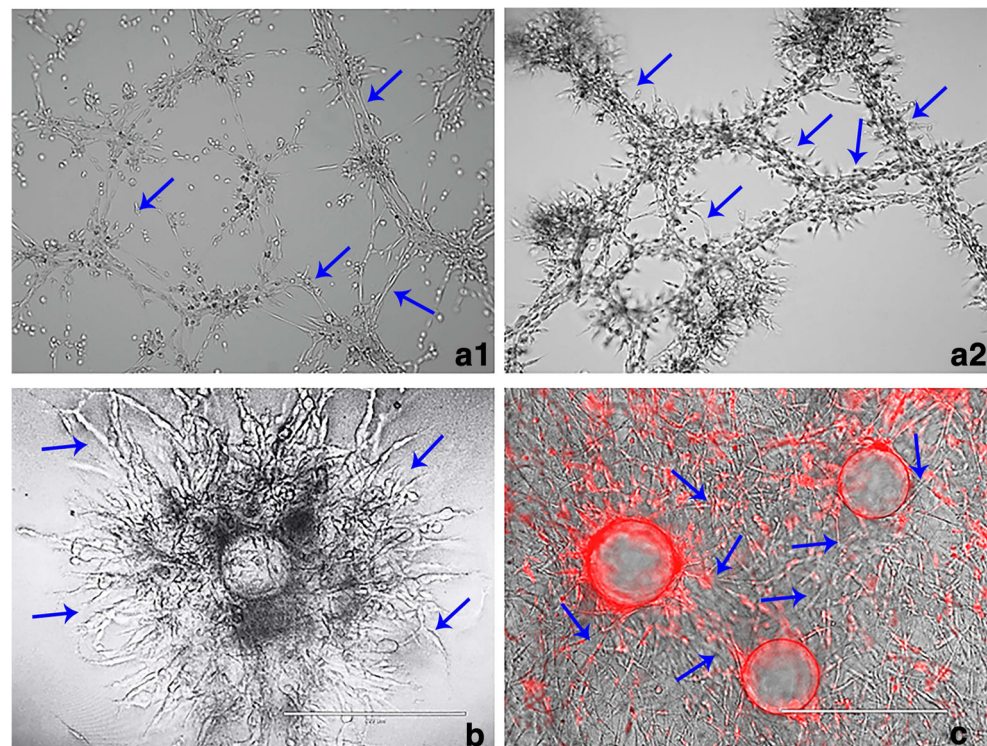
For decades angiogenesis through vascular sprouting and intussusception followed by vascular myogenesis was believed

to be the sole mechanism for the development of new vascular networks [139]. Since the 1930s, endothelial/capillary-like formations were observed in leukocyte cultures [140]; these and other reports suggested uncharacterized circulating precursor cells with roles in blood vessel formation. In 1997, Asahara *et al.*, described the first putative isolation of bone-marrow derived, circulating endothelial progenitor cells from human peripheral blood using surface marker CD34 and a polyclonal antibody to endothelial cell marker VEGFR-2 [141]. Since then, EPC isolation has been widely reported from human and other animal sources, including bone marrow and adipose tissue (data from our lab; Fig. 4c and Fig. 5). However, despite great interest and ongoing work, significant controversy has surrounded the precise definition and function of EPCs. It is now widely accepted that EPCs represent heterogeneous cells that exhibit differing functions depending on isolation and culture methodology. A comprehensive account of this still unfolding story and specific cell makers is left to dedicated reviews [140, 141, 142–155]. The acknowledged CD34⁺/VEGFR-2⁺ EPC initially isolated by Asahara incorporated en-masse into sites of neovascularization due to induced ischemia, whereas CD34⁻/VEGFR-2⁻ cells only rarely incorporated [140]. By 2000, Gehling *et al.*, reported that CD133⁺ cells from granulocyte colony stimulating factor-mobilized peripheral blood differentiated to exhibit various endothelial markers when cultured with VEGF and stem cell growth factors [156], implicating CD133 as a strong selective marker for EPC identification. Also, in 2000, Peichev *et al.*, reported that a small subset of CD34⁺/AC133⁺/VEGFR-2⁺ cells were found in several different hematopoietic sources. When incubated with VEGF, FGF-2, and collagen, these cells differentiated into CD133⁻/VEGFR-2⁺ mature endothelial cells, suggesting that EPCs with angioblast potential may be marked selectively through CD133 [157]. In 2005, Romagnani *et al.*, reported CD34⁺ cells also expressing either CD133 or CD14 additionally express embryonic stem cell markers Nanog and Oct-4. Accordingly, they proliferated in response to stem cell growth factors, and could differentiate into endothelial cells, adipocytes, osteoblasts, and neural cell lineages [158]. To date, there is no exclusive EPC marker, and it is impossible to differentiate immature EPCs from primitive hematopoietic stem cells due to overlapping surface markers [140]. However, ‘early’ EPCs and ‘late’ EPCs have been distinguished, with the former being spindle-shaped with peak culture growth at 2-3 weeks and secreting many angiogenic and neuroregulatory cytokines prior to dying after approximately four weeks in culture. These ‘early’ EPCs have been characterized as being myeloid angiogenic cells – hematopoietic lineage and the ‘late’ cells being endothelial colony forming cells (ECFC) [155]. ECFCs appear as cobblestone-shaped cells after 2-3 weeks in culture which can be maintained for up to 12 weeks [140]. Clinically, ECFCs are characterized by a high proliferative potential and vasculogenic capacity as was

recently documented by Reid *et al.* in a model of ischemic retinopathy [159]. Despite nomenclature discrepancies, their potential application in the formation of new blood vessels has spawned much enthusiasm among researchers and clinicians. Recently, Liang *et al.*, utilized human bone marrow derived EPCs which expressed CD133, CD34, VEGFR2, VE-cadherin, E-selectin, and vWF to demonstrate enhanced angiogenesis when co-cultured in the presence of mesenchymal stem cells (MSCs). The effects were mediated through PDGF (platelet derived growth factor) and Notch signaling [100]. Liu *et al.*, used cell sheets to co-culture EPCs and MSCs which proved advantageous for integration into an irradiated field [160]. In this study, EPCs emerged as cobblestone colonies in primary culture and expressed CD31, VE-Cadherin, and VEGFR2 [160]. In addition to surface marker expression the source of EPC isolation may affect vasculogenic potential. For example, it has been suggested that adult peripheral blood derived EPCs may have lower vasculogenic potentials when compared to other sources [161]. However, Melero-Martin *et al.*, demonstrated that postnatal EPCs have an inherent vasculogenic ability when co-cultured with mesenchymal progenitor cells [162]. The authors utilized both cord blood derived EPCs and adult peripheral blood EPCs which were purified by CD31 selection and formed EC colonies with a cobblestone appearance. EPCs expressed CD31, CD105, CD44, CD29, and VEGFR2 but failed to express CD90 and CD45 with flow cytometry characterization. In the presence of mesenchymal stem cells (60%), EPCs from either source demonstrated robust vascularization [162]. MSCs are known as

being able to differentiate into pericytes through EC influences and paracrine effects [163]. However, the isolation and culture of EPCs from blood is time consuming and inefficient. In 2004, Planat-Benard *et al.*, described using adipose tissue for EPC isolation, with their procedure being the same as for isolation of MSCs from adipose [164]. In 2013, Hager *et al.*, described using magnetic activated cell sorting with CD133, CD34, and CD31 cell markers to isolate EPCs from human lipoaspirate [165]. Zhou *et al.*, showed that adipose derived EPCs (adEPCs) formed capillary-like structures in Matrigel []. Other groups have differentiated ADSCs into EPCs by pretreating them with endothelial media for three days then subjecting them to laminar shear stress [166]. These cells had significantly increased expression of endothelial markers and formed tube-like structures in Matrigel. However, some studies reject this theory suggesting that ADSCs do not differentiate into EPCs, but rather the SVF including EPCs [167]. Amerion *et al.*, showed that rat adEPCs change morphology over time [168], which is consistent with other studies describing early and late EPCs [169]. They showed that cells grew more rapidly after seven days in endothelial induction medium, which led to formation of a cell sheet after three weeks. VEGFR2 expression was significantly increased in the early stage and continued; whereas, TIE-2 (tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 2) signaling increased in the third week and other genes had fluctuating expression. Our laboratory has demonstrated that human adipose derived EPCs can organize into tubule formation within 24 hours in 3D collagen culture (Fig. 5)

Fig. 5 Human adipose tissue isolated endothelial progenitor cells: EPCs (CD34⁺/CD31⁺) cultured on 3D collagen scaffolds in endothelial microvascular media (MV media) demonstrated vasculogenesis. **a** Live imaging was performed using an Evos FL Auto (ThermoFisher) microscope with arrows showing early tube formation (A1) and excessive growth at 21 hours (A2). **b** A Cytodex 3 microcarrier bead coated with EPCs for 12 hours and cultured in collagen demonstrating sprouts (arrows) at 72 hours using live imaging and z-stack reconstruction. **c** EPCs stained with CellTracker dye and coated on Cytodex 3 beads demonstrating network/tube formation (arrows) after six days in 3D collagen culture (Unpublished work from our lab)



without any pretreatment. ECM production appears to be vital to the structural integrity of engineered tissues including the stabilization of the microvascular network. It has been shown that EPCs demonstrate superior ECM deposition and assembly when compared to mature ECs and should be considered for microvascular engineering [170]. The importance of ECM deposition for microvascular network stabilization can further be witnessed that co-culture with mural cells offers significant advantages.

Mural and Perivascular Cells

The absence of pericytes is a limiting factor in maintaining engineered microvessels in culture for an extended period. However, pericytes themselves are a rare occurrence and their isolation and characterization is imprecise. First discovered in the late 19th century as contractile cells surrounding the endothelium of small blood vessels, the term “pericyte” was ascribed by Zimmerman in 1923 [103]. Since then their wide-ranging importance has been documented in vascular development, stabilization, maturation, and remodeling [171], along with ECs. Transforming growth factor beta 1 (TGF- β 1) has been shown to play a central role in the pericyte-EC interaction, especially in regards to mesenchymal→mural cell differentiation and the suppression of EC proliferation and migration [172]. Additionally, recent studies have suggested pericyte similarities to multipotent stem cells and progenitor cells [173]. Validated pericyte markers include PDGFR- β , NG2 (chondroitin sulfate proteoglycan 4), CD13 (alanyl aminopeptidase), α -SMA (alpha-smooth muscle actin), and desmin. However, these markers are dependent on multiple factors and are dynamic in their expression [103]. Angiopoietin-1 (ANG1) is expressed on pericytes and its interaction with the TIE-2 receptor on ECs provides vessel stabilization and maturation. Therefore, its importance to vascularization of engineered tissues has been probed [174]. Morin *et al.*, used human brain pericytes and HUVECs to fabricate aligned microvessels in a 3D fibrin gel by day three [175]. The authors noted that both pericytes and a defined medium were required to achieve a highly interconnected microvasculature with the presence of pericytes increasing the total vessel length [175]. Chen *et al.*, used pericytes with HUVECs in Matrigel to generate capillary like structures [176]. The authors isolated microvascular pericytes (CD146⁺/CD34⁻/45⁻/56⁻) from human skeletal muscle. Pericytes were combined with an equal number of HUVECs in either 2D or 3D culture. Co-culture with pericytes significantly improved and hastened microvascular formation in both culture conditions [176]. Cultured pericytes exhibited sustained high inflammatory gene expression, including HIF-1 α (hypoxia-inducible factor 1-alpha). HIF-1 α is a subunit transcriptional factor that has been demonstrated to play a significant role in angiogenesis. The peri-endothelial location of pericytes is frequently

confused with other adjacent mesenchymal cells including vascular smooth muscle cells and fibroblasts. Secondary to the difficulty in pericyte isolation and the importance of supporting cells in the developing microcirculation other perivascular cells have been used for microvascular engineering endeavors.

The utility of fibroblasts in microvascular tissue engineering has been well documented. They are ubiquitous in connective tissue and therefore are located throughout the body. Since they are responsible for the generation of extracellular matrix and collagen, they contribute to the structural support of tissues, including the vascular tree. Additionally, they have been shown to be vital to angiogenesis through the secretion of soluble mediators, such as tissue inhibitor of metalloproteinase-1 (TIMP-1) [177]. However, unlike pericytes, they are relatively easy to isolate and are present in large numbers. This has led to their extensive investigation in tissue engineering endeavors. Chen *et al.*, co-cultured normal human lung fibroblasts (NHLFs) and HUVECs in fibrin gels to generate *in vitro* prevascularized constructs [178]. Furthermore, a high density of fibroblasts accelerated functional anastomosis following animal implantation by 2-3 days. Costa-Almeida *et al.*, co-cultured human dermal fibroblasts with ECs and showed that they promoted vascularization, which was thought to be due to their higher expression of α -SMA and podoplanin as well as increased synthesis of ECM [179]. Newman *et al.*, also showed that fibroblast-derived ECM components play a critical role in EC lumen formation via expression of five genes: collagen I, procollagen C endopeptidase enhancer I, secreted protein acidic and rich in cysteine, TGF- β -induced protein Ig-h3, and insulin growth factor-binding protein 7 [180]. Other groups have also shown that growth factors and matrix proteins from fibroblasts modulate EC sprouting into capillary networks *in vitro* [181, 182].

Likewise, MSCs have also been shown to support vascular tube formation. They can serve as a source of vasculogenic growth factors and have the ability to differentiate into mural cells via communication with ECs [183]. HUVECs cultured with BM-MSCs versus fibroblasts showed a higher number of cell junctions and slower vessel formation with a higher number of branches, simulating physiological vessel growth [184]. Pill *et al.*, showed that MSCs from both adipose and bone marrow promotes tubulogenesis in co-culture with ECs, although via distinct mechanisms [185]. Verseijden *et al.*, showed that prevascularized HUVEC/ADSC constructs resulted in significantly higher vessel formation after *in vivo* implantation in mice [186]. Melero-Martin *et al.*, showed combination of EPCs and MSCs from blood or bone marrow allowed rapid formation of functional anastomoses *in vivo*, which remained patent at four weeks [163]. Rao *et al.*, found that a high ratio of EC:BM-MSCs (5:1) led to unstable vessel formation compared to lower ratios [187]. Other

groups have also shown that both BM-MSCs [188–190] and ADSCs [191–193] co-cultured with ECs support vascular formation.

Microvascular Fragments

The uncertainty of cellular isolates and their correct ratios for microvascular engineering has led to an interest in the utility of multicellular vessel segments. Hoying *et al.* reported that microvascular fragments (MF) isolated from adipose tissue undergo expansion and sprouting when placed in a collagen gel [194]. These features have been demonstrated in mouse, rat, and human sources [195]. MF retrieval relies on the isolation of intact short vessel segments that includes arterioles, capillaries, and venules. In addition to luminal ECs, these fragments are rich in smooth muscle cells, progenitors, and perivascular cells. By containing native cell ratios, MFs eliminate the uncertainty of recombining the correct cellular portions (e.g. ECs, MSCs, fibroblasts) *in vitro* while offering a simpler isolation protocol than that of individual cells. This can be appreciated where the retrieval of cell subpopulations from adipose tissue involves extensive collagen digestion but MF isolation only requires a fraction of time [196]. Although, it has been demonstrated that angiogenic MFs can be isolated from a variety of tissues [197], the ubiquity and accessibility of adipose tissue has made it a preferred choice. Specifically, MFs can be obtained from patients via minimally invasive liposuction techniques that can be performed under local anesthesia in a clinic setting. In culture, MFs have been shown to release proangiogenic mediators, such as VEGF [118], potentially obviating the need for exogenous angiogenic supplementation. Furthermore, they have demonstrated the ability for inosculation and perfusion following animal implantation. Laschke *et al.* demonstrated that the incorporation of adipose derived MFs into porous scaffolds contributed to enhanced inosculation following implantation (Fig. 4d) [118]. Shepard *et al.* engineered prevascularized cardiac patches *in vitro* using microvascular fragments which demonstrated inosculation with the coronary microcirculation in a mouse myocardial infarct model within seven days [198]. It has also been suggested that MFs demonstrate substantial plasticity with microvessels of arterial identity being able to give rise to both arterial and venous phenotypes [199] despite the luminal dimensions becoming somewhat uniform in culture with an average diameter of ~23 μm [200]. While the maintenance of native cell ratios in a multicellular fragment is advantageous, the large size of MFs may limit their utility in additive manufacturing technologies, such as vascular bioprinting. Furthermore, directed manipulation with miRNAs may prove cumbersome in a multicellular fragment [201]. However, continued investigation of MFs is warranted to determine if their heterogeneous cell populations offer an

Fig. 6 Cylindrical scaffold fabrication approach for microvascular engineering: **a** Schematic diagram adopted from Tung *et al.* [209], demonstrating HUVEC culture on a degradable cylindrical PLGA scaffold for microvessel formation. **b** Cylindrical PLGA scaffolds were made from a PDMS mold that had been generated using soft-lithography. **c** HUVEC-cultured cylindrical scaffold stained for F-actin (red) and nucleus (blue) using 3D confocal microscopy [209]

advantage to the *in vitro* recombination of ECs and mural cells for microvascular engineering.

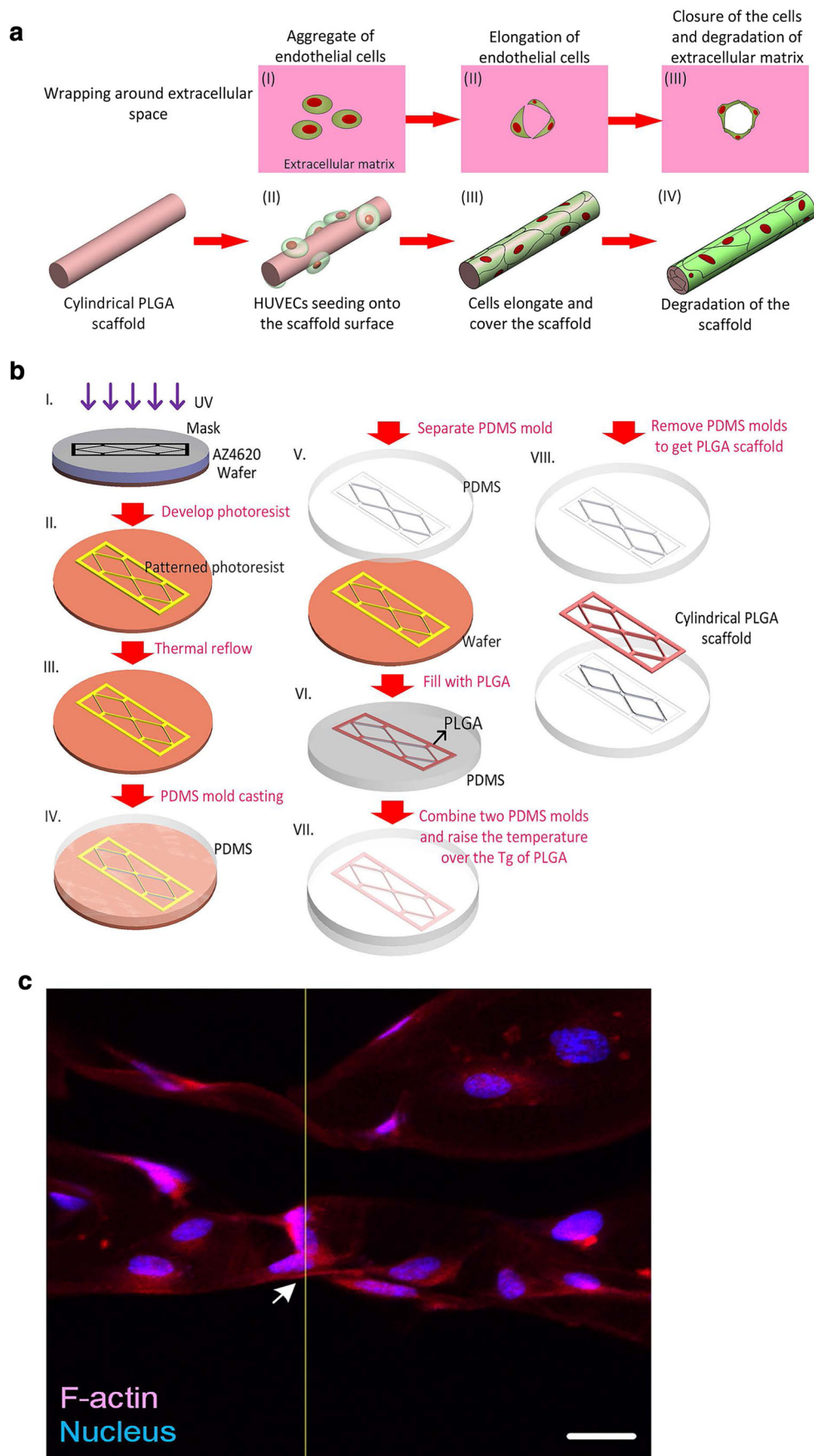
Cell Seeding

Cell-based therapies were the earliest approaches in healing damaged tissue in which single cell suspensions were injected into defect areas to help regeneration and organ recovery. However, this approach has limitations such as difficulties in retaining cells at the target site [202]. As microvascular dysfunction is generally not limited to a confined region, as is the case in diabetes [203], direct cellular injections for microvascular regeneration may not prove beneficial. Additionally, engineered tissues need to rapidly integrate upon implantation. Therefore, other approaches have been developed to optimize engraftment upon implantation.

In Vitro Prevascularization

Prevascularization is a concept in which engineered tissue has an established microvasculature prior to implantation facilitating vascular integration and perfusion prior to the onset of hypoxia induced cell damage. Current strategies generally rely on random cell/spheroid seeding, cell sheets, and bioprinting technologies.

Seeding cells onto scaffolds has been widely used for *in vitro* prevascularization, using all the cellular sources outlined above. The scaffold chosen must consider material biocompatibility, biodegradability, mechanical properties, and scaffold architecture. Numerous scaffold materials have been utilized in tissue engineering with the general goal of imitating the native ECM [204] required for native cell function. Most of the scaffolds used in microvascular engineering endeavors can be broadly grouped as hydrogels. Hydrogels are a group of natural (e.g. polysaccharide or polypeptide-based) or synthetic (e.g. polyethylene glycol, polyacrylamide) polymeric materials whose hydrophilic structure allows them to hold large amounts of water [205]. Natural hydrogels such as fibrin, collagen, and alginate have all been used as ECM mimics in tissue engineering. Fibrin is formed during wound coagulation and is used extensively in clinical applications for hemostasis. It can either be obtained whole or separately in its fibrinogen and thrombin components which can increase tunability of its final application. While useful in angiogenic assays, its susceptibility to protease mediated degradation



mitigates its utility in *in vitro* prevascularization approaches. Collagen based hydrogels have a deep history in 3D culture applications [206] with Matrigel being extensively used in microvascular engineering as it best resembles the complex ECM found in living tissues. It is unique in that in addition to collagen type IV it contains laminin, entactin, and various other proteoglycans and growth factors [206]. While it robustly supports cell growth and vascular network formation it is derived from mouse sarcoma cells, thereby potentially limiting clinical translation. This has led to a variety of other collagen sources being investigated (e.g. type 1 bovine) [207] that can be fashioned in different concentrations allowing for mechanical stability at the expense of porosity. To further expand the hydrogel-based scaffold platform, sourcing has also transitioned from natural to synthetic sources such as poly ethylene glycol (PEG), dimethacrylate, methacrylated gelatin [208] and poly(lactide-coglycolide) [209]. Scaffolds have been fashioned in various patterns to reproduce microcirculatory architecture [208] and into cylindrical shapes (Fig. 6) [209]. Likewise, scaffolds have also widely been used as a platform for cell seeding and random tubulogenesis. While each scaffold has its own advantages and disadvantages, the materials which best resemble native ECM have fragile mechanical properties which complicate the transfer of *in vitro* prevascularized constructs into an *in vivo* model. This further muddles the cell seeding process in addition to the optimal ratio of vascular and mural/stromal cells remaining ill-defined [210]. Furthermore, the ability to precisely control the complex microvascular architecture is not possible yet.

Cell sheet engineering is a new scaffold-free method that overcomes the drawbacks of scaffold-based techniques [211]. In this method, cell aggregates can be harvested without proteolytic enzymes, allowing cells to be manipulated without disrupting their native ECM [212, 213]. Intact cell sheets, including the deposited ECM, can be harvested using thermoresponsive hydrogels, such as poly *n*-isopropyl acrylamide (PNIPAAm) and methylcellulose (MC), simply by changing the temperature. In these systems, cells adhere and proliferate on the substrate at 37°C and when the temperature is decreased to room temperature, confluent cells can be released as an intact sheet. MC and PNIPAAm hydrogels have been utilized in many studies for cell sheet fabrication. Shimizu *et al.*, showed that cell sheets can be stacked to create a thick construct with vascularity [214]. Sekiya *et al.*, showed that layered EC sheets produced lumens and vascular structures [215]. Asakawa also showed that stacked cell sheets of HUVECs and dermal fibroblasts created vascular networks [216]. Another study described vessel formation in a five-layer myoblast sheet construct by stacking HUVECs and myoblast cells [217]. They observed the anastomosis of the endothelial network in the stacked construct to host vessels after transplantation *in vivo*. These studies clearly indicate the potential for cell sheet engineering techniques in microvascular

endeavors. While manipulation of the engineered microvascular network may prove more stable with this approach, it offers limited benefit over traditional scaffold cell seeding with regards to controlling the vascular architecture.

Bioprinting is one of the recently described techniques that allows direct fabrication and preparation of complex 3D vascular networks [218]. The major advantage of this technique is the ability to fabricate a complex tissue construct with fine spatial control over the cell pattern and requisite growth factor deposition [219] with both scaffold-based and scaffold-free techniques. In bioprinting, living cells are delivered in a bioink, which forms the initial ECM following either extrusion-, droplet-, or laser-based bioprinting. Ideal bioinks remain stable during the bioprinting process while exhibiting a viscosity in the range of 10–150 mPa, which eases deposition and prevents nozzle clogging [220]. Natural biopolymers such as gelatin, collagen, agarose, alginate; and synthetic materials such as poly ethylene glycol have been used as bioinks [221]. Bioinks with thermally reversible gelation, such as gelatin-based hydrogels, are preferred due to their ability to demonstrate both solid and semisolid states [222]. Kolesky *et al.*, used a cell laden ink composed of gelatin and fibrinogen to bioprint thick vascularized tissues which could eventually be perfused with growth factors to promote osteogenic differentiation of human MSCs *in situ* [222]. Byambaa *et al.*, developed a bone-like construct by arranging individual bioprinted rods including VEGF-functionalized gelatin-methylated rods and hMSC laden gels loaded with silicate nanoparticle cylinder rods in the form of a pyramidal construct [223]. Ozbolat and co-workers introduced co-axial bioprinting to directly extrude perfusable vasculatures in the range of 1 mm in external diameter, which was then further advanced by other groups [224–227]. While patterned vascular formations can be formed, bioprinting still lacks the resolution to print an intact vasculature from the capillary level (~5 μm diameter) to one that permits reliable surgical anastomosis (~1 mm diameter). Therefore, without the ability for rapid perfusion prevascularized scaffolds still suffer from some degree of ischemia following implantation. To limit this, it may be beneficial to incorporate growth factors into the implanted construct or recipient sites.

Angiogenic factors play critical roles in vascularization after implantation when incorporated into biomaterial scaffolds [2, 123, 228, 229]. Angiogenic growth factors accelerate vascularization by activating and stimulating ECs/ EPCs, which results in migration of these cells toward the factor gradient. These factors not only cause vascular organization and network formation, but they have great impact on vessel maturation by recruiting mural cells and stabilizing the implanted ECs [229]. The main growth factors involved in upregulating angiogenesis and vascular formation include: VEGF, basic fibroblast growth factor and hepatocyte growth factor (HGF). Other cytokines such as platelet-derived growth

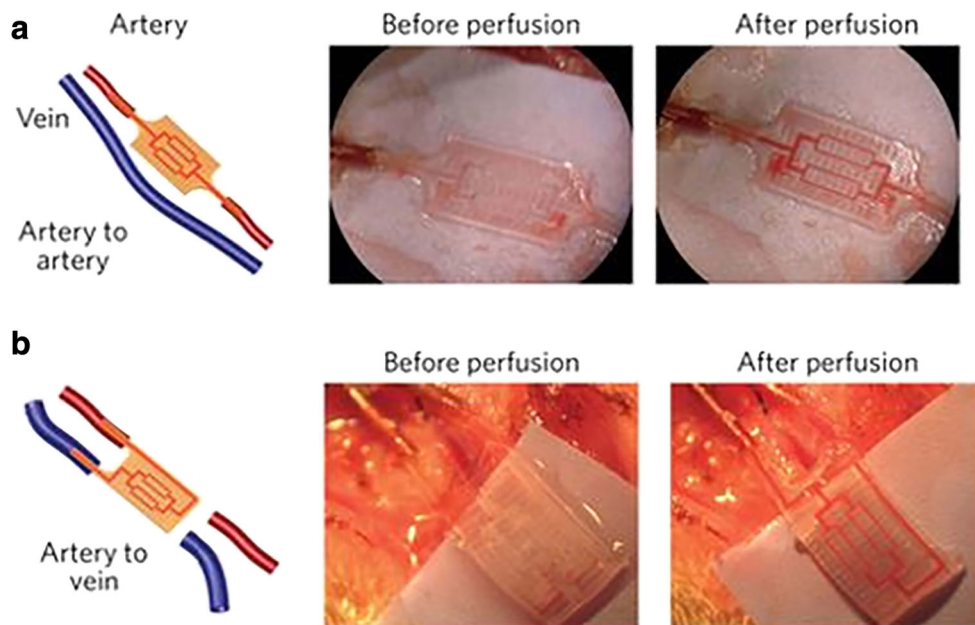


Fig. 7 Direct surgical anastomosis of engineered cardiac tissue: The AngioChip is a citric acid-based scaffold that supports the assembly of parenchymal cells (e.g. cardiac, hepatic) adjacent to a perfusable, branched 3D microchannel network that can be coated with endothelial cells. Furthermore, the AngioChip can be surgically anastomosed for

rapid perfusion. Schematic diagram adopted from Zhang *et al.* [241] demonstrating surgical anastomosis of the AngioChip cardiac tissue on the rat femoral vessels in the configuration of an artery-to-artery graft (a) and artery-to-vein graft (b) exhibiting a rapidly perfused engineered microcirculation

factor, TGF- β , and angiopoietin also affect angiogenesis indirectly and are involved in the stabilization of newly formed vessels. The major drawback of these factors is that they are not highly stable and have high degradation rates *in vivo*, which makes delivery challenging. Therefore, spatial and temporal release of cytokines is essential. Immobilizing these factors within the scaffold and tissue engineered construct is a promising approach which can provide fine control over the delivery of angiogenic growth factors [123, 229]. In one study, Poldervaart *et al.*, investigated temporally controlled release of VEGF from gelatin microspheres which resulted in prolonged release of VEGF for three weeks *in vitro* [230]. In another study, Campagnolo *et al.*, investigated how conjugating polycaprolactone (PCL) constructs with heparin binding peptide increases accumulation of vascular endothelial growth factor [231]. Growth factor incorporation may prove useful in further augmenting the potential of cell stacking and bioprinting technologies for functional microvascular creation.

In Vivo Prevascularization

In vivo prevascularization is an approach where the engineered construct is implanted into well vascularized tissue in order to induce ingrowth of surrounding blood vessels into the scaffold prior to final transfer into the defect site [232]. Inducing vascularization *in vivo* using arteriovenous loops (AVLs) was first described by Erol in 1980 [233]. In this technique, grafts (e.g. skin) are implanted adjacent to a large

caliber vascular conduit (1-3 mm) that has been fashioned into a loop shape. Following a week of implantation, the AV-loop has inoscultated the implanted graft through vascular ingrowth. The entire AV-loop/graft can now be transferred into a desired remote site with the AV-loop permitting a direct anastomosis to the final vascular bed. This approach has been used clinically when there is no free-flap option to reconstruct challenging wounds [234]. AVLs have also been used to lengthen the vascular pedicle [235], decrease risk of thrombotic events at the anastomosis [236], and reduce ischemia time [237], which all improve the chance of tissue transfer survival. This has prompted its utility in the vascularization of prevascularized engineered tissue, utilizing either autogenic or allogenic vascular grafts. For this purpose, tissue constructs and arteriovenous loops are placed into a chamber which could be empty or filled with ECM. After vascular formation the construct can be surgically anastomosed at the site of the defect [238]. Arkudas *et al.*, used an AVL model to assess vascular formation of sintered 45S5 Bioglass [239]. They observed a dense network of capillaries three weeks after implantation in the medial thigh of rats. In another study by Beier *et al.*, hydroxyapatite-beta tri-calcium phosphate (HA/ β -TCP) constructs were vascularized by implanting ceramic constructs placed in an AVL into the groins of merino sheep after six and 12 weeks [240]. A drawback of this strategy is the number of operations that are needed to complete the process. However, when integrated with cellular based strategies and novel microfabrication techniques, it may demonstrate utility in the rapid perfusion of an engineered microvasculature.

Conclusions

Tremendous advances in stem cell biology, materials science, and additive manufacturing have drastically propelled the field of tissue engineering recently. A major limiting factor towards clinical translation remains vascularization and immediate or near-immediate perfusion. However, endothelial cell-based co-cultures or microvessel fragments have demonstrated significant potential in replicating the vasculogenic process. It is interesting that with both approaches adipose tissue has emerged as a substantial donor depot for cellular based strategies for microvascular engineering. Advances in *in vitro* prevascularization strategies have led to more rapid perfusion upon implantation. This has led to promising platforms such as vascularized organ-on-a chip with direct surgical anastomosis (Fig. 7) as was recently detailed by Zhang *et al.* [241]. The authors of this study highlighted the importance of a cross-disciplinary collaboration with input from materials science, cell biology, additive manufacturing, and surgery. This is particularly exciting as it represents a step forward in the clinical translation of engineered tissues. However, it is likely that both the vasculogenic and angiogenic processes of engineered tissue will need to be optimized for large volume tissue transfer or the correction of microvascular dysfunction. In clinical surgery, it is well established that a period of warm ischemia greater than four hours will lead to significant tissue necrosis and poorer patient outcomes. Therefore, we are not only challenged with the task of striving for perfusion within this time frame but in also fabricating a microcirculatory bed that will function appropriately for the engineered tissue or organ.

Adult ECs along with multipotent and pluripotent stem cells have been cultured in various scaffolds and conditions to demonstrate *in vitro* and *in vivo* possibilities

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Compliance with ethical standards

Conflict of interests Authors declare no conflict of interests.

Abbreviations AdEPC, Adipose derived EPC; ANG, Angiopoietin; AVL, Arteriovenous loop; bFGF, Basic fibroblast growth factor; BM, Bone marrow; Dll4, Delta-like 4; DMVEC, Dermal microvascular endothelial cell; EB, Embryoid body; EC, Endothelial cell; ECFC, Endothelial colony forming cell; ECM, Extracellular membrane; eNOS, Endothelial nitric oxide synthase; EPC, Endothelial progenitor cell; ESC, Embryonic stem cell; FGF, Fibroblast growth factor; FLK-1, Fetal liver kinase-1; HA, Hydroxyapatite; HGF, Hepatocyte growth factor; HIF, Hypoxia-inducible factor; HUVEC, Human umbilical vein endothelial cell; iPSC,

Induced pluripotent stem cell; MC, Methylcellulose; MF, Microvessel fragment; MMP, Matrix metalloproteinase; MSC, Mesenchymal stem cell; NHLF, Normal human lung fibroblast; PCL, Polycaprolactone; PDMS, Polydimethylsiloxane; PECAM, Platelet endothelial cell adhesion molecule; PEG, Poly ethylene glycol; PLGA, Poly(lactide-co-glycolide); PNIPAAm, Poly n-isopropyl acrylamide; SMA, Smooth muscle actin; SVF, Stromal vascular fraction; TCP, Tri-calcium phosphate; TGF, Transforming growth factor; TIMP, Tissue inhibitor of metalloproteinase; VE, Vascular endothelial; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; vWF, von Willebrand factor

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