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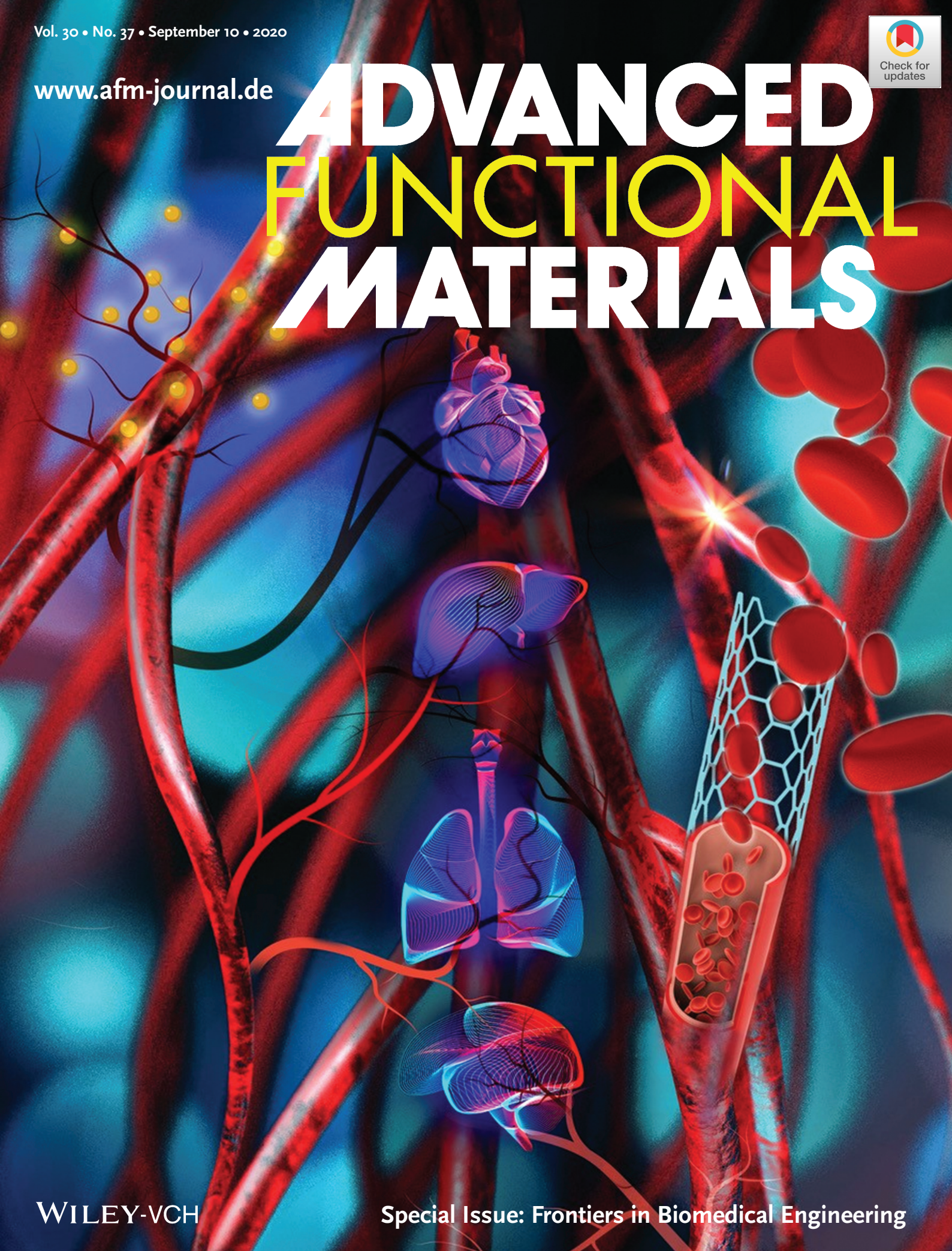

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From Arteries to Capillaries: Approaches to Engineering Human Vasculature

*Sharon Fleischer, Daniel Naveed Tavakol, and Gordana Vunjak-Novakovic**

From microscaled capillaries to millimeter-sized vessels, human vasculature spans multiple scales and cell types. The convergence of bioengineering, materials science, and stem cell biology has enabled tissue engineers to recreate the structure and function of different hierarchical levels of the vascular tree. Engineering large-scale vessels aims to replace damaged arteries, arterioles, and venules and their routine application in the clinic may become a reality in the near future. Strategies to engineer meso- and microvasculature are extensively explored to generate models for studying vascular biology, drug transport, and disease progression as well as for vascularizing engineered tissues for regenerative medicine. However, bioengineering tissues for transplantation has failed to result in clinical translation due to the lack of proper integrated vasculature for effective oxygen and nutrient delivery. The development of strategies to generate multiscale vascular networks and their direct anastomosis to host vasculature would greatly benefit this formidable goal. In this review, design considerations and technologies for engineering millimeter-, meso-, and microscale vessels are discussed. Examples of recent state-of-the-art strategies to engineer multiscale vasculature are also provided. Finally, key challenges limiting the translation of vascularized tissues are identified and perspectives on future directions for exploration are presented.

1. Introduction

The human circulatory system is comprised of a dense, intricate vascular network that is optimized to efficiently transport gas, nutrients, and metabolites to and from cells. Its hierarchical levels span from the centimeter-scale aorta to micrometer-scale capillaries (**Figure 1**). Over the past few decades, significant progress has been made to individually recapitulate the different components of the vascular tree. Large and small caliber vessels have been engineered to reconstruct or bypass occluded arteries.[1] Fabrication of in vitro vascular networks has received considerable attention with the development of "organ-on-a-chip" platforms to model human patho/physiology and patient-specific drug responses.[2] In addition, developing techniques to embed meso- and microvascular networks within large-scale engineered tissues has been pursued to support their prolonged survival and functionality in vitro and postimplantation.[3]

Although all vascular components are blood-carrying lumens, their heterogeneity in size and functionality require different design criteria and fabrication methodologies.[4] While engineered large vessels are single-lumen structures designed to support fluid flow with high pulsatile pressure, for engineered microvasculature the emphasis needs to be placed on mimicking the complex capillary network organization and endothelial barrier functionality.[5] Numerous strategies combining developments in engineering, biomaterials, and stem cell biology have helped capture the complexity of each component of the vascular niche for tailored approaches toward translational applications.[6] Foundational studies in vascular biology have provided invaluable insights to tissue engineers that enabled the field, since its early beginnings in the 1990s, to advance to where we stand today.[7] Continued research on the mechanisms of vascular formation and functionality in both health and disease will be critical for further progress in the field.

In this review, we briefly summarize the structure of the vascular tree, its formation during embryogenesis, and essential functions in the body. We then explore methodologies to fabricate distinct hierarchal levels of the vasculature, starting from large and small caliber vessels used for implantation, to meso- and microscale channels in organ-on-a-chip platforms and engineered tissues. We further discuss recent technologies to fabricate multiscale vasculature. Throughout, we will recognize a number of seminal papers that influenced the development of these sub-fields as well as highlight the major advancements over the past few years in pushing the field forward (**Figure 2**). Finally, we delineate our perspective on the challenges the field faces and our perspective on future research and fabrication strategies that will hopefully advance the translation of engineered tissues.

Dr. S. Fleischer, D. N. Tavakol, Prof. G. Vunjak-Novakovic
Department of Biomedical Engineering
Columbia University
622 west 168th Street VC12-234, New York, NY 10032, USA
E-mail: gv2131@columbia.edu
Prof. G. Vunjak-Novakovic
Department of Medicine
Columbia University
622 west 168th Street VC12-234, New York, NY 10032, USA
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2. Human Vasculature In Vivo

The body's vascular network is organized in hierarchal, tree-like structures with complex and diverse branching configurations designed to efficiently exchange oxygen, nutrients, and waste within and between tissues throughout the body. Large arteries (>6 mm) carry oxygenated blood to smaller arteries (1–6 mm), and then to the arteriolar network (100–1000 μm), and finally into capillary beds (10–15 μm).[8] Venules drain oxygen-depleted blood from the capillaries into larger veins, eventually reaching the heart, where the blood is transferred through the pulmonary artery to the lung for reoxygenation (Figure 1).[9]

Two distinct processes are responsible for the formation of these complex networks in the developing embryo: *vasculogenesis*, the formation of new blood vessels, and *angiogenesis*, the formation of vasculature from pre-existing blood vessels. During vasculogenesis, mesodermal cells differentiate into angioblasts, also known as endothelial precursors, that give rise to blood islands. Remodeling and fusion of blood islands leads to the formation of the primary capillary plexus. At this early stage, cells already acquire an arterial or venous cell fate. During angiogenesis, the vascular plexus grows and remodels to form a branched network of capillary beds, arteries, arterioles, veins, and venules. Following this process, nascent endothelial channels are supported by mural cells, including pericytes and smooth muscle cells (SMCs), and extracellular matrix (ECM) is deposited to stabilize the newly formed vessels (Figure 1).[4b]

Endothelial cells (ECs) line the interior surfaces of all blood vessels and serve as a selective barrier for circulating immune cells, nutrients, hormones, and ions. The endothelium of larger blood vessels, including arteries and veins, is surrounded by a thick basal lamina and one or more layers of SMCs, conferring strength and durability to the vessels. Capillaries are only surrounded by a thin basal lamina and scattered pericytes, which act as "scaffolding" during angiogenesis. Solute exchange takes place primarily through these small capillaries by either paracellular or transcellular transport.[10] SMCs, in contrast to pericytes, function as vasoconstrictors and vasodilators, altering mechanical strain on blood vessels to allow for the pulsatile flow of blood and can act in response to stress or injury. In regeneration, SMCs play a vital role in matrix secretion as well as the propagation of diseases such as atherosclerosis and hypertension.[11]

In different organs, endothelium acquires tissue-specific phenotypes, giving rise to varying architectures, barrier functions, and molecular signatures according to the needs of the tissue. For example, in organs such as the kidney, small intestine, and liver, the endothelium is fenestrated or discontinuous, allowing easy exchange of fluids and molecules. On the contrary, the blood–brain barrier is composed of tight endothelium, surrounded extensively by pericytes and astrocytes, to restrict paracellular transport and enable transcytosis only by specialized transporters (Figure 1).[4b] When dysregulated, the endothelium can contribute to the pathogenesis of numerous diseases such as cancer and diabetes. In contrast, neovascularization may also have a positive effect following injury, through the formation of new vessels to provide nutrients and oxygen and enable wound regeneration.

Sharon Fleischer received her B.Sc. in Biotechnology, M.Sc. and Ph.D. from Tel-Aviv University. Her doctoral research focused on developing advanced biomaterials for cardiac tissue engineering to promote heart tissue regeneration. She joined the laboratory for Stem Cells and Tissue Engineering directed by Dr. Gordana Vunjak-Novakovic at Columbia University as a Rothschild Postdoctoral Fellow in 2018. Her current research interests as a postdoctoral fellow are developing new methodologies for tissue vascularization, tissue engineering for disease modeling and drug screening and optogenetics.

Daniel Naveed Tavakol is a biomedical engineering Ph.D. student in the Laboratory for Stem Cells and Tissue Engineering at Columbia University under the advisement of Dr. Gordana Vunjak-Novakovic. As a National Science Foundation graduate research fellow, he is working on translational approaches to integrated organ-on-a-chip systems. Previously, he was a Whitaker International Fellow at the Ecole Polytechnique Federale de Lausanne in Switzerland (2017–2018) working on models of the hematopoietic niche. He graduated from the University of Virginia with a B.S. in biomedical engineering in 2017, where he studied the role of perivascular cells in angiogenesis.

Gordana Vunjak-Novakovic is University Professor, the highest academic rank at Columbia University, as the first engineer to receive this distinction. She is also the Mikati Foundation Professor of Biomedical Engineering and Medical Sciences. Her laboratory is working on engineering functional human tissues (heart, lung, bone, muscle, neurons, tumors, and vasculature) for regenerative medicine and study of disease.

Deep understanding of vascular biology is required to engineer blood vessels, recapitulate their structural components and functionality, and promote integration between engineered vessels and

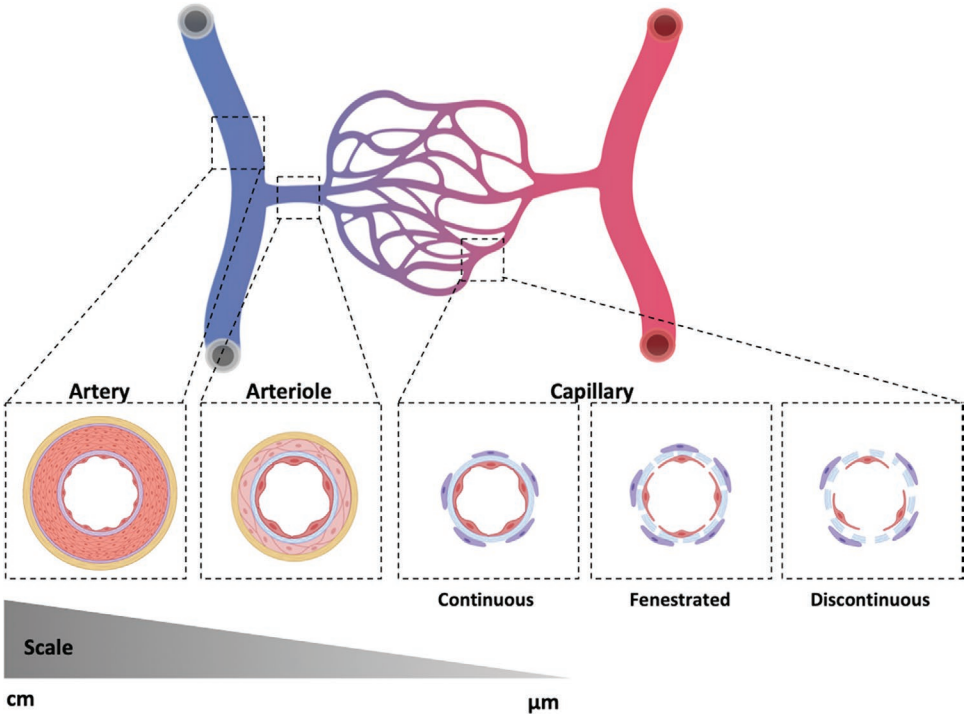

Figure 1. Organization of the vascular tree. The vascular tree is organized into a hierarchical network of arteries, arterioles (blue), capillary beds, veins, and venules (red) that span several orders of magnitude in diameter. All vessels are characterized by an inner layer of endothelium and an outer layer of basement membrane. Arterioles and venules are further bound by a second layer of SMCs as well as elastin and collagen fibers. Capillaries have a varying extent of basement membrane and pericyte coverage and can be continuous, fenestrated, or discontinuous. Created with BioRender.com.

vascularized tissues to the host vasculature. Interestingly, the progress in vascular biology has also been stimulated by the advances in vascular tissue engineering, by provision of sophisticated and controllable models of human vasculature, emphasizing the importance of the crosstalk between the two disciplines.

3. Tissue Engineered Vascular Grafts

Cardiovascular diseases are the number one cause of death in the developed world, affecting more people than all types of cancer combined.[12] Common disorders are associated with stenosis or occlusion of blood vessels, often requiring bypass or reconstructive surgery. Large-caliber vessel replacements (>6 mm) are usually needed for aortic, iliac, and femoral artery repairs. Smaller-caliber vessels are required for more common vascular diseases such as atherosclerosis or embolism that affect the coronary arteries (1–6 mm), for example.[5a,8]

To address the constantly growing clinical demand for vascular conduits and improve clinical outcomes for cardiovascular patients, the development of engineered vessels has been rigorously pursued over the past few decades. Tissue engineers have developed numerous methods to fabricate functional vessels with diameters ranging from 1 to 10 mm.[13] Certain design considerations are similar between the different types of vasculature, regardless of size, but some considerations, including cell source and structural complexity, vary greatly between scales. Both small and large-vessel grafts are designed as single tubes that must withstand high pulsatile pressure without deforming or bursting.[5a] In addition, the composition and geometry of the luminal surface must prevent thrombosis and encourage remodeling and regeneration postimplantation to ensure proper cellular and mechanical integration with the host vasculature.[14] However, the heterogeneity in size-scale is coupled with heterogeneity in function, therefore requiring distinct biomaterial design and engineering strategies.

3.1. Large-Caliber Vessels

For engineering large caliber vessels, their size and high blood flow rates reduce the risk for occlusion and therefore reduce the complexity of fabrication.[8] Synthetic nonbiodegradable materials such as polyethylene terephthalate (i.e., Dacron) and expanded polytetrafluoroethylene (ePTFE) have been widely used to generate large vascular grafts since the 1950s.[1c] These grafts demonstrated promising long-term results, primarily due to their antithrombogenic nature and capability to withstand strong mechanical loads.[15] A major limitation after implantation of these grafts is their susceptibility to bacterial infection that can evoke inflammatory response and lead to graft rejection.[16]

Material modifications have been developed to further improve the clinical success rates of biomaterial-based large vascular grafts. For example, it was demonstrated that covalently linking heparin to the luminal surface of transplanted ePTFE grafts led to reduced bleeding, improved patency rates, and lowering overall adverse events.[17] In a different approach, tubular biodegradable scaffolds, composed of polyglycolide knitted fibers and an L-lactide and ε-caprolactone copolymer sponge were shown to enable graft remodeling. Two years after

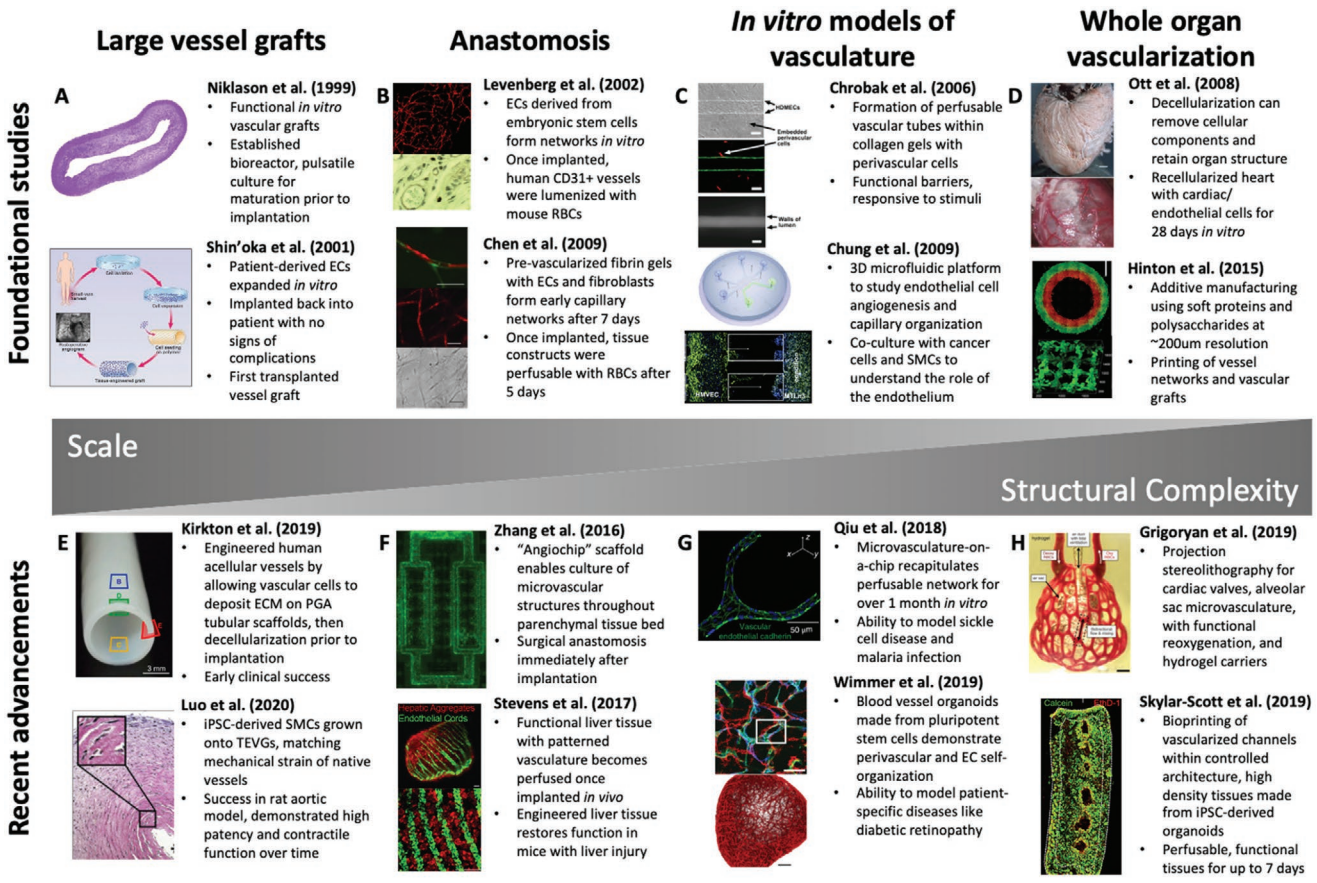

Figure 2. Overview of fundamental and more recent developments in vascular bioengineering; sub-fields are broken down into engineered large vessel grafts, anastomosis, in vitro models of vasculature and whole organ vascularization. A) Top: Engineered blood vessel postimplantation. Reproduced with permission.[63] Copyright 1999, American Association for the Advancement of Science (AAAS). Bottom: Schematic for translation of engineered vessels. Reproduced with permission.[13b] Copyright 2001, Massachusetts Medical Society. B) Top: Human CD31+ staining in vitro and once implanted in vivo. Reproduced with permission.[139d] Copyright 2002, National Academy of Sciences. Bottom: Stromal support of endothelial cells in fibrin gels. Reproduced with permission.[98] Copyright 2009, Mary Ann Liebert. C) Top: Perfusable mesovasculature in collagen gels. Reproduced with permission.[72] Copyright 2006, Elsevier. Bottom: Microfluidic platform to study endothelial cell interactions with supportive populations during angiogenesis. Reproduced with permission.[94] Copyright 2009, Royal Society of Chemistry. D) Top: Macroscopic view of whole heart decellularization and recellularization with ECs and cardiomyocytes. Reproduced with permission.[145] Copyright 2008, Springer Nature Limited. Bottom: Bioprinting of soft proteins like collagen in various organizations. Reproduced with permission.[3a] Copyright 2015, AAAS. E) Top: Acellular vessel grafts from bioreactor-based tissue culture from Humacyte, Inc. Reproduced with permission.[1e] Copyright 2019, AAAS. Bottom: Incorporation of iPSC-derived SMCs strengthens engineered vessel organization once implanted. Reproduced with permission.[13e] Copyright 2020, Elsevier. F) Top: Macroscopic view of "Angiochip" scaffold for improved anastomosis. Reproduced with permission.[84] Copyright 2016, Springer Nature Limited. Bottom: Patterned endothelial cells within hepatocyte aggregates prior to implantation. Reproduced with permission.[155] Copyright 2017, AAAS. G) Top: Perfused endothelial cells in microvasculature-on-a-chip. Reproduced with permission.[2a] Copyright 2018, Springer Nature Limited. Bottom: iPSC-derived blood vessel organoids show cell crosstalk and overview of organoid. Reproduced with permission.[6a] Copyright 2019, Springer Nature Limited. H) Top: Bioprinted vascular structures emulate alveolar capillary structure. Reproduced with permission.[152] Copyright 2019, AAAS. Bottom: Live/dead staining in high density organoids within vascularized tissues. Reproduced with permission.[151] Copyright 2019, AAAS.

implantation into the inferior vena cava of canines, these grafts were both endothelialized and able to maintain their mechanical properties while avoiding stenosis and calcification.[18] A year later, the same group demonstrated that when the same graft was used to replace the pulmonary artery, well-formed vasculature without marked stenosis was observed after only 12 months.[1d]

Engineered large-vessel grafts are also needed for surgical reconstruction of congenital defects in pediatric patients. A prerequisite for large-vessel replacements for pediatric populations is the ability for continuous growth and remodeling that would eliminate the need for multiple interventions. Since commonly used large-caliber vessel grafts are nonliving and are composed of synthetic materials, their remodeling capabilities are rather limited. In an attempt to address this clinical need, several groups have developed cellularized, biodegradable large-vessel grafts that can mature with a pediatric patient after implantation by promoting the generation of new vessel tissue in vivo.[5c] The feasibility of this approach has been demonstrated in the replacement of pulmonary artery and aorta in aging ovine models.[19] The first successful clinical trial of living large-caliber vessels in a pediatric patient was reported in 2001 by Shin'oka and colleagues. A 1 cm diameter biodegradable tube was seeded with autologous myofibroblasts and SMCs, cultured for 1 week in vitro, and transplanted as a replacement to an occluded pulmonary artery. The authors reported on high patency rates with no signs of graft occlusion 7 months postimplantation.[13b]

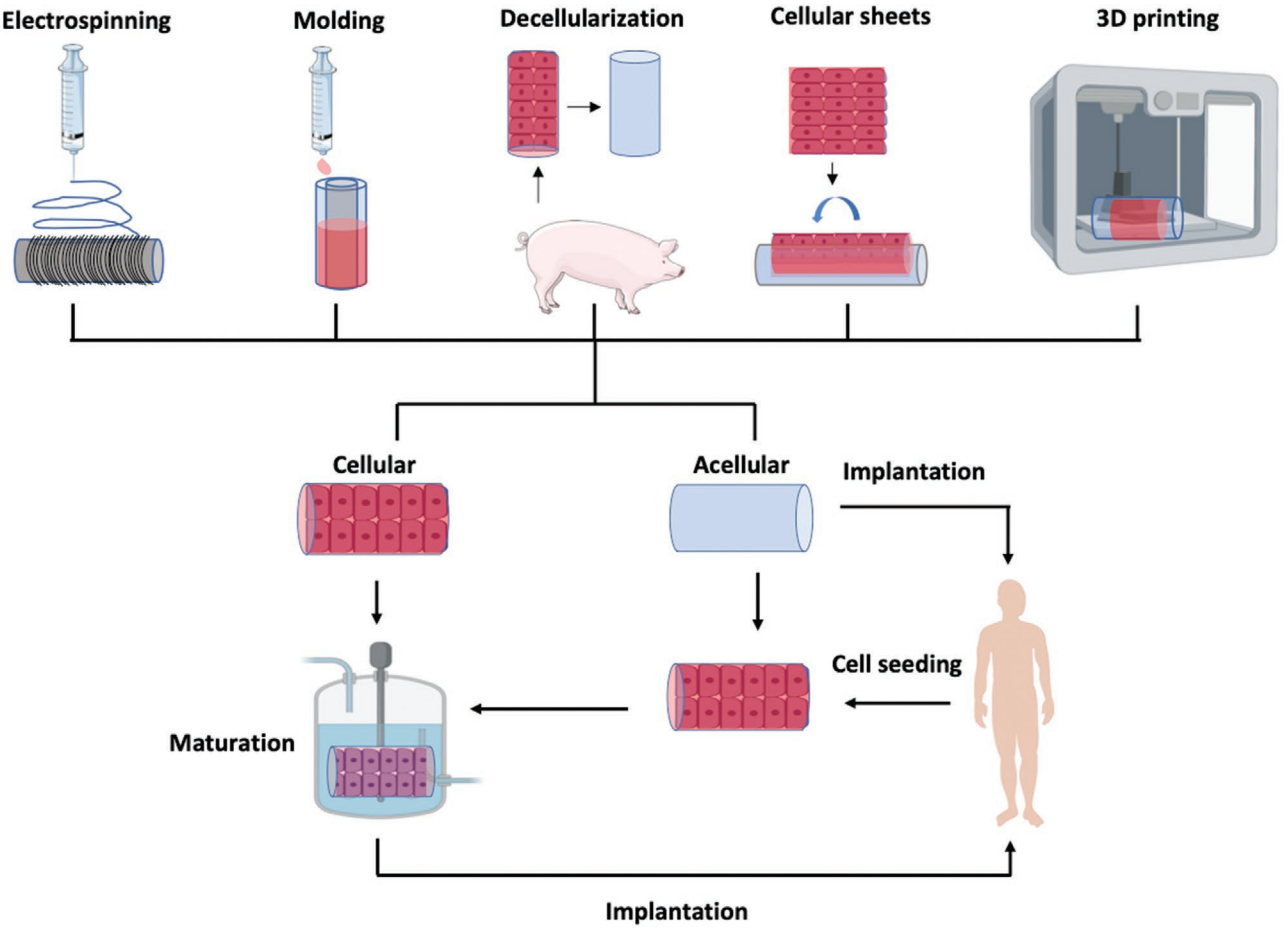

Figure 3. Fabrication approaches for small-caliber vessels. Tissue engineered small-caliber vessels are fabricated by electrospinning, molding, decellularization, cellular sheets or 3D printing to generate either cellular or acellular grafts. Acellular grafts are either implanted immediately postfabrication or seeded with an autologous cell source. Cellularized grafts could be further stimulated in bioreactors to mature vessels prior to implantation. Created with BioRender.com.

A few years later, the same group conducted a large clinical trial of these engineered vessels and reported implantation in 25 pediatric patients with no graft-related mortality.[20]

3.2. Small-Caliber Vessels

While synthetic materials demonstrated promising clinical efficacy in large-caliber vessels, their efficacy in small-vessel grafts has been limited, primarily by the poor patency rates. To date, autologous veins or arteries are considered to have the best clinical outcome in bypass or replacement surgeries of small-diameter vessels, outperforming synthetic grafts.[8] Given the constant increase in the numbers of cardiovascular patients, limited availability, and variable quality of the autologous grafts, there is an urgent need to develop clinical alternatives.[8]

The first tissue engineered blood vessel was reported by Bell and Weinberg in the mid 1980s. In their pioneering work, they engineered a tissue construct that recapitulated the multilayered arterial structure and demonstrated its capability to function as a selective barrier. Collagen gel containing SMCs was casted around a mandrel and cultured for 1 week to generate the central layer of an artery. A Dacron sleeve was then slipped around the construct, seeded with fibroblasts, and cultured for two more weeks to enable the cells to contract the outer layer and provide mechanical support. Finally, the tube was slipped off the mandrel and the lumen was seeded with ECs to form endothelium.[1a]

While this landmark study demonstrated ability to capture the structural and some functional properties of native arteries, the mechanical properties of the engineered vessels were poor, with a burst strength tenfold lower than required to withstand arterial pressure postimplantation.[21] Although this approach was never applied in the clinic, it laid the foundation for the development of numerous tissue engineering approaches to generate small-caliber vessels.

Engineered small-caliber vessels must meet a few critical design criteria and exhibit several postimplantation responses: i) withstand high-pressure flow without bursting or deforming; ii) prevent thrombus formation and occlusion through the choice of the appropriate composition, topography, and cell population residing the luminal surface of the vessels; and iii) enable graft remodeling to properly integrate with the host vasculature and promote regeneration.[14,22] Current approaches aiming to address these needs can be divided into three main groups: i) scaffold-based fabrication methods, ii) decellularized matrix, iii) cellular sheets, and iv) 3D bioprinting (**Figure 3** and **Table 1**).

Table 1. Overview of fabrication strategies.

Fabrication approach	Vascular architecture and resolution	Advantages	Disadvantages	References
Synthetic scaffold	Single tube (mm-scale)	• Source readily available • Highly reproducible • Tunable mechanical properties • Low cost	• Lack of cell binding site • Extended in vitro culture periods	[15,18,33,36]
Natural scaffold	Single tube (mm-scale)	• Source readily available • Supports cell adhesion and proliferation • High remodeling rates in vivo	• Inferior mechanical properties • High compaction rate • More susceptible to degradation • Potential transmission of disease if animal-derived	[25d,26,39,40]
Cellular sheets	Single tube (mm-scale)	• Nonimmunogenic (if fabricated from an autologous cell source) • Biomimetic composition and mechanical properties	• Extended in vitro culture periods • Limited reproducibility • Final properties are highly dependent on cell type	[1b,51,52]
Decellularization	Single tube (mm-scale)	• Conservation of native biochemical and structural properties • Native mechanical properties • Nonimmunogenic	• Not readily available • Invasive • Low reproducibility • Low cell infiltration • Incomplete decellularization could provoke immune response • Excessive decellularization can lead to the loss of biochemical and mechanical properties	[43,45,46]
3D printing (large vessels)	Single and branched tubes (mm-scale)	• User-defined • Control over cellular organization of multiple cell types • High cell densities (extrusion) • Low cost (inkjet)	• Reduced mechanical stability • Cell damage (inkjet) • Nozzle clogging (inkjet)	[55,57–59]
Micromolding and soft lithography	Single tube (needle molding) and branched channels (soft lithography, 50–200 µm)	• Highly reproducible • Perusable vessels • Controlled shear stress • Controlled transmural pressure • Cylindrical geometry (needle molding)	• Need perfusion to form endothelium • Low resolution • Vessel geometry cannot be altered postfabrication • Rectangular channels (soft lithography) • Control over vessel structure in 2D	[72,76,80,82]
3D printing (mesoscale vessels)	Complex vascular geometries, with either extrusion (100–500 µm) or Inkjet (50–150 µm)	• User-defined • Control over vascular structure in 3D • Applicable for large-scale constructs	• Reduced mechanical stability • Low resolution • Cell damage (inkjet) • Low speed (extrusion)	[88,89,91,93]
Laser degradation	Highly Complex vascular geometries (5–50 µm)	• High resolution • User-defined • Control over vascular structure in 3D • Dynamic manipulation of vessel geometry and organization (4D control) • Cytocompatibility	• Limited scaling • Time consuming • Expensive	[3d,6d,83]
Self-assembly	Capillary networks (5–15 µm)	• Perfusable capillary networks • Recapitulates in vivo microvessel formation processes • Vascular organization could be dynamically manipulated by GFs and mechanical and physical cues	• Long fabrication periods • Lack of control over vascular geometry and organization • Low reproducibility	[2d,80,94c,96,97,99]

3.2.1. Scaffold-Based Fabrication Methods

Commonly used fabrication methods to generate small-caliber vessels from synthetic or natural polymers are electrospinning and tubular molding.

In electrospinning, a polymer is dissolved in an organic or aqueous solution and dispensed through a strong electrical field onto a conductive collector to draw nano- to microscale fibers.[23] This technique offers precise control over the pore size, fiber diameter, composition, and fiber alignment, thus

enabling to mimic the native ECM of a wide range of tissues.[24] A range of synthetic polymers such as poly(lactic-*co*-glycolic) acid, polycaprolactone (PCL), poly(ethylene oxide), and natural polymers such as collagen, chitosan, silk fibroin, and elastin have been used to generate electrospun vascular grafts.[25] Electrospun scaffolds composed of synthetic polymers have higher mechanical durability in terms of burst strength and compliance, though the use of naturally based polymers significantly improves cell engraftment.[26]

Electrospinning of hybrid solutions or more advanced electrospinning techniques such as co-electrospinning, coaxial electrospinning, and sequential electrospinning can be used to generate composite scaffolds with superior properties.[27] For example, an asymmetric vascular graft was generated by electrospinning an inner layer composed of PCL/carboxymethyl chitosan and an outer layer composed of PCL/chitosan. The PCL provided both layers of the scaffold with mechanical strength while the inner layer provided it with anticoagulant properties and the outer layer with antibacterial properties to reduce scaffold rejection.[28] In a different approach it was shown that wrapping electrospun fibrin tubes along a thin layer of electrospun PCL fibers significantly improved suture retention and long-term survival of the graft.[29]

Advanced electrospinning techniques could also be used to load various drugs and growth factors (GFs).[30] For example, PCL nanofibers loaded with rapamycin were electrospun outside a decellularized vascular graft to prevent intimal hyperplasia.[31] In a different study, vascular endothelial growth factor (VEGF) and heparin were crosslinked to a multilayered vascular graft. Matrix metalloproteinase-2 was used for programmed release of the molecules, leading to an initial fast release of VEGF to promote endothelialization in the first 12 d followed by a more sustained release of heparin that lasted up to 50 d to ensure long-term anticoagulation effects.[32] Programmed release strategies for gradual integration of a vascular graft may prime the host environment for more efficient graft survival.

Small-caliber vessels can also be generated by tubular molding, in which a polymer in a liquid form is casted into a mold and crosslinked to form a stable structure.[8,33] However, these vascular grafts often show inferior mechanical properties when compared to native blood vessels.[34] Methods such as applying centrifugal forces or incorporation of porous meshes can be used to enhance mechanical stability.[35] To improve cellular migration, attachment, and remodeling postimplantation, techniques such as phase separation and porogen leaching are harnessed to generate porous scaffolds.[36] These tubular structures can later be wrapped with electrospun fibers to reinforce the structures for increased cellular compartmentalization.[37] Surface patterning can further support cell adhesion and orientation. For example, it was shown that micropatterning channels on the exterior surface of vascular grafts led to SMC alignment and increased expression of contractile proteins.[38]

In a different approach, the immediate fabrication of cellularized grafts can be achieved by suspending cells within a combinatory solution of fibrinogen and thrombin and casting them into a mold.[39] Syedian and colleagues used this approach to fabricate fibrin tubular structures cultured with fibroblasts. Instead of immediate implantation, they decellularized the grafts after 5 weeks in culture, to generate acellular off-the-shelf grafts consisting primarily of collagen produced by ovine fibroblasts during the in vitro cultivation. One year after implantation in lambs, the grafts were completely recellularized and reinforced with matrix proteins, without evidence of calcification or aneurism, indicating the clinical potential of this technique.[40] In a later study, they demonstrated the efficacy of these grafts in baboons.[41]

3.2.2. Decellularized Matrix-Based Approaches

In efforts to harness the native ECM, vessels are harvested from a xenogenic source and decellularized, leaving only the underlying matrix and structural integrity. A variety of techniques have been developed to remove the cellular components by the use of various detergents, chelators, and enzymes together with physical methods such as freeze/thawing and agitation.[42] The advantage of this technique is that the native composition, architecture, and mechanical properties of the vessel are preserved. Decellularized grafts from various sources such as canine, porcine, and bovine have been studied over the past decade.[43] Although xenogenic grafts are readily available, incomplete removal of the cellular components and immunological ECM components could elicit a severe inflammatory response and hinder success of this approach.[44] To overcome this challenge, allogenic human decellularized vessels from deceased donors have been used and reported to have successful clinical outcome after implantation in phase I clinical trials.[45]

To further improve in vivo outcomes, decellularized vascular grafts have been modified with various molecules to reduce thrombogenicity, inhibit calcification, and promote endothelialization.[46] In an attempt to generate an off-the-shelf vessel graft, decellularized rat aortas were seeded with thrombospondin-2 knockout cells which secrete a nonthrombogenic and promigratory extracellular matrix protein and decellularized again after 10 d in culture. Four weeks following implantation in rats the grafts demonstrated increased cell recruitment and decreased failure rates.[46a] In a different study, it was shown that incorporation of an antioxidant polymer to decellularized grafts led to reduced oxidative tissue damage and calcification and therefore improved long-term maintenance in vivo.[46c] To shield the underlying thrombogenic ECM, click-chemistry can be harnessed to amplify heparin and deposit it in an oriented manner on the luminal surface to generate a continuous shielding layer.[47]

3.2.3. Cellular Sheets

In this approach, cell sheets are rolled over a mandrel to form tubular structures and are cultured to enable the cells to deposit ECM proteins and remodeling of the graft into a more stable, native-like structure.[13d] The first successful self-assembly method to generate vascular grafts was reported by L'Heureux and colleagues in the late 1990s. SMCs and dermal fibroblasts were cultured in monolayers to form two separate sheets containing the deposited ECM. The sheets were rolled around a mandrel to form both the medial and adventitial

layers and cultured for 8 weeks to allow the layers to fuse with cohesive spatial organization. The mandrel was then removed and a third layer of ECs was seeded within the lumen of the tube to form an endothelium. The burst pressure of these engineered vessels (2600 mm Hg) exceeded that of native veins (1800 mm Hg) and when implanted into canines, these grafts demonstrated patency and capability to withstand physiological pressures.[1b] Since blood infiltration between the layers was observed, L'Heureux and colleagues further optimized this technique by only using fibroblasts sheets and increasing maturation time.[48] A clinical trial using these grafts was performed in ten patients with end-stage renal disease. Patency rates were promising in six patients at six months. Graft failures in the remaining patients were associated with rejection, thrombosis, or aneurysms.[49]

Tubular vessels can also be generated from cell sheets composed of mesenchymal stem/stromal cells (MSCs) and saphenous vein fibroblasts.[50] To enhance mechanical properties and handling of the grafts, cell sheets can be combined with synthetic polymer sheets fabricated from PCL, poly-L-lactic acid, and poly(dimethylsiloxane) (PDMS).[51] Micropatterning of the polymer layer can further promote cell alignment to recapitulate the anisotropic properties of native blood vessels.[51a] In a different approach, anisotropic cell sheets could also be fabricated by seeding cells on aligned fiber matrices.[52]

3.2.4. 3D Printing

Additive manufacturing, also known as 3D printing, has been frequently used in the past few years to fabricate vascular grafts based on computer-aided-designs (CAD). To generate more accurate CAD models of human vasculature, medical images obtained from computer tomography or magnetic resonance imaging can be utilized.[53] Following the printing process, the 3D vessels can be implanted immediately or seeded with cells prior to implantation. Extrusion, inkjet, and stereolithography are the three main techniques currently used for 3D printing of vascular grafts.

In extrusion-based bioprinting, bioinks are loaded in cartridges and extruded through a needle using either pneumatic pressure, a piston, or a rotating screw. After extrusion, bioinks are then fused to the previously printed layer to form a stable tubular structure.[54] Acellular 3D printed vessel grafts could be generated by extrusion of thermoplastics such as PCL, polyvinyl alcohol (PVA), and polyethylene glycol diacrylate.[55] The polymers could further be functionalized with molecules such as nitric oxide to improve hemocompatibility and peptides to support cell adhesion.[56] Extrusion of cell-laden hydrogels could also be used to directly print cellular vessel structures composed of materials such as alginate gelatin, and fibrin.[57]

In inkjet printing, cell aggregates are deposited dropwise onto a substrate and subsequently crosslinked to form stable vascular structures. This technique is capable of faster printing speed rates with higher resolution when compared to extrusion printing. However, it is limited to low viscosity bioinks and lower cell densities to avoid nozzle blockage and enable droplet deposition.[54] Thus, resulting constructs often have reduced mechanical stability. To overcome this, droplets can instead be deposited into a reservoir with a crosslinker; by using this method, more complex structures can be generated, such as zig-zag tubes and channels with both horizontal and vertical bifurcations.[58] As an alternative to a reservoir, ice could be printed dropwise under ambient conditions of −30 °C to form a sacrificial scaffold that could be coated with numerous materials to form the vessel wall. Using this approach, free-standing complex vessel structures with diameters ranging from 1–8 mm and several bifurcations had been generated.[59]

Stereolithography bioprinters use a light projector to crosslink cellular or acellular bioinks. In this process, the bioink is solidified layer-by-layer on a collector to create complex 3D structures. This method offers the advantage of high printing resolution and increased throughput in thick tissues.[60] In addition, it allows the fabrication of vascular grafts with adequate mechanical properties and complex geometries while maintaining cell viability.[61] Melchiorri and colleagues used CAD designs based on images of the recipient aorta to mimic the specific curvature of the recipient. In addition they demonstrated that vascular grafts printed by this technique can sustain patency and functionality for up to 6 months after implantation.[53b] Bioinks used in stereolithography printing could further be optimized to improve cell viability and support cell attachment and proliferation.[62]

3.2.5. Graft Maturation

To improve the clinical efficacy of engineered grafts and their ability to replace native vessels, a further maturation step is required. Niklason and Langer were the first to report on a bioreactor system designed to enhance engineered blood vessels functionality. In their seminal study, pulsatile flow was applied to tubular vessels composed of polyglycolic acid (PGA) and seeded with SMCs and ECs. It was demonstrated that mechanical stimulation promoted cell migration and secretion of ECM proteins and led to the formation of a more contracted and firm tissue structure when compared to static conditions.[63] Although the mechanical strength of these grafts was significantly improved, they did not yet withstand physiological arterial pressures.

In later work by the Vacanti group, it was shown that when gradually increasing pulsatile pressure and flow to mimic the growth process of vessels from fetus to adult, engineered vessels demonstrated mechanically equivalent strength to native arteries and enhanced elastin production.[13d] Cyclic strain has also been reported to improve cell distribution, increase collagen secretion, and guide circumferential alignment of ECM proteins.[64] In a different study, a bioreactor was developed to simulate more native-like loading conditions by providing both circumferential and axial stretch. Biaxial loading led the development of more mature elastin and collagen fibers, leading to improvements in compliance and suture retention.[65]

Maturation of engineered vessels further improves their translation to the clinic; in early clinical efforts by the Humacyte, Inc., a spin-off company for vessel grafts from the work presented by Niklason and Langer in 1999, high patency rates and graft stability were maintained as hemodialysis

ports for patients with end-stage renal failure. In the current approach, Humacyte developed off-the-shelf grafts derived from seeding vascular cells onto PGA tubular scaffolds, grown for 8 weeks in perfusion bioreactors where cellular ECM deposition constituted the structural integrity of the vessels, and then decellularized prior to implantation. Recovered vessels demonstrated re-endothelialization in vivo, along with recruitment of SMCs and other perivascular support cells.[1e]

3.2.6. Cellular Components

Determining the appropriate type and source of the cells used to engineer vascular tissues may hold the key to their clinical translation. The use of autologous vascular cells such as ECs and SMCs extracted from blood vessel biopsies has been motivated by the clear advantage of using a cell source which will alleviate immune rejection. However, there are several disadvantages in using these cells that compromise their clinical utility, including the invasiveness of the procedure causing trauma to the patient, and limited passaging cycles that could restrict the final size or density of the engineered graft.[66] Moreover, each batch of autologous cells needs to undergo validation and quality control, significantly increasing the time and cost involved in this approach.

The use of autologous cells from other sources that do not require a highly invasive procedure, such as the bone marrow and adipose tissue, has also been explored.[20,67] Given the ability to rapidly expand and differentiate these cells, they could be a promising alternative to native endothelial populations. Human induced pluripotent stem cells (hiPSCs), generated from reprogramming somatic cells by ectopic expression of transcription factors, also provide a promising source of autologous cells.[68] They have the potential to propagate indefinitely while retaining their capability to differentiate into almost any cell type, including vascular cells, therefore the advantage of using them in vascular tissue engineering is clear. Pluripotent stem cell-derived vascular differentiation protocols have been developed over the past decade, with improvements on yield, specification, and application over time.[69] However, the ability to recapitulate vessel functionality is limited by the immature state of the iPSC-derived vascular cells. Therefore, further development of maturation protocols is required.

It was reported that iPSC-SMCs seeded within PGA tubular scaffolds and then cultured for additional 2 months in a bioreactor are capable of generating enough collagenous matrix to form grafts with no evidence of rupture following implantation.[70] However, their low mechanical strength and radial dilation have hampered their clinical potential. To further improve the properties of these grafts, Luo et al. utilized an optimized culture medium and incremental pulsatile radial stretching regimen. The vascular grafts presented mechanical strength similar to that of native vessels and excellent patency rates 4 weeks postimplantation in rats. The use of biodegradable scaffolds, pluripotent cell sources, and pulsatile mechanical stimulation have provided the optimal set of cues for vessel graft formation, envisioning functional vessel substitutes for patients with dysfunctional vascular function.[13e]

3.2.7. Clinical Outlook

The emergence of clinical trials for vascular grafts represent a major milestone in the field, demonstrating how a bold idea, proposed 30 years ago, could ripe into a clinical procedure.[1d,e,20,71] However, while major advancements have been achieved, further developments are required for widespread clinical realization. An important undertaking in the next few years will be to develop strategies to prevent thrombosis (associated with early graft failure), intimal hyperplasia (associated with mid-term graft failure), and prolonged inflammatory responses, the main adverse events associated with clinical failures of vessel grafts.

Early graft failure is usually driven by platelet adhesion to collagen, leading to thrombosis and consequently low patency rates. In vivo, this process is prevented by the endothelium which shields the blood from the vascular wall matrix. Therefore, to improve clinical outcome of acellular or decellularized grafts, further advancements in surface functionalization and coating may be imperative to inhibit platelet activation and recruit circulating endothelial progenitors. Furthermore, since incomplete decellularization can lead to an immunogenic response provoked by cellular material and access decellularization could harm the biochemical and mechanical properties of the native ECM, further optimization of decellularization protocols and quality control processes will be required.

Cellularized synthetic or natural based polymer scaffolds provide a promising alternative to overcome this challenge. Emphasis should be placed on tailoring material properties to prevent cell colonization and access ECM production that could lead to intimal hyperplasia while enabling cell infiltration and remodeling. A deep understanding of the mechanisms activated at the blood-tissue surfaces would be beneficial to develop advanced biomaterial systems to achieve this goal. Biomaterials with adaptive control, to sense and actuate cells and drive cell-specific behaviors such as migration and proliferation, or those that can sense localized changes in microenvironments, to dynamically control vessel architecture and stiffness, would be of great interest.

Recent studies have shown the potential of iPSC-derived vascular cells to support functional vascular graft formation to generate patient-specific or allogenic implants and avoid an immunological response.[13e] Advances in 3D printing and imaging technologies can now enable personalized vessel grafts to prevent dimension mismatch at the site of anastomosis. However, extended fabrication time and lack of well forged regulatory pathways make acellular off-the-shelf grafts, as in the case of the acellular ECM-rich vessels from Humacyte, Inc., a more attractive solution for the clinic, offering a readily available option for emergent procedures.[1e]

4. Meso- and Microvascular Engineering

The microvasculature is composed of a dense, high-aspect ratio network of capillaries (10–15 μm) located within <100 μm from one another. This structure provides optimal conditions for the diffusion of gasses and transport of nutrients, metabolites, and circulating cells to the tissues.[4b] In recent years, fabrication of microvascular networks to generate in vitro models of vasculature and vascularized tissues have gained significant interest (Figure 2

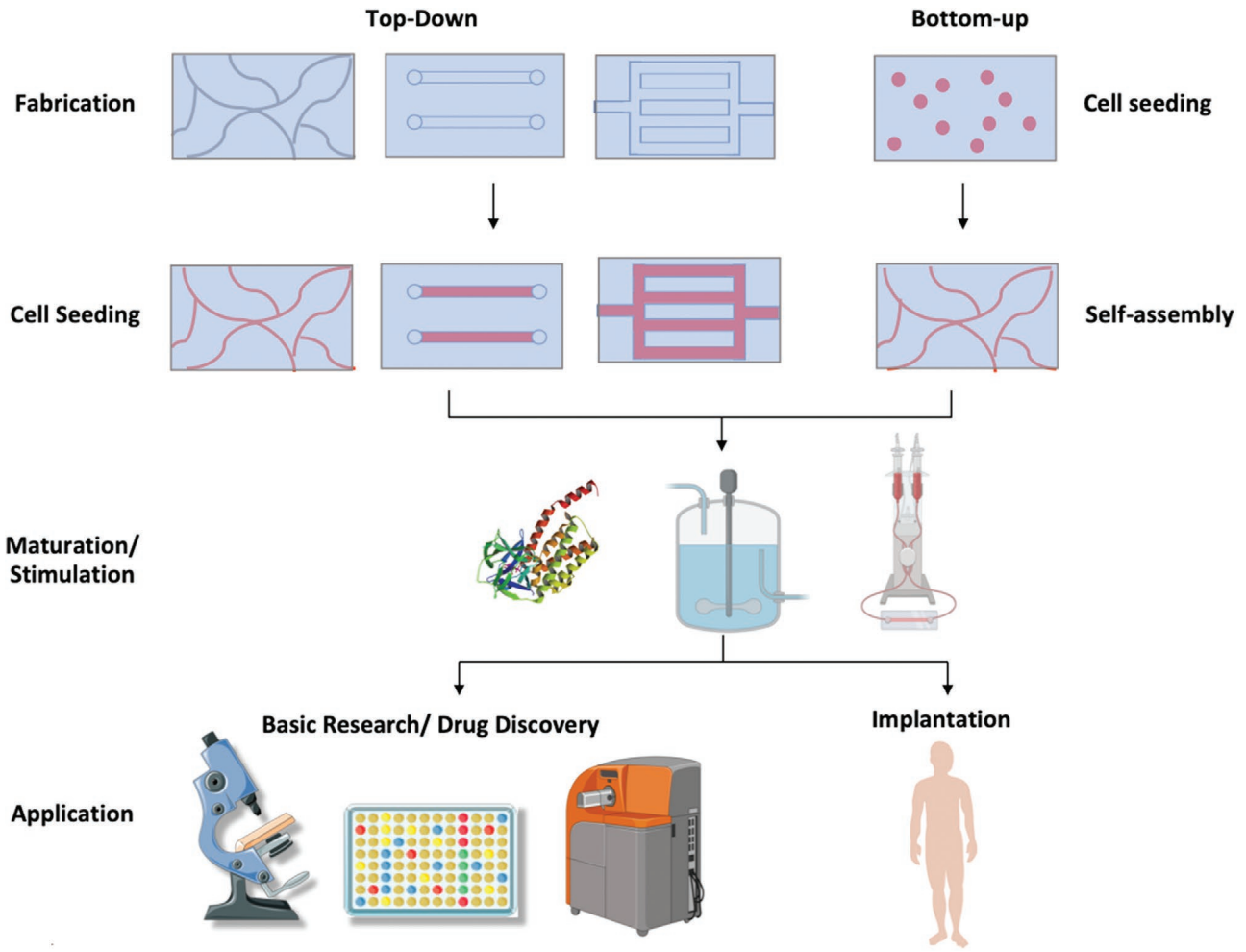

Figure 4. Engineered meso- and microvasculature. Approaches to engineering meso- and microvasculature can be divided into top-down and bottom-up approaches. In top-down the vasculature is predesigned, while bottom-up relies on cellular and extracellular stimuli to promote vessel formation. The vasculature can be further matured or stimulated to induce sprouting to then generate a stable and functional vascular network. Both approaches are utilized to generate in vitro vasculature models for basic research and drug screening applications and/or to vascularize tissues for regenerative medicine applications. Created with BioRender.com.

and **Figures 4** and **5**). Clearly, the design considerations and fabrication techniques to recapitulate the function and architecture of the microvascular networks are unlike those used for engineering large vessels. Here, the goal is to fabricate fine capillaries with high-resolution, with diameters of 5–10 µm, a dimension that is two to three orders of magnitude lower than for large vessels.

Historically, the relatively small scale of the capillaries and their complex geometry limited the ability to recapitulate them in vitro. However, in the past few years, a variety of biofabrication techniques have emerged, exploiting advances in lithography, laser degradation, and 3D printing, to engineer vasculature with resolution ranging from meso- to microscale. Figure 5 and Table 1 provide a summary of these strategies.

4.1. Micromolding and Soft Lithography

Needle molding, one of the earliest techniques developed to generate perfusable, vessel-like structures, was pioneered by the Tien group in 2006.[72] By casting a hydrogel around a removable needle, a cylindrical channel is formed and connected to a flow loop to allow medium perfusion. The hydrogel surrounding the engineered vasculature must be mechanically stable to support formation of a lumen upon removal of the needle and must resist deformation during perfusion. To form endothelium, ECs in suspension are introduced into the channel and allowed to adhere to the lumen walls.[72] To mimic the bilayer nature of capillaries or the trilayer nature of the arterial and venous wall, concentric needles could be used for the assembly of layers comprising ECs and perivascular cells.[73] Ingram and colleagues demonstrated the utility of needle molding coupled with iPSC-derived ECs to generate lumens with functional barriers, advancing the field toward the fabrication of patient-specific vascularized tissues.[74] These developments have been harnessed to generate perfused vascularized tissues such as adipose and skin.[6e,75] While this method provides a relatively cheap and simple methodology to vascularize tissues, it is limited to the construction of single tubular mesochannels with

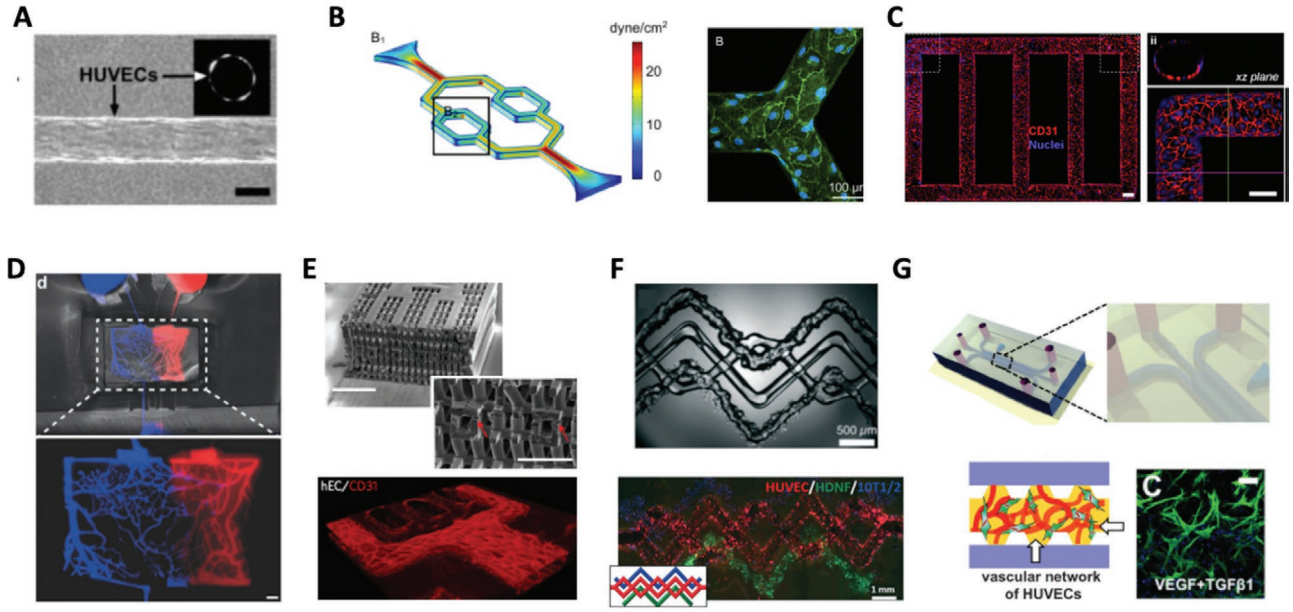

Figure 5. Overview of approaches for engineering meso- and microscale vasculature. A) Uniaxial mesochannel fabricated by needle molding. Reproduced with permission.[72] Copyright 2006, Elsevier. B) Patterned mesochannels in PDMS. Reproduced under the terms of the Creative Commons CC-BY License.[77c] Copyright 2015, Public Library of Science. C) Mesochannel casting within collagen hydrogels. Reproduced with permission.[80] Copyright 2012, National Academy of Sciences. D) Laser patterning microvascular networks within polyethylene glycol hydrogels. Reproduced with permission.[3d] Copyright 2016, Wiley-VCH. E) Layer-by-layer assembly of a branched 3D mesochannel network. Reproduced with permission.[84] Copyright 2016, Springer Nature Limited. F) Mesovasculature 3D printing using sacrificial bioinks. Reproduced with permission.[91c] Copyright 2014, Wiley-VCH. G) Vasculogenesis-based vascular self-assembly. Reproduced with permission.[100] Copyright 2014, Oxford University Press.

no branches, therefore lacking the capability to recapitulate the resolution and complex nature of the microvasculature.

For generating more complex geometries, lithography techniques have been adopted from the microchip industry. Molds are used to fabricate channels with specific architectures in PDMS and closed channels are generated by bonding two layers of PDMS and subsequently seeding ECs to form a confluent endothelium.[76] Further maturation of the endothelium could be achieved by applying shear stress.[77] Fluid flow through the channel can be mathematically modeled and controlled by channel dimensions, to establish flow rates providing physiological shear stress and oxygen diffusion representative of the tissue of interest (e.g., shear stress variations in capillaries versus arterioles).[78] Of note, PDMS has clear benefits such as ease of use, biocompatibility, and optical transparency to allow real-time monitoring. However, it has nonselective absorption of oxygen and other hydrophobic molecules therefore compromising its utility in various applications including drug testing.[79]

Soft lithography can also be utilized to generate vessel-like structures in natural hydrogel materials to provide a more biologically relevant environment compared to PDMS. These biomaterials support cell attachment and infiltration and allow the degradation and remodeling of the surrounding matrix.[80] A drawback of this technique is the nonphysiological rectangular shape of the channels. To address this issue, advanced lithography techniques such as backside and dual-projection lithography have been harnessed to generate microvessels with circular cross-sections.[81]

A different approach to generate 3D complex vascular networks within 3D hydrogels is sacrificial molding. In this approach, sacrificial materials can be casted into molds to form a desirable microvasculature design. Subsequently the material is embedded within a hydrogel and washed away after its solidification. For example, sacrificial templates with complex architectures composed of PVA or gelatin can be embedded within hydrogels and dissolved by washing with water or incubation in 37 °C, respectively.[77c,82] Importantly, this method enables researchers to generate mesovasculature within monolithic hydrogels, eliminating the risk for improper alignment and adhesion between two layers generated by soft lithography.

4.2. Laser Degradation

Laser energy can be utilized to selectively degrade or ablate complex microchannel networks within 3D hydrogels. It allows for high resolution patterning and mm-scale z-depth penetration while maintaining cell viability. For example, Brandenberg and Lutolf demonstrated that by photodegradation, network structures within cell-laden gels could be manipulated overtime, allowing 4D control.[3d] In a different study, it was shown that laser degradation accurately recapitulated the complex architecture and density of the in vivo microvasculature with a resolution that enabled to form capillaries as small as 3 μm.[6d] Microchannels could further be modified by laser degradation to form intraluminal topographies and dynamically regulate cell function.[83] While these studies stress useful tools, creating

user-defined biomimetic architectures with high resolution, they are limited in both scale and time, and require expensive multiphoton systems, which may not be effective for vascularizing full tissues.

4.3. Layer-by-Layer Assembly

Layer-by-layer assembly offers another tactic to generate 3D structures with high architectural complexity. In this approach, single layers are patterned separately and stamped by biological, chemical or photo-crosslinking to form 3D structures. Using this technique, the Radisic group reported on the development of Angiochip, a 3D cardiac tissue with mesoscale vessels. The tissue surrounding the channels remained functional and underwent extensive remodeling while an open-vessel lumen had been maintained.[84] In a different study that similarly used layer-by-layer assembly, electrospun sheets were photopatterned to form mesogrooves and bonded together, generating lumens defined between fibrillar walls to mimic the ECM surrounding the endothelium.[85]

4.4. 3D Printing

3D printing could be categorized into two major groups: i) direct printing in which a cell-laden material is deposited onto a surface and ii) indirect printing in which printed sacrificial materials are embedded within larger scale cell-laden materials.

Direct printing can be used by coaxial extrusion nozzle to generate freestanding vascular structures. The outer tube disperses a crosslinkable polymer, while the inner tube dispenses the crosslinker.[86] After the construct is printed, a further crosslinking step can be performed to ensure stability.[87] Vessel wall thickness and lumen diameter can be controlled by varying nozzle diameter and extrusion flow rate.[88] Further advancements in coaxial extrusion technologies enable the fabrication of more complex vascular structures with multiple layers and cell types.[87b,89] For example, by controlling the on–off status of coaxial extrusion nozzles, intermittent filaments composed of heterogenous cell populations can be generated.[89a]

Characteristics of the bioink such as shear thinning properties, crosslinking mechanism, gelation behavior, and chemical composition are important determinants of cell viability and function in extrusion. Optimizing these parameters has been of high interest in the field, to improve cell survival and proliferation over time.[90] For example, Colossi et al. developed a composite bioink composed of alginate and gelatin-methacryloyl (GelMA). They demonstrated that the special properties of the bioink improved gelation time, increased printing resolution, and reduced shear stress applied on the cells.[88a] In a different study, a hybrid bioink composed of alginate, vascular ECM, and a proangiogenic drug was developed. It was demonstrated that the bioink was capable of not only enhancing endothelial progenitor cell differentiation and survival, but promoting neovascularization in vivo as well.[90a]

In the simplest case, indirect printing can be utilized to fabricate uniaxial channels within hydrogels by extrusion of a sacrificial material to form a template structure within a bulk material. Selective removal of the sacrificial template leaves a lumen within the gel that retains its original architecture. ECs can either be incorporated within the sacrificial material and adhere to the wall of the channel upon its removal, or introduced after channel formation.[91] The resolution of the channels generated by using sacrificial materials is on the mesoscale, limiting the capability to mimic the dimension scales of capillary beds.

Vascular networks composed of various sacrificial materials could also be printed separately and subsequently embedded within hydrogels.[6c,92] For example, a carbohydrate glass formulation was developed with adequate mechanical stiffness to enable extrusion of free-standing 3D lattices. The lattices were embedded within various ECM materials and simply dissolved with water to form mesoscale vasculature able to withstand applied pulsatile pressure.[93] In a later study, carbohydrate glass was used to generate centimeter-long lumens with uniaxial orientation. On implantation it was reported that this vasculature was able to integrate to host vasculature and rescue perfusion better than unpatterned structures, suggesting that the geometry of the vessel is a critical factor in supporting anastomosis.[6c]

4.5. Self-Assembly

Vascular networks can also be formed by a bottom-up approach. Instead of seeding cells into predefined vascular structures, mechanisms of angiogenesis and vasculogenesis can be exploited to form microvasculature. Microfluidic devices have provided scientists with unprecedented opportunities to study these processes in vitro and investigate regulators of EC sprouting, tubular formation, perivascular cell recruitment, and secretion of matrix proteins.[94] It was found that factors such as growth factor gradient, fluid flow, matrix stiffness, and degradability are critical players in the initiation and stabilization of microvasculature.[95]

4.5.1. Angiogenesis-Driven Vascular Self-Assembly

Angiogenesis is the process of growth of new capillary beds from pre-existing blood vessels. This process can be recapitulated by utilizing microfluidic devices with parallel endothelialized channels separated by hydrogels.[94] For example, Zheng and colleagues used this platform to study the role of both perivascular cells and proangiogenic factors on EC sprouting. They found that the presence of proangiogenic factors inhibited the recruitment of pericytes by microvessels, an observation that is consistent with the angiogenesis process in vivo.[80] In a different study, Nguyen and colleagues were able identify two distinct cocktails of proangiogenic factors that were able to direct EC sprouting and assembly into tubular structures mimicking key features of angiogenesis in vivo.[96]

4.5.2. Vasculogenesis-Driven Vascular Self-Assembly

Similar to in vitro angiogenesis, microfluidic chambers are often used as platforms to promote and study vasculogenesis.

Typically, ECs are seeded within collagen or fibrin gels, placed in a microfluidic device and self-assemble into vascular networks within a few days.[2d,94c] The George group was first to report the in vitro formation of a perfusable capillary network capable of withstanding physiological flow and shear rates and maintain a metabolically active stroma.[97] By implanting microvessels formed in vitro within fibrin hydrogels, perfusion into the host tissue, along with the presence of murine red blood cells (RBCs), was found after 5 d[98] (Figure 2). In a different study by the Kamm group, a fibrin gel containing human umbilical cord endothelial cells (HUVECs) and human lung fibroblasts was casted in a microfluidic chamber (Figure 2). They reported that patent lumens were able to form after only 4 d in culture and that vessel diameter and permeability could be manipulated by varying fibroblast cell density, concentrations of growth factors, and hydrogel stiffnesses.[99] Other studies have demonstrated the crucial role of pericytes and MSCs to support the formation of stabile capillaries in vitro.[77a,100]

The knowledge acquired in these early studies by bioengineers, together with advancements in vascular biology, is currently translating into vascularizing 3D tissues. Emphasis is placed on creating dense capillary beds, patent and perusable lumens, controlled architectures, and enhanced structural and functional maturation. Self-assembly of vascular networks, although in the early stages of microvessel formation in vitro, may provide us with insights into how to make functional, vascularized building blocks of smaller tissue sections prior to in vivo implantation. To achieve these crucial goals and advance the field, various factors of the vascular microenvironment must be recapitulated in vitro, including the i) growth factor milieu, ii) mechanical signals, and iii) topographical cues.

4.6. Growth Factors

In vivo, the ECM acts as a depot for GFs that are stored and released according to certain physiological and pathological triggers. GFs bind to the ECM in varying affinities and at distinct locations.[101] When released from the ECM, they bind to cellular receptors to activate downstream signaling pathways and guide the formation of new blood vessels during angiogenic sprouting.

Numerous strategies have been developed to mimic this process in vitro by the controlled release of GFs from biomaterials. Commonly used GFs to promote neovascularization include VEGF, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF).[102] GFs can be incorporated into matrices by chemical and biological covalent binding reactions or loaded within microparticles for sustained delivery.[85,103] For example, to capture the different physiological release kinetics of GFs, Freeman and colleagues fabricated alginate-sulfate scaffolds and VEGF, PDGF-BB, and TGF-β with varying affinities. They demonstrated that the sequential delivery led to enhanced blood vessel formation with increased recruitment of SMCs, indicating early vessel maturation.[104] Instead of sustained delivery strategies, GFs can also be released on-demand in vivo by using minimally invasive stimuli such as heat and ultrasound.[105] Spatial presentation of growth factors has a critical role in guiding neovascularization.[77a,106] By patterning GFs in certain geometries within scaffolds to generate gradients, cell migration and branching in preferential directions can be achieved.[107] A recent study reported that interstitial flow can eliminate spatial GF gradient within hours, suggesting that GFs and fluid forces work in concert to direct new blood vessel growth.[108]

In other approaches, cells have been engineered to secrete proangiogenic paracrine factors. For example, it was demonstrated that targeted genetic manipulation of MSCs to express high levels of endogenous VEGF significantly improved their angiogenic-supportive capabilities in vivo.[109] Our group has demonstrated that preconditioning mesenchymal progenitor cells with low concentrations of TGF-β promotes arteriogenic gene expression profiles. When implanted in infarcted hearts in a rat model of myocardial infarction, these cells were able to enhance vascular formation by the secretion of stromal cell-derived factor 1.[110] A critical limitation of GF-mediated microvascular assembly is that these approaches often lead to the formation of leaky, naïve microvessels and that network organization is not carefully controlled.[111]

4.7. Mechanical Cues

ECs are constantly exposed to mechanical signals in the form of matrix stiffness, shear stress, and changes in matrix tension.[112] In studies investigating the effect of hydrogel mechanical properties on EC self-assembly it was demonstrated that softer substrates induced sprouting and promoted microvascular assembly and stabilization; whereas in rigid substrates, EC migration was inhibited and capillary formation was restricted.[113] Matrix stiffness can be manipulated by varying polymer or crosslinker concentrations. However, it subsequently results in alterations in other factors such as density and pore size, limiting the ability to decouple its role in angiogenesis and vasculogenesis.

To tune matrix stiffness while keeping other factors constant, hydrogels can be placed within physical boundaries. In this setup, when cellular contraction imposes constraint on the hydrogel, the boundaries prevent the gel from deforming, resulting in increased cellular tension. By culturing hydrogels containing ECs within rectangular matrix boundaries, they align along the long-axis where they experience greater effective stiffness and can eventually form orientated microvessels.[114] The ability to control microvessel orientation is especially important in tissues such as the heart, skeletal muscle, and bone, which are characterized by aligned vasculature.[115] In a recent study utilizing these methods, it was shown that ECs and pericytes can self-assemble into highly aligned microvessels that could be perfused for up to 6 d in vivo. In addition, it allowed for the formation of exceptionally dense lumens, a critical demand for highly metabolic tissues such as the heart.[116] In a similar approach, Chang and colleagues generated highly aligned gels and implanted them into mice with or without the physical frame. They reported that while microvessel alignment was lost after 30 d in the unconstrained gels, it was kept aligned in the constrained ones, highlighting that in vitro topologies are not kept after transplantation and that constant orientation cues are needed in vivo to sustain alignment.[117]

The pulsatile nature of blood flow exposes vascular cells to cyclic tensile strain which can be uniaxial or multiaxial, depending on the blood vessel location.[118] Applying cyclic stretch in the physiological regime (5–10%) can significantly enhance proangiogenic GF secretion, microvessel sprouting, and lumen orientation.[119] In addition, the degree of cell-induced forces directly correlates not only with vessel elongation but with network quality as well.[120] Higher cyclic stretch, mimicking pathological conditions, is associated with activation of endothelial inflammatory pathways and apoptosis.[121]

Unidirectional laminar blood flow constantly exerts shear stress on ECs lining vessel walls. Numerous studies devoted to studying the effect of physiological shear stress on ECs, demonstrated that it has a critical role in promoting angiogenesis sprouting, cell–cell interactions, EC alignment, and increasing barrier functionality and vessel maturation.[3c,77a,122] On the contrary, nonphysiological fluid flow shear-stress can lead to cytoskeleton disassembly, leaky vasculature, and inflammatory gene expression.[122a,123] The use of microfluidic devices provides an excellent model to study the role of shear stress in angiogenesis while decoupling the impact of other factors.[77a,80] Galie et al. used a microfluidic device to independently investigate the role of shear stress exerted by luminal and transmural fluid flow. They reported on the existence of a shear stress threshold, that when surpassed, induced angiogenic sprouting regardless of whether it was luminal or transmural, emphasizing the importance of shear stress to initiate angiogenesis.[124] Advances in microfabricated flow bioreactors now enables the investigation of shear stress on multiple cell constructs simultaneously in a facile, time, and media efficient manner.[125] Recently, a perfusion flow bioreactor was developed to apply flow-induced shear stress to vascularized 3D tissues. It was reported that direct flow conditions induced the formation of microvasculature and increased vessel length, density, and maturation.[3c]

4.8. Topographical Cues

In the vascular microenvironment the ECM plays a critical role in regulating the organization of vascular cells into functional blood vessels. It has been shown that cells can sense micro- and nanoscale topographical features and translate them, by mechanosignaling, into different cellular behaviors such as elongation, alignment, chemokine secretion, collagen production, and remodeling capacity.[126] In an attempt to promote the assembly of microvascular networks within tissues, numerous studies have fabricated scaffolds mimicking different structural aspects of the vascular ECM.

Microporous scaffolds have been utilized to support EC infiltration and self-assembly into vascular structures within the pores.[127] For example, it has been shown that when ECs were seeded within scaffolds with average pore size of 200 µm, they formed microcapillary-like structures with lumens.[128] In another study, pore geometry was tailored to generate randomly oriented and uniaxially aligned pores. It was demonstrated that the aligned pore architecture and increased pore interconnectivity resulted in improved cell–cell interactions and formation of perfusable microvessel networks.[129]

While the role of porous scaffolds to support the formation of capillary structures has shown promising results, their utility in tissues that require vascular alignment such as skeletal and cardiac muscle remains limited. To address this goal, scaffolds composed of aligned fibers could be fabricated by electrospinning synthetic or natural polymers and collecting the fibers on a rotating drum. When seeded with ECs, the cells align according to fiber orientation and form anisotropic structures.[130] In a different approach, hydrogels composed of aligned collagen fibers can promote vascular network formation composed of thick and more aligned channels.[131] Qian and colleagues recently reported on the fabrication of aligned nanoscale fibrous ECM sheets mimicking the cardiac ECM. Cocultured MSCs and ECs within these scaffolds organized into native-like myocardial vascular networks, recapitulating vessel alignment, diameter, and density.[6f]

4.9. Cellular Components

In many bioengineering strategies to fabricate meso- and microscale vasculature, primary endothelial populations, such as HUVECs, have been employed.[99,132] Interestingly, Bersini et al. reported on the respecification of primary endothelial cells into muscle-specific endothelium in their 3D human muscle model, the first study to demonstrate this phenomenon for in vitro endothelial cultures.[133] Although some studies attempt to use primary vascular endothelial cells, these cells can be heterogenous and are just starting to be characterized in adult human organs.[134] Recently, the Carmeliet group published a seminal study identifying the single-cell transcriptional profiles of murine endothelium, demonstrating the heterogeneity in endothelial populations that may provide a key to engineering meso- and microscale vascular models and regenerative cues.[135]

In the microvasculature, pericyte abundance, specification, and plasticity regulate capillary formation and regeneration. Pericytes play a critical role in paracrine and autocrine signaling to guide and support the microvasculature.[136] Their varying surface marker expression levels (ex. smooth muscle actin, neural-glial antigen 2, platelet derived growth factor receptor-beta) and potential for plasticity toward smooth muscle cell and other stromal cell types have demonstrated some challenges in recapitulating these cells in vitro.[137] Critical in bioengineering approaches, perivascular cells have often been replaced for primary fibroblasts or bone marrow-derived MSCs in engineered models; these cells are able to provide some of the supportive scaffolding for angiogenic sprouting in microvascular models, though they lack the tissue-specific and plastic hallmarks of pericytes.[77c,138]

Therefore, it is of significant interest in engineering microvascular disease models or in vivo regeneration to employ novel cell sources, including iPSC methodologies. Many groups have attempted the derivation of iPSC-ECs (CD31+, CD144+ antigen expression), many of which have resulted in generic arteriole- or venous-like endothelial populations.[69e,139] When compared to HUVECs, some studies have highlighted the lack of endothelial maturation to cause functional deficits once ectopically implanted in vivo.[134] In early work by Levenberg et al., embryonic stem cell (ESC)-derived ECs in embryoid body form were

able to anastomose into native murine tissue, demonstrating the first instance of human CD31+ vessels with intraluminal murine blood cells (Figure 2).[139d] In a recent study by the Duh and Gerecht groups, iPSC-ECs were able to revascularization the ischemic retina significantly better than human primary endothelial cells, as shown by a higher CXCL12/CXCR4 chemotactic relationship in iPSC-ECs.[140] Many groups have also attempted to generate iPSC- and ESC-derived perivascular cells, with many being functionally tested through in vivo implantation in regenerative injury models.[139b,141] Approaches for recent directed differentiation protocols for pericytes, as well as protocols to mimic endothelial progenitor plasticity, have been summarized by Gerecht and colleagues in 2018.[142] Relying on potentially autologous, high yield, and unlimited cell sourced iPSCs in engineering meso- and microscale vasculature may suggest to be the most effective avenue toward generating in vitro models and vascularizing tissues.

5. Engineering Complex and Multiscale Vascular Networks

Almost all tissues in the body are vascularized, with the exception of the cornea, cartilage, and the skin epithelium. Only tissues with a cell density of up to 3×10^5 cells cm^{-3} and thickness of <200 μm can function properly without vasculature.[143] However, a physiologically relevant tissue for transplantation should be comprised of at least 1×10^8 cells cm^{-3}.[144] These dimensions require exceptionally high levels of oxygen supply. Therefore, engineered tissues or organs with high metabolic demand cannot remain viable for more than a few hours without a vascular network supporting them. To date, most strategies to address this goal have focused on the incorporation of micro- or mesoscale vascular networks and demonstrated limited success. Fabrication of vascular networks within engineered tissues that could meet these demands remains a fundamental challenge in the field.

In vivo, the unique structure of the vascular tree allows optimal transport of oxygen and nutrients through capillaries while minimizing the energy cost required to deliver blood from the heart to the entire body. It is composed of vessels that span several orders of magnitude in diameter and form complex intertwined configurations. The promise of tissue engineering may be realized by developing advanced methods to recapitulate the structural and functional properties of the vascular tree. While many advanced fabrication techniques exist to generate discrete components of the vascular tree, spanning multiple lengths in scale, only a few attempts have been made to capture the complex functional and structural hierarchy of the vascular network (**Figure 6**). Currently, trends in developing fully vascularized tissues is moving toward recapitulating the complex multiscale vascular architecture, yet we are only beginning to understanding how we may address this major engineering challenge.

One of the early strategies that was developed to meet this challenge was decellularization of whole organs followed by repopulation with cells. The rationale behind this approach is that instead of developing engineering solutions to recapitulate the complexity of the organ vasculature, one can use native vasculature as a template. In the pioneering work by Ott and colleagues, whole rat hearts were decellularized and repopulated with ECs and cardiomyocytes. The constructs were then perfused in a bioreactor that simulated physiological conditions. After 7 d, ECs formed single layers in both small and large coronary vessels and the cardiac construct was able to generate pump function.[145] Following this seminal work, a similar approach has been demonstrated in many additional organs, including the lung, liver, and kidney.[146]

An important milestone was achieved when recellularized lungs, an organ with extreme vascular complexity, were transplanted into rats and were able to connect to the host circulation and perform functional gas exchange; long-term success was stunted, however, as lung failure was observed a few hours after transplantation, largely due to blood clotting and pulmonary edema.[146a,b] Clinical translation of this approach will require the decellularization process to be optimized to remove any residual cellular traces that could provoke inflammatory immune responses. In addition, gentle structures must be preserved to ensure the organization of a functional endothelium that will serve as a tight barrier.

Recently, Arakawa and colleagues reported on the development of a laser-degradation technique that enabled fabrication of multiscale complex vascular networks with channel cross-sections ranging from 10×10 to 300×300 μm. This technique provides exquisite spatial control in a user-defined manner and allows researchers to explore multiscale geometries that have been previously unattainable. However, it is a low throughput method, therefore limiting its capability to generate large-scale vascularized tissues.[83] Projection-based 3D printing allows fabrication of complex 3D structures by patterning light through a digital micromirror device chip for crosslinking photosensitive biomaterials. A recent study harnessed this technique to fabricate a vascular tree-like structure composed of intricate channels with widths ranging from 1 mm to several micrometers. The printed structure enabled perfusion through all length-scale without the need for an external pump, thus mimicking passive capillary flow. While this technique enables generation of complex structures in one short pulse of light (a few seconds), its ability to generate vasculature in thick 3D tissues is limited to the depth of light penetration into the polymer.[147]

In a different approach, high levels of electric charge can be implanted inside a polymer dielectric and then discharged to locally fracture the material, generating a multiscale network with dimensions ranging from 10 μm to 1 mm. While this technique allows the instantaneous fabrication of multiscale channels in large-scale constructs, its potential to accommodate cells has not yet been explored.[148] A sacrificial template-based strategy could be harnessed to embed solvent-spun microscale fibers and mm-scale sacrificial rods within a cell-laden hydrogel and subsequently dissolving them to form a multiscale network. While this approach enables control over vascular diameter and density by manipulating spinning parameters and time, respectively, control over their spatial organization is limited.[149]

With the goal to engineer the human vascular tree, Wimmer and colleagues cocultured iPSC-derived ECs and pericytes to form a 3D blood vessel organoid. They reported that within these settings, cells self-assembled into an interconnected vascular network with proper localization of pericytes. Transplantation

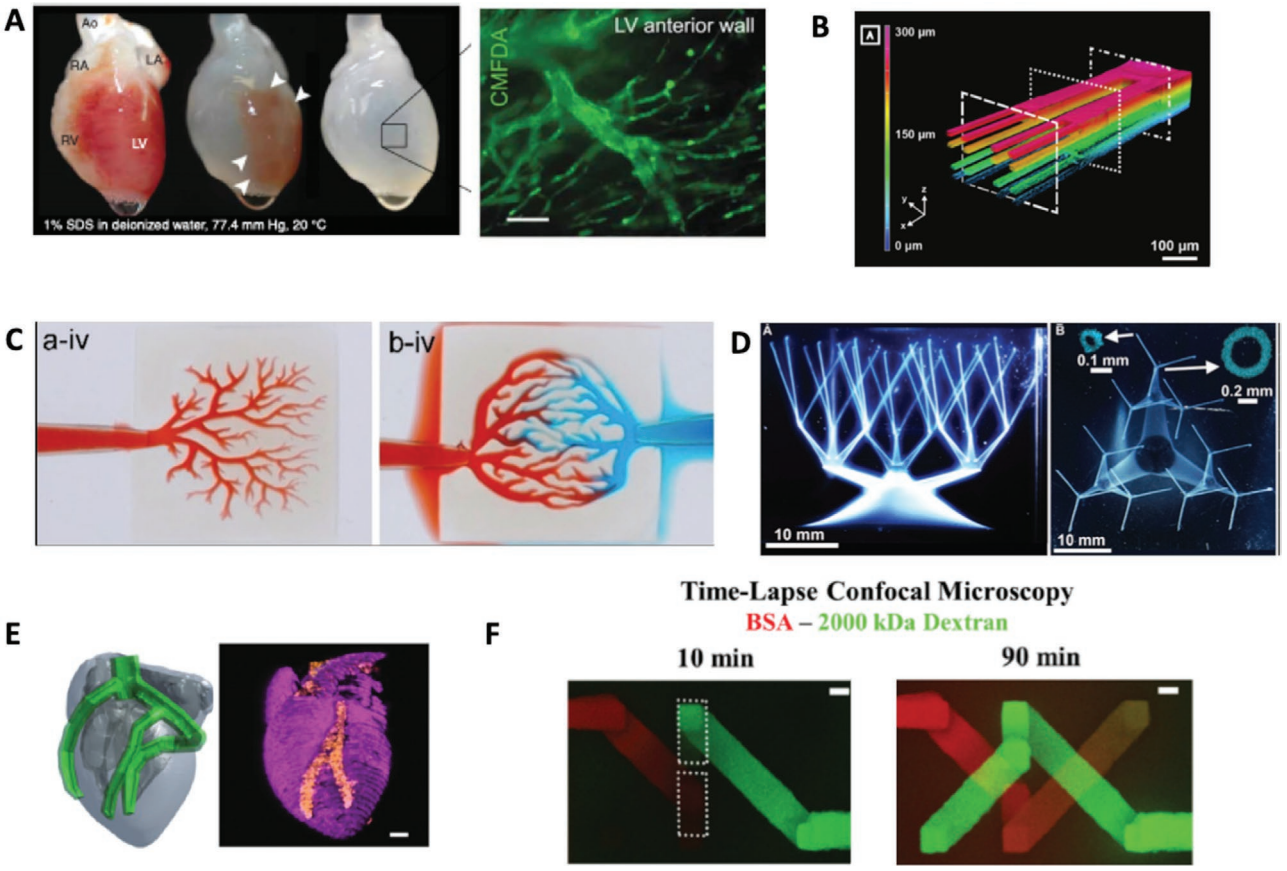

Figure 6. Overview of approaches for engineering complex and multiscale vasculature. A) Decellularized (left) and reendothelialized (right) whole heart. Reproduced with permission.[145] Copyright 2008, Springer Nature Limited. B) Hierarchical vasculature generated by programmable photodegradation. Reproduced with permission.[83] Copyright 2017, Wiley-VCH. C) Projection based 3D printing of multiscale channels. Reproduced with permission.[147] Copyright 2018, American Chemical Society. D) 3D printed hierarchically branched tubular networks. Reproduced with permission.[150] Copyright 2015, AAAS. E) 3D printed perfusable model of the heart. Reproduced with permission.[3b] Copyright 2019, Wiley-VCH. F) Transport between 3D intertwining microchannels generated by laser degradation. Reproduced with permission.[6d] Copyright 2016, Wiley-VCH.

blood vessel organoid into mice led to further differentiation and the formation of a vascular tree composed of arteries, arterioles, capillaries, and venules.[6a] These organoids could contribute to understanding how cell organization and paracrine signaling drive patterns of mesoderm development and vascular specification. This knowledge could then be harnessed to develop advanced biomaterial systems to promote the organization of hierarchical vascular networks within 3D tissues.

Direct extrusion into a granular gel could be used to print finely detailed multidimensional structures.[3a,150] For example, multiscale tubular networks composed of 40 connected vessels and 12 junctions have been fabricated by extruding PVA within a slurry of Carbopol particles and subsequently crosslinked.[150] The laboratories of Tal Dvir and Adam Feinberg recently reported on the fabrication of 3D heart models with complex vasculature by direct extrusion. Patency of the printed vessels was demonstrated by perfusion.[3b,6b] Importantly, while these studies demonstrated major advancements in the fields of tissue engineering and bioprinting, the capability to fabricate microscale vasculature using this technique is limited.

Another major hurdle hampering the clinical translation of these techniques is generating tissues with physiologically relevant cell densities (1×10^8 cells cm^{-3}) while maintaining stabilized and patent vascular networks. To address this challenge, Jennifer Lewis and colleagues recently reported on the development of a new 3D printing technique utilizing highly dense constructs via embryoid body aggregation within ECM bioinks. They patterned a sacrificial ink within the constructs to form vascular structures and demonstrated that upon its removal they were able to generate perfusable tissue constructs and maintain their functionality for over 7 d in culture.[151]

In the body, tissues usually contain intertwined distinct vascular networks, creating complex transport regimes. For example, most tissues contain arterial networks for oxygen and nutrient delivery as well as venous and lymphatic networks, which are responsible for waste and carbon dioxide removal. To mimic the close interaction between the cardiovascular and lymphatic system, Heintz and colleagues have utilized laser-degradation methods to fabricate two independent intertwining networks that allow internetwork transport, unlocking previously limited design freedom.[6d] Similarly, in the lung, the circulatory and pulmonary systems are entangled but physically distinct. Recently, Grigoryan and colleagues used stereolithography to reconstruct a 3D entangled vascular network within

hydrogels to generate a perfusable model of an alveolar sac. They investigated the transport of oxygen from a source vessel to deoxygenated red blood cells which were perfused in a separate channel located 300 μm away. After ventilating the source channel with gaseous oxygen, red blood cells became oxygenated, suggesting functionality of the model.[152] Importantly, this study represents a milestone in the field, enabling to generate intervascular networks with unprecedented functionality.

6. Towards Clinical Translation of Vascularized Tissues

An impressive spectrum of complex fabrication techniques and advanced engineering solutions has been developed over the past few years to enable and promote tissue vascularization. While tremendous progress has been achieved, there are still quite a few challenges that must be addressed toward clinical translation of large-scale engineered tissues.

To date, most preclinical and clinical studies relied on the ability of the host vasculature to either sprout into the engineered tissue and vascularize it or to anastomose to preexisting engineered vasculature. In a study that utilized an in vitro angiogenesis model and time-series microscopy, it was suggested that the rate of angiogenesis sprouting is ≈5 μm h^{-1}.[153] This rate is too slow to provide a thick, metabolically active tissue with the necessary nutrient and oxygen exchange, and the tissue would necrose before an intricate dense network would form.

To overcome the reliance on the rate of spontaneous anastomosis, direct surgical anastomosis to the host vascular supply will most likely be critical. Direct surgical perfusion of vascularized tissues has previously been reported by the use of resected arteries and veins, yet this strategy is invasive and requires multiple interventions.[154] In a more recent study, Zhang and colleagues developed a multilayered scaffold containing a synthetic fluidic network connected to a single inlet and outlet that was able to establish immediate perfusion on implantation; however, patency rates were not determined.[84] Moving forward, it will be important to harness the methods and knowledge acquired in the development of tissue engineered small-caliber vessels, such as material selection, surface functionalization, and cell seeding to reduce the risk for thrombosis, intimal hyperplasia, and inflammation and improve host integration and vascular remodeling within the engineered tissue. In addition, it would be interesting to investigate how a host's luminal fluid flow will affect the formation of a mature vascular network in vivo, and if it will exhibit appropriate vasodilation and vasoconstriction properties.

For ideal oxygen and nutrient delivery, engineered tissues require a dense network of microscale capillaries placed within <100 μm from each other. A large scale vessel capable of surgical anastomosis does not efficiently provide nutrients such as capillary beds and surgical anastomosis of microvessel networks is not feasible. Therefore, the solution for direct and rapid integration with host vasculature and efficient oxygen and nutrient exchange will most likely require the recapitulation of the structural hierarchy of the vascular tree within engineered tissues. Although a few studies have recently attempted to generate multiscale vasculature on certain levels, generating such a complex structure in vitro, with the scales that span over several orders of magnitude with functional vascular specifications, has not been achieved yet and will require new translational solutions. Capturing this complexity will most likely benefit from the integration of engineering-based approaches to generate predefined large and mesoscale vessels followed by environmental signals to stimulate dense microscale capillary bed formation.

In vitro models of angiogenesis and vasculogenesis have taught us that ECs are capable of sprouting from predefined lumens and self-assembling within hydrogels into complex perfused microvasculature which could ultimately bridge between multiple tissue sources.[80,96,97,99,138a] Stevens et al. showed that patterning endothelial channels along hepatocyte aggregates in an engineered liver tissue was essential for establishing perfusion in vivo; once implanted, these engineered constructs with microvascular networks were able to restore liver function in mice with liver injury, as well as grow over 50-fold in size, demonstrating their ability to bridge early regeneration in vivo.[155] Importantly, these studies provide a proof-of-concept that the convergence of bottom-up and top-down approaches can be harnessed to mimic the multiscale nature of the vasculature. Further investigation into the scaling-up of these techniques will be required. Criteria such as spatial organization of the predefined vessels, cellular composition, and type of stimulating factors will need to be defined to ensure the formation of a functional vascular network with distinct architectures.

The hierarchal nature of the native vascular tree allows distribution of blood to the entire body while minimizing the energy cost. To recapitulate this capability, the diameters of the parent and daughter vessels can be calculated and allocated according to Murray's law.[156] While previous studies have not reported on impaired vascular function in engineered tissues as a result of atypical branching, in-depth investigation into the role of hemodynamic forces to support vascular integration, stabilization, and guided vessel specification will be necessary to rationally design complex vasculatures.

Bottom-up approaches usually result with the formation of microvessel networks that exhibit a dense torturous architecture, typical to tumor vasculature in vivo. While this could be advantageous to certain organs with high metabolic demand such as the liver, its use in organs such as the heart and skeletal muscle, where highly aligned vasculature is found, might be limited. Previous studies have demonstrated the importance of vascular geometry in promoting anastomosis and improving tissue integration to host vasculature.[6c,120] Therefore, further high-resolution fabrication strategies should be developed to control the architecture of the microscale capillary beds connecting larger predefined vessels. For example, integration of image-guided laser positioning and laser induced degradation or laser scanning lithography can be harnessed to pattern specific structures with high fidelity resolution to form lumens or immobilize cell adhesion peptides. Furthermore, with advancements in high resolution microscopy and high-level image processing techniques, patient-specific blue-prints of the defected organ can be generated to fabricate vascularized tissues that could precisely match the patient's vasculature.

Microvasculature embedded within engineered tissues needs to form a stable, mature, and functional endothelium that will serve as a selective barrier for transport of cells and soluble factors.[10] Recently, a vessel-on-a-chip was made by vascular self-assembly, recapitulating physiological paracellular and transcellular transport pathways through the endothelium.[157] Qiu and colleagues reported on the microvasculature-on-a-chip that was able to not only maintain barrier function for months but also to restore it after injury.[2a] However, achieving this level of functionality in large-scale tissues, with comprehensive vasculature, will require the use of more sophisticated engineering strategies. Further maturation could be achieved by the recruitment of perivascular cells for support and establishing ECM for maintained stability.[80,99,138b] Similar to maturation protocols utilized in engineered large blood vessels, advanced bioreactors must be developed to mature meso- and microscale vasculature. In addition, further development of imaging technologies to enable online monitoring of vascular formation and maturation in vivo will be highly beneficial to better understand these processes and recapitulate them in vitro.[158]

Future engineered tissues will most likely contain various cell types generated from autologous or matched iPSCs. The success of this approach will rely on the maturation level and commitment of the iPSC-derived populations, and their ability to withstand dedifferentiation postimplantation. Current iPSC differentiation protocols result in nonspecialized ECs with insufficient levels of maturity.[69d,e] Therefore, development of advanced iPSC differentiation protocols, for both endothelial and supportive populations, will be essential in promoting vascular network maturation for patient-specific applications.

Lastly, there are marked differences in endothelium phenotypes in different tissues.[4b] Therefore, each type of engineered tissue will most likely require a specialized endothelium that will fulfill tissue-specific requirements. For example, a fenestrated or discontinuous endothelium that will enable fast and efficient filtration and secretion will be necessary for engineered kidney or liver tissues, while a continuous, highly selective barrier will be crucial in tissues such as the heart, lung, or brain. Recently established differentiation protocols have demonstrated first attempts at generating tissue-specific ECs such as brain, corneal, and liver sinusoidal ECs.[69f,159] In addition, some groups have attempted specification of iPSC-derived endothelial cells into arteriole versus venous lineages which may be important for large- and small-caliber vessel applications.[139a,160] Perhaps tissue-specific phenotypes can be acquired over coculture with multiple cell types; until further characterization of iPSC-ECs in multicellular constructs is widespread, these specifications need to be validated prior to implantation. Going forward, the field will need to scale-up these advances toward directed specialization of tissue-specific hierarchical networks in thick 3D tissues.

7. Summary

Engineering large-scale vessels has been a central challenge for the tissue engineering community for many years. From various fabrication methods to cell sources, efforts in engineering large vessels have already reached early stages of clinical translation. In engineering meso- and microvasculature, the focus has turned to using more advanced biofabrication technologies to enable higher structural complexity and resolution. Many groups have shown remarkable efforts in modeling the meso- and microvasculature in health and disease, transforming the fields of vascular biology and applications in drug development. In tandem, though novel bioengineering approaches have enabled to vascularize engineered tissues, major roadblocks still prevent clinical translation of these tissues. Future efforts must harness a more comprehensive understanding of fundamental vascular biology, as well as innovations in material science, engineering, and stem cell biology, to develop engineered tissues with functional and multiscale vasculature that could be immediately perfused upon implantation and efficiently supply oxygen and nutrients.

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Conflict of Interest

The authors declare no conflict of interest.

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