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Vascular tissue engineering: progress, challenges, and clinical promise

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Abstract

Although the clinical demand for bioengineered blood vessels continues to rise, current options for vascular conduits remain limited. The synergistic combination of emerging advances in tissue fabrication and stem cell engineering promises new strategies for engineering autologous blood vessels that recapitulate not only the mechanical properties of native vessels but also their biological function. Here we explore recent bioengineering advances in creating functional blood macro and microvessels, particularly featuring stem cells as a seed source. We also highlight progress integrating engineered vascular tissues with the host following implantation, as well as the exciting pre-clinical and clinical applications of this technology.

Introduction

Ischemic diseases, such as atherosclerotic cardiovascular disease (CVD), remain the leading cause of mortality and morbidity across the world (GBD 2015 Mortality and Causes of

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Author Contributions

HHGS, RTR, and CSC: Wrote and edited the paper; CKO and ERE: Edited the paper.

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Death Collaborators, 2016; Mozaffarian et al., 2016). These diseases have resulted in an ever-persistent demand for vascular conduits to reconstruct or bypass vascular occlusions and aneurysms. Synthetic grafts for replacing occluded arterial vessels were first introduced in the 1950's following surgical complications associated with harvesting vessels, the frequent shortage of allogeneic grafts, and immunologic rejection of large animal derived-vessels. However, despite advances in pharmacology, materials science, and device fabrication, these synthetic vascular grafts have not significantly decreased the overall mortality and morbidity (Nugent and Edelman, 2003; Prabhakaran et al., 2017). Synthetic grafts continue to exhibit a number of shortcomings that have limited their impact. These shortcomings include low patency rates for small diameter vessels (<6mm in diameter), a lack of growth potential for the pediatric population, necessitating repeated interventions, and the susceptibility to infection. In addition to grafting, vascular conduits are also needed for clinical situations such as hemodialysis, where several times a week for several hours, large volumes of blood must be withdrawn and circulated back into a patient.

In addition to large scale vessel complications, ischemic diseases also arise at the microvasculature level (<1mm in diameter), where replacing upstream arteries would not address reperfusion needs of downstream tissues (Hausenloy and Yellon, 2013; Krug et al., 1966). Microvascularization and perfusion have proven to be a critical step during regeneration and wound healing, where the delay of this process (in diabetic patients, for example) significantly slows down the formation of the granulation tissue and can be a risk factor for severe infection and ulceration (Baltzis et al., 2014; Brem and Tomic-Canic, 2007; Randeria et al., 2015).

In order to design advanced grafts, it is important to take blood vessel structural components into consideration, as understanding these elements is required for rational biomaterial design and choosing an appropriate cell source. Many of the different blood vessel beds also share some common structural features. Arteries, veins and capillaries are all trilaminate with tunica intima comprised of endothelial cells (EC), which regulate coagulation, confer selective permeability, and participate in immune cell trafficking (Herbert and Stainier, 2011; Potente et al., 2011). Arteries and veins are further bound by a second layer, the tunica media, which is composed of smooth muscle cells (SMC), collagen, elastin and proteoglycans, conferring strength to the vessel and acting as effectors of vascular tone. Arterioles and venules, which are smaller caliber equivalents of arteries and veins, are comprised of only a few layers of SMCs, while capillaries, which are the smallest vessels in size, have pericytes abutting the single layer of ECs and basement membrane. Vascular tissue engineering has evolved to generate constructs that incorporate the functionality of these structural layers, withstand physiologic stresses inherent to the cardiovascular system, and promote integration in host tissue without mounting immunologic rejection (Chang and Niklason, 2017).

A suitable cell source is also critical to help impart structural stability and facilitate in vivo integration. Patient-derived autologous cells are one potential cell source that has garnered interest because of their potential to minimize graft rejection. However, isolating and expanding viable primary cells to a therapeutically relevant scale may be limited given that patients with advanced arterial disease likely have cells with reduced growth or regenerative

potential. With the advancement of stem cell (SC) technology and gene editing tools such as CRISPR, autologous adult and induced pluripotent stem cells (iPSCs) are emerging as promising alternative sources of a variety of cell lineages including ECs and perivascular SMCs that can be incorporated into engineered vasculature (Chan et al., 2017; Wang et al., 2017).

Importantly, a viable cell source alone is not sufficient for therapeutic efficacy. Although vascular cells can contribute paracrine factors and regenerative capacity, simply delivering a dispersed mixture of ECs to the host tissue has shown limited success at forming vasculature or integrating with the host vasculature (Chen et al., 2010). Therefore, recent tissue engineering efforts have instead focused on recreating the architecture and the function of the vasculature *in vitro* before implantation, with the hypothesis that pre-vascularized grafts and tissues enhance integration with the host. In this review, we explore recent advances in fabricating blood vessels of various calibers, from arterial vessels to vascular beds comprised of microvessels, and how these efforts facilitate the integration of the implanted vasculature within a host. We also discuss the extent to which SC-derived ECs and SMCs have been incorporated into these engineered tissues.

Engineering of Arterial Vessels

Within the vasculature hierarchy, blood vessels can markedly vary in size. Large vessels (arteries and veins) are arranged to ensure efficient transport through the lumen to sites far afield, while smaller vessels (capillaries) are arranged for optimal transmural exchange of nutrients, oxygen, and waste. Not surprisingly, design requirements and approaches for engineering large vessels have been distinct from those in making smaller-caliber vessels less than 1mm in diameter, such as arterioles and capillaries. For large vessels, tissue engineers are particularly interested in arteries (>6 mm in diameter with 1 mm wall thickness), since occlusions due to atherosclerosis or embolism usually occur at this level, leading to end-organ ischemia.

Early Tissue-Engineered Blood Vessels

Since the 1950s, vascular grafts constructed from synthetic polymer materials like polyethylene terephthalate (PTFE) could be clinically used as conduits for large diameter arteries but complications arose when they were used for smaller vessels (DeBakey et al., 1965).This issues initiated the development of tissue-engineered blood vessels (TEBV) as an alternative (Canver, 1995; Chard et al., 1987).

Weinberg and Bell reported the first TEBV that mimicked all three arterial histologic layers., They constructed a vessel by seeding cultured bovine aortic ECs, SMCs, and adventitial fibroblasts on a collagen and thin Dacron mesh scaffold (Weinberg and Bell, 1986). They then cast the medial layer with collagen and SMCs mixed with culture media, lined the cast with adventitial fibroblasts in Dacron mesh for mechanical support, and lined the lumen with ECs. This process formed a contiguous endothelial lining that offered a large-molecule permeability barrier and was noted to be biochemically active with von Willebrand factor and prostacyclin production. However, only 120 to 180 mmHg burst strength was achieved in comparison to the physiologic burst strength of approximately 2000 mmHg and 3000

mmHg in human saphenous vein and internal mammary artery respectively (Konig et al., 2009; Yow et al., 2006).

To leverage the mechanical strength of synthetic grafts in conjunction with the ability of ECs to modulate vascular response to injury, Herring et al. reported the first EC-lined synthetic graft. The authors seeded a 6 mm Dacron graft with canine venous EC suspensions and observed a 76% patency rate vs 22% in controls (Herring et al., 1979). Seed cell yields were improved using crude whole vein homogenates, omental adipose tissue microvasculature, and other xenogeneic sources (Noishiki et al., 1990; Pennell et al., 1986; Williams and Wick, 2004).

The ability to withstand physiologic pressures without employing a synthetic mesh within the scaffold, however, remained a mystery until L'Heureux et al. reported the construction of a completely biodegradable TEBV in 1998 (L'Heureux et al., 1998). The authors constructed a trilaminate TEBV by inducing collagen production from human umbilical vein SMCs and human skin fibroblasts. The TEBV was seeded with ECs to line the lumen and it displayed a burst strength of nearly 2600 mmHg, but with a patency that lasted only up to 7 days in the best three out of six constructs.

Fabrication Techniques

The main criteria for engineering a long-lasting artery include high compliance/tensile mechanical properties, low thrombogenicity, and regenerative remodeling that ultimately leads to successful integration (Dimitrievska and Niklason, 2017). After immediately branching off the aorta, arteries must be compliant enough to accommodate flow with highly pulsatile pressure and tensile enough to prevent rupture. Once grafted, the implanted vessel must survive the host's response that often results in inflammation and the formation of thrombus and graft failure. Finally, remodeling events are needed in order to encourage regenerative integration with the host tissue, including 1) EC migration and formation of cell-cell contact between host and graft ECs, 2) blurring the host-graft interface via SMC migration across the interface, and 3) reestablishment of mechanical homeostasis through circumferential graft ECM rearrangement (Huang and Niklason, 2014; Roh et al., 2010; Sapoznik et al., 2015). The overview of engineering arterial vessels is illustrated in Figure 1.

Source of biomaterials—Collagen and elastin, which are the two major components of an artery responsible for its characteristic mechanical properties, have been widely used as the biomaterial backbone of the graft (Miranda-Nieves and Chaikof, 2017). Usually, these natural ECMs are harvested from xenogenic, digested ECMs that can be triggered to re-crosslink by adjusting temperature and pH (Stenzel et al., 1974) or by adding aldehydes or crosslinking enzymes (Delgado et al., 2017; Makris et al., 2014). For collagen, microscopic orientation and fibrillar thickness dictate the macroscopic mechanical properties of the ECM and the behavior of embedded cells (Doyle et al., 2015; Wolf et al., 2013). Therefore, significant efforts have been invested in exploring different techniques such as electrospinning, stretching, and microfluidics to gel collagen with some degree of control over orientation. Alternatively, ECM materials can also be sourced from cells cultured in vitro. Fibroblasts in particular, are known for their capacity to deposit a variety of ECM such

as collagen, elastin, and fibronectin (Kutys et al., 2013). This method takes advantage of the native composition and architecture of ECM components that has induced physiologically relevant cell behavior (Cukierman, E., Pankov, R., Stevens, D. R., Yamada, 2001).

Synthetic polymer materials that are biocompatible, non-immunogenic, and degradable are also being explored for use as scaffolds, including polyglycolic acid (PGA), polycaprolactone (PCL), poly-L-lactide (PLLA), and mixtures thereof. In these materials, the mechanical strength of the resulting graft is dependent on not only the initial mechanical properties of the composite material but also the new collagen that is produced and deposited by seeded cells such as fibroblasts and/or SMCs.

Engineering vascular tubes—Creating tubular structures with biomaterials involve three commonly used methods: sheet rolling, tubular molding, and direct scaffolding. In sheet rolling, a sheet of the desired biomaterial with or without cells is fabricated and then rolled over a mandrel into a tubular structure. In the presence of cells, the sheet layers within the tube are remodeled to a more homogenous structure that prevents unrolling of the sheet. For acellular grafts, a bonding layer can be added on top of the sheet to help the graft stay tubular. As utilized by Weinberg and Bell, the tubular molding method shapes materials that are injected and crosslinked inside the annular molds. Syedain et al. recently generated a cell-derived graft that was molded from a glass annular mold and showed potential in a lamb artery replacement model (Syedain et al., 2016). For most synthetic materials, polymer materials are directly formed into tubular scaffolds by physical carving or electrospinning on the surface of a rotating cylinder.

Grafts can also be sourced from decellularized xenogenic tissues, often from pigs. The advantage of decellularization over reconstitution of soluble ECMs or synthetic materials is that the native architecture of the vessel ECMs is largely preserved and may provide a more advanced starting point for vessel remodeling. The idea of using non-human tissues has appealed to the tissue engineering field due to their availability compared to human tissues. However, severe and sometimes fatal immune responses to xenogeneic cells and materials such as galactose-alpha-1,3-galactose (alpha-gal) has significantly hindered progress (Ayala et al., 2017; Galili, 2005). Advances in decellularization techniques have reduced these responses, but complete removal of immunogenic foreign biomolecules still remains as a challenge (Shirakigawa and Ijima, 2017). For example, attempts have been made to inactivate the immunogenic region of foreign molecules such as alpha-gal by crosslinking the tissue with glutaraldehyde, which had shown promise by reducing immunogenicity. However, glutaraldehyde treatment can not only lead to cytotoxicity in the tissues surrounding the graft if not washed out completely, but it can also make grafts more thrombogenic and susceptible to calcification (Roosens et al., 2016; Simionescu et al., 2011).

With the rise of clustered regularly interspaced short palindromic repeats (CRISPR) gene-editing technology, a new momentum has emerged for “humanizing” animals to decrease, and one day completely remove, xenograft immunogenicity. Recently, the Yang and Church groups successfully inactivated all genetic copies of porcine endogenous retroviruses (PERVs), which can integrate within the human genome when infected, causing safety

concerns for xenotransplantation (Niu et al., 2017; Patience et al., 1997; Yang et al., 2015). If a similar approach can inactivate other immunogenic biomolecules or mutate them into human-compatible ones, arterial xenografts could become a feasible reality.

Cell seeding and graft conditioning—One challenge in generating these TEBV is how to distribute cells within the graft, as ECs and SMCs need to locate to the wall surface and "tunica media" space, respectively. Passive or static seeding introduces cells onto the graft by simply allowing a suspension of cells to stochastically sediment onto the surface by gravity. Static methods of seeding can only achieve suboptimal distribution of SMCs across the surface of the graft and rely on cell migration for penetration into the depths of the construct, which in turn is largely dependent on construct porosity (Ravi et al., 2009). A variety of biological glues like fibronectin, collagen, or biomimetic surfactant polymers from heparin-binding sites of fibronectin have offered improved adherence of seeded ECs by mimicking the structure of ECM when coated on the scaffold pre-seeding (Pawlowski et al., 2004; Sagnella et al., 2005; Salacinski et al., 2001).

An alternative to static seeding is dynamic seeding using a rotating technique. Rotational seeding utilizes centrifugal force to insert cells onto scaffolds. Human bladder SMC suspension spun at 2500 rpm with porous PGA scaffolds reportedly have greater seeding efficiency than static or spinner flask techniques and have led to more homogenous deposition (Godbey et al., 2004). Seeding grafts along a pressure gradient, by applying an internal or an external vacuum pressure, has offered an inexpensive alternative with rapid processing and higher seeding efficiency compared to a rotational seeding technique (Nieponice et al., 2008; Williams and Wick, 2004). However, its impact on cellular viability and distortion of normal cellular morphology remains uninvestigated.

Once the graft is fabricated and seeded, a TEBV may not yet possess sufficient strength to support physiologic blood pressures and requires further "maturation" in a bioreactor. A strategy to induce this adaptation involves a pulsatile pump to introduce flow through the inner wall of the graft. In their seminal work, Niklason et al. demonstrated that pulsatile flow is necessary for the SMCs to migrate throughout the graft scaffold (Niklason et al., 1999). External stimuli such as shear stress and matrix stretching also have recently been reported to enhance SMC senescence, matrix deposition, contractility, and differentiation from progenitor cells (Eoh et al., 2017; Kang et al., 2017; Ratz et al., 2015; Ueba et al., 1997). ECs also respond to fluid shear stress and stretching through signaling pathways that regulate vascular barrier function, survival, proliferation, maturation, and even quiescence of neighboring SMCs (Polacheck et al., 2017; Wang et al., 2013, 2016; Zhou et al., 2014). These conditioning responses may further improve vascular graft function and therefore justifies the use of mechanical preconditioning for TEBVs.

Stem Cells as Seed Sources

For vascular tissue engineering, seed cells need to possess an ability to proliferate, avoid an immunologic reaction, be harvestable, and preserve the synthetic functionalities performed by the relevant cell type in native vessels (Rabkin and Schoen, 2002). Historically, autologous vascular cells harvested from primary tissues like vein segments have historically

been a common source. However, there are significant difficulties to using this cell source, including technical harvesting challenges, a reduced proliferative capacity demanding prolonged *ex vivo* expansion, and overall lack of availability in the most prevalent patient demographic who are elderly and have systemic atherosclerotic disease (Seifu et al., 2013). Advancements in stem cell technology have made adult and induced stem cells promising alternatives (Wang et al., 2017).

The ground breaking work of Yamanaka and colleagues demonstrating conversion of adult human fibroblasts into human iPSCs has offered the latter as an exciting source of a variety of cell lineages (Takahashi and Yamanaka, 2006). iPSCs have shown superior proliferative capacity *in vitro* in comparison to both adult primary and stem cells. Studies with embryonic stem cells (ESCs) derived from human ESC lines have laid the groundwork for *in vitro* induction of pluripotent stem cells to differentiate into ECs and SMCs. Selective media coupled with ECM like Matrigel, as well as induction of CD34+ progenitor cells with platelet-derived growth factor (PDGF-BB), retinoic acid (RA), and transforming growth factor β (TGF-β) have promoted differentiation of cells with a SMC phenotype (Hill et al., 2010; Xie et al., 2007). Recently, Sivarapatna et al. demonstrated that exposure to shear stress can further specify iPSC-derived ECs into an arterial lineage as evidenced by upregulation of EphrinB2 and Notch1 (Sivarapatna et al., 2015). Adams et al. also demonstrated phenotypic plasticity of iPSC-derived ECs (permeability, leukocyte binding, and expression of atheroprotective genes) in response to humoral, biomechanical, and pharmacological stimuli, similar to what would occur with adult ECs (Adams et al., 2013). Direct comparison with primary cells was not performed in these studies, but they highlight the amenability of iPSC-ECs to vessel-specific functions and physiological cues.

Not only do stem cells offer a more readily available source of seed cells, they also produce ECM components like collagen and elastin with higher yields in comparison to autologous cells. The limited replicative and ECM productive capacity of adult cells may be a result of telomere shortening and senescent downregulation of ECM production, respectively (Johnson et al., 1995; Sundaram and Niklason, 2012). While ectopic expression of telomerase has been used to reverse telomere shortening and achieve autologous cell proliferation beyond their normal life spans in SMCs and ECs, the accumulated ECM components were noted to be inversely proportional to donor age regardless of telomerase extension (Poh et al., 2005). Additionally, the use of retroviral vectors to induce telomerase expression raises safety concerns pertaining to random oncogene activation (Klinger et al., 2006).

In a recent report, Gui et al. utilized tubular PGA scaffolds seeded with SMCs differentiated from human iPSCs that revealed an abundance of collagen deposited by the iPSC-SMCs, and the resulting TEBV was mechanically strong enough to be surgically sutured as a graft *in vivo* (Gui et al., 2016). However, it could only withstand up to 500 mmHg of burst pressure after 8–9 weeks of culture and dilate *in vivo* over the period of 2 weeks, suggesting that further differentiation or maturation is needed, perhaps by conditioning the grafts with pulsatile flow and mechanical stretching before subjecting them to in vivo conditions.

Clinical Applications

The first reported successful clinical application of TEBV in patients was performed by Shin'oka et al., who implanted a biodegradable construct as a pulmonary conduit in a child with pulmonary atresia and single ventricle anatomy (Shin'oka et al., 2001). The construct was composed of a synthetic polymer mixture of L-lactide and e-caprolactone, and it was reinforced with PGA and seeded with autologous bone marrow-derived mesenchymal stem cells (BM-MSCs). The authors demonstrated patency and patient survival 7 months post-implant, and expanded their study to a series of 23 implanted TEBVs and 19 tissue patch repairs in pediatric patients with single ventricle physiology that were noted to have no graft-related mortality and four patients requiring interventions to relieve stenosis at a mean follow-up of 5.8 years (Hibino et al., 2010). The first sheet-based technology to seed cultured autologous cells, developed by L'Heureux et al., was iterated by the group to induce cultured fibroblast cell sheet over a 10-week maturation period and produce tubules of endogenous ECM over a production time ranging between 6 and 9 months. They dehydrated and provided a living adventitial layer before seeding the constructs with ECs (L'Heureux et al., 2006). Their TEBV, named the Lifeline graft, was implanted in 9 of 10 enrolled patients with end-stage renal disease on hemodialysis and failing access grafts in a clinical trial. Six of the nine surviving patients had patent grafts at 6 months, while the remaining grafts failed due to thrombosis, rejection and failure each (McAllister et al., 2009). An attempt to create an "off the shelf" version of this graft where prefabricated, frozen scaffolds were seeded with autologous endothelium prior to implantation led to two of the three implanted grafts failing due to stenosis and one patient passed away due to graft infection (Benrashid et al., 2016).

Most recently, the decellularized engineered vessel, Humacyte® reported results of their phase II trial in end-stage renal disease patients surgically unsuitable for arterio-venous fistula creation (Lawson et al., 2016). This clinical scenario offers a relatively captive patient population where graft complications are unlikely to be limb or life threatening, and infectious and thrombotic event rates for traditional materials such as ePTFE are high (Haskal et al., 2010). The manufacturers seeded a 6mm PGA scaffold with SMCs from deceased organ and tissue donors, and decellularized the scaffold following ECM production in an incubator coupled with a pulsatile pump prior to implantation. Humacyte® demonstrated 63% primary patency at 6 months, 28% at 12 months, and 18% at 18 months post-implant in 60 patients. Ten grafts were abandoned. However, twelve-month patency and mean procedure rate of 1.89 per patient-year to restore patency were comparable to PTFE grafts, while higher secondary patency rates were observed (89% vs 55-65% at 1 year) (Huber et al., 2003; Lok et al., 2013). Although Humacyte® revealed no immune sensitization, and a lower infection rate than PTFEs (reported up to 12%) (Akoh and Patel, 2010), there remains much work to be done to improve primary patency and reduce the need for interventions.

Harnessing the regenerative functions reported in ECs derived from adult stem cells and iPSCs offer the promise of improving TEBV patency. Mcllhenny et al. generated ECs from adipose-derived stromal cells, transfected them with adenoviral vector carrying the endothelial nitric oxide synthase (eNOS) gene, and seeded the ECs onto decellularized

human saphenous vein scaffolds (McIlhenny et al., 2015). They hypothesized that through inhibition of platelet aggregation and adhesion molecule expression, NO synthesis would prevent thrombotic occlusion in TEBV. Indeed, they reported patency with a non-thrombogenic surface 2 months post-implantation in rabbit aortas. While introducing additional complexities, engineering ECs and SMCs with other regenerative, anti-inflammatory, anti-thrombotic genes could perhaps bridge the functional difference between SC-derived cells and native primary cells.

Engineering of Microvessels

Biomaterial Source

Small vessels like arterioles, venules, and capillaries have diameters in the sub-millimeter scale, and thus present a different set of challenges for bioengineers. Fabrication methods for larger arteries are not applicable for micro-vessels, in part because the clinical need is not for single tubes of smaller vessels but instead a network of a nutritive (high endothelium surface-area-to-volume ratio) vascular “bed”. In delivering a network of microvessels, hydrogels or scaffolds are used to provide regenerative cues that induce formation and maintenance of the vascular bed, as well as encase and retain the network of engineered microvessels at the target implant site.

For similar reasons as discussed in the arterial vessels section, natural materials such as collagen and fibrin are commonly used for engineering microvessels. In particular, fibrin, one of the main components of the provisional matrix in wound healing, has been used widely due to its naturally angiogenic properties (reviewed in depth recently by Ceccarelli and Putnam) (Ceccarelli and Putnam, 2014). Other naturally derived materials such as hyaluronic acid, dextran, agarose, and gelatin (Burdick and Prestwich, 2011; Hanjaya-Putra et al., 2012; Park and Gerecht, 2014; Trappmann et al., 2017), and synthetic materials such as polyethylglycol (Cuchiara et al., 2012) have been chemically remodified to support vascularization and even make the material more angiogenic. For example, Li et al. recently developed a hyaluronic acid-based hydrogel chemically modified with fibronectin motifs that specifically promotes EC binding of $\alpha 3/\alpha 5\ \beta 1$ integrins, and this resulted in better vascularization compared to a non-modified hydrogel in a mouse stroke model (Li et al., 2017). Biomaterials that are similarly inspired to support microvascular function have great potential to accelerate the transition away from xenogenic materials for clinical application.

Top-down fabrication

Recent approaches in creating microvasculature can be divided into two basic strategies: top-down and bottom-up (Figure 2). In the top-down approach, the resulting geometry and architecture of the vascular bed are pre-designed and pre-fabricated before cells are introduced. Much effort has been spent on developing scalable 3D fabrication technologies that can pattern biomaterials at a high resolution.

3D Printing—Three-dimensional printing has garnered increasing attention because of its potential for high scalability. Early strategies utilized cellular 3D printing, where a tissue is printed with “bio-ink” composed of a suspension of living cells in viscous pre-polymer

(Mironov et al., 2009; Visconti et al., 2010). Though this may be adequate for creating tissues with a more-or-less homogenous cell population, creating microvessels using this technique has proven to be difficult because of the low resolution (hundreds of micrometer voxels) and low cell viability (due to shear damage from ink extrusion) of the technique. Several attempts demonstrated that the overall perfusability and function of the resulting vessels remain poor (Norotte et al., 2009; Skardal et al., 2010).

Recent strategies have focused on developing acellular “sacrificial” materials, or fugitive inks, which can be printed with fine resolution into filamentous network structures and then later removed from the construct (by dissolution) to create microfluidic channels where the ECs can later be seeded (Figure 2Ai). Use of sacrificial 3D printing as a vascular fabrication platform was first demonstrated by Miller et al. with a water-soluble sugar ink (Miller et al., 2012). In this work, the 3D schematic of the vascular architecture is first printed with carbohydrate glass material, which can be fabricated to diameters as small as 150μm. Once hardened, this lattice is uniformly embedded in a soluble ECM which crosslinks around the print. The sacrificial print is then dissolved and flushed with water, which then leaves hollow microfluidic channels. ECs can then be seeded on the wall of the channels, creating a final vascular bed. Other sacrificial materials have improved on this initial work, but the overall workflow has generally been similar (Bertassoni et al., 2014; Kolesky et al., 2014). This method is particularly attractive due to its flexibility in accommodating a variety of stromal/parenchymal cell type as well as ECM material. Recently, Kolesky et al. utilized sacrificial printing to generate an interconnected vascular network that perfuses throughout a thick (centimeter-scale) engineered construct of human MSCs and human fibroblasts in fibrin (Kolesky et al., 2016). With continuous perfusion of growth factors through the printed, human umbilical vein endothelial cell (HUVEC)-lined vasculature, they successfully differentiated the stem cells into an osteogenic lineage, demonstrating feasibility and scalability of the technology to vascularize an engineered tissue that has the potential to replace diseased tissue. In some cases, stem cells also offer the advantage of acting as a source of trophic factors to induce host vascular cell migration to endothelialize 3D bioprinted tissues post-implantation. For example, Skardal et al. bioprinted amniotic fluid-derived cells and BM-MSCs in fibrin-collagen gel that endothelialized post-implantation at full-thickness skin wounds in nu/nu mice (Skardal et al., 2012).

In addition to using stem cells as a source of parenchyma, they are also being investigated for their potential to contribute as vascular cells within 3D-printed microvasculature. Abaci et al. employed alginate as a sacrificial mold that could be washed with a sodium citrate solution once embedded in collagen gel containing neonatal human dermal fibroblasts (Abaci et al., 2016). Once washed, the hollow channels were seeded with CD31+ VECad+ ECs differentiated from iPSCs through activation of WNT pathways. Skin constructs seeded with HUVECs and iPSC-ECs both established an endothelial barrier function with similar vascular permeability to 70kDa dextran (0.045 and 0018 μm s^{-1}, respectively) that were significantly higher than reported permeability of rodent microvessels (0.008 μm s^{-1}) (Corovic et al., 2015). The authors do not discuss potential reasons for the disparity, but this could be due to many factors including the absence of mural cells such as pericytes and SMCs, difference in endothelial lineage (venous vs. microvascular ECs), and lab-to-lab variance. Regarding the endothelial lineage, ECs of different organs are known to have

different molecular markers and barrier functions (Potente and Mäkinen, 2017), but the precise molecular mechanism of organ-specific barrier regulation is largely unknown.

Laser-degradation—A vascular network can also be fabricated top-down by selectively degrading regions within a bulk crosslinked ECM (Figure 2Aii). In this method, a biomaterial with or without cells is first crosslinked into a hydrogel, and a laser is scanned in a pre-designed geometry to locally degrade the material (Brown et al., 2017). Termed "laser-degradation," this technique was first used for vascular patterning by Heintz et al. The authors patterned a photodegradable material with a structure that was derived from scanned *in vivo* cerebral cortex vasculature (Heintz et al., 2016). They reported that channels could be fabricated with diameters as small as 3μm, which is in the scale of the smallest physiologic capillary vessels. However, they experienced challenges flowing HUVECs through these channels without clogging, and the smallest fabricated microchannel that they could flow and seed with ECs was about 50μm in diameter. Nonetheless, the strategies provide attractive possibilities.

Layer-by-layer fabrication—In a layer-by-layer technique, the pre-designed, 3-dimensional structure is fabricated additively (Figure 2Aiii). Often, each layer is fabricated separately through lithography, and all the components are aligned and fixed together one-by-one mechanically (Morgan et al., 2013; Zheng et al., 2012) or chemically through UV crosslinking (Zhang et al., 2016). For example, Zhang et al. recently reported their top-down vascular scaffold made with a degradable synthetic polymer, poly-(octamethylene maleate (anhydride) citrate) (POMaC) (Zhang et al., 2016). The scaffold contains microfluidic channels that branch out from an inlet channel and merge into an outlet channel like the hierarchical vasculature found in the body. These structures were fabricated by photo-crosslinking POMaC layer-by-layer in poly(dimethylsiloxane) (PDMS) silicone molds. Each layer was then bonded together to create hollow channels where ECs can flow through and eventually attach.

Endothelial Characterization—These top-down approaches have shown that the seeded ECs eventually spread and form a monolayer along the walls of the microfluidic channel. Some characterization of the engineered vasculature's barrier function was performed by observing the morphology of cell-cell junctions, such as vascular endothelial cadherin, and quantifying the permeability of fluorescently labeled molecules (such as dextran) introduced in the luminal space (Alimperti et al., 2017; Kolesky et al., 2016; Zhang et al., 2016). The polarity of the ECs lining the pre-formed channels has also been assessed by staining for basement membrane proteins deposited by the ECs at the basolateral side (Nguyen et al., 2013). However, other aspects of polarization, such as the placement of certain receptors and transporters, remain unclear in these engineered models. In addition, the ability of the pre-designed vasculature to intrinsically remodel in response to different physical and biomolecular cues after implantation has not yet been explored in depth. Nonetheless, top-down approaches give tissue engineers opportunities to systematically investigate the effect of vascular geometry on the viability of lab-grown tissues and ultimately successful integration with the host.

Bottom-up fabrication

Bottom-up approaches rely on encouraging cells to recapitulate the physiological mechanisms for new vessel formation that occurs during development and tissue regeneration events such as wound healing. In the body, neovascularization occurs in two ways: angiogenesis, or formation of new vessels from a preexisting vessel, and vasculogenesis, or formation of new vessels by self-assembly of individual nearby ECs and/or endothelial progenitor cells that are recruited to the region after injury (Isner and Asahara, 1999; Luttun and Carmeliet, 2014; Song et al., 2014).

Angiogenesis-driven vascularization—Several recent studies have recapitulated angiogenic sprouting *in vitro* using a chemical gradient (Kim et al., 2013; Nguyen et al., 2013) or fluid shear stress (Galie et al., 2014; Song and Munn, 2011). In these mimics, ECs seeded on the walls of parental microfluidic channels collectively migrate toward the chemical gradient as often observed in vivo (Figure 2Bi). Using a similar approach, Nguyen et al. identified an optimal, synergistic cocktail of angiogenic factors which include monocyte chemoattractant protein-1 (MCP-1), VEGF, phorbol 12-myristate 13-acetate (PMA), and sphingosine-1-phosphate (S1P), and demonstrated that the these neovessel sprouts are often lumenized and can be perfused (Nguyen et al., 2013). With this setup and modified dextran hydrogel, we have recently demonstrated that matrix degradability affects the 3D collective migration of sprouting HUVECs during angiogenesis and provides new insights on angiogenic biomaterial design (Trappmann et al., 2017).

In addition to chemical and mechanical stimuli, low oxygen tension also aids vascularization through the regulation of hypoxia inducible factor (HIF) in parenchymal cells (Pugh and Ratcliffe, 2003). Under hypoxia ($pO_2 < 5\%$), the hypoxia-inducible factor (HIF) transcriptional complex is translocated into the nucleus to induce expression of multiple proangiogenic factors, including VEGF, PDGF, Ang and FGF (Krock et al., 2011; Semenza, 2012). Hypoxic tissues can therefore be vascularized and reperfused by ECs. There are also some reports suggesting hypoxia as a promoting factor for differentiation of stem cells and progenitor cells into the endothelial lineage (Bekhite et al., 2014; Kusuma et al., 2014; Ong et al., 2010; Prado-Lopez et al., 2009). To our knowledge, hypoxia has not yet been incorporated in these angiogenesis-driven fabrication platforms for therapeutic applications but has a potential for shortening the differentiation timeframe and increasing the extent of vascularization.

Vasculogenesis-driven vascularization—The second type of bottom-up fabrication is vasculogenesis-driven, which involves self-assembly of ECs and/or endothelial progenitor cells into multicellular networks while forming vacuoles that fuse into a vascular lumen (Davis et al., 2015; Luttun and Carmeliet, 2014) (Figure 2Bii). The ability of ECs to self-assemble into a network structure was first described in 1988 when Kubota et al. described the aggregation of ECs into “capillary-like” structures when grown on a 2D matrigel surface (Kubota et al., 1988). Since then, it has been exploited as a popular, although somewhat inaccurate and incomplete, assay in the vascular biology field to evaluate the angiogenic potential of ECs. Often, cell types such as fibroblasts (Moya et al., 2013; Whisler et al., 2014), pericytes (Kim et al., 2013; Lin et al., 2008), or MSCs (Jeon et al., 2014) are

cocultured with ECs to accelerate self-assembly and prolong the stability of the resulting vasculature. Perivascular cells that reside next to the endothelium and often share the basement membrane regulate vascular barrier function, integrity, and stability through paracrine and adhesive interactions with ECs through cadherins (Alimperti et al., 2017; Bowers et al., 2014; Saunders et al., 2006; Stratman and Davis, 2012). For example, Davis and Camarillo demonstrated improved human EC 3D capillary network and lumen formation using HUVECs embedded in a collagen matrix together with a mesenchymal cell that provides pericyte-like function (Davis and Camarillo, 1996). Chen et al. created vascular networks in a similar manner, but with fibrin gel mixed with HUVECs and human lung fibroblasts (Chen et al., 2009). Perfusability of the network was demonstrated when these networks were implanted in mice and mouse erythrocytes were found within the HUVEC vessels.

While hypoxia has not yet been incorporated in angiogenesis-driven platforms as discussed above, its role in vascular self-assembly has recently been investigated using a vasculogenesis-driven platform. Park and Gerecht developed a gelatin-based hydrogel that induces acute hypoxia and used isolated endothelial progenitor cells to demonstrate that hypoxia induces a HIF-dependent increase in the expression of VEGF, VEGF receptor, Ang, and matrix metalloproteinases (MMPs) that ultimately accelerate vasculogenesis in vitro (Park and Gerecht, 2014). This suggests that vasculogenesis-driven fabrication of a vascular bed can be achieved sustainably by the activation of HIF in ECs without the use of high-dose angiogenic drugs or additional perivascular cell types.

Stem cells have also been used as a source of cells to create microvascular beds from the bottom-up. Kusuma et al. induced differentiation of human iPSCs (hiPSCs) into early vascular cells (composed of VECad+ early ECs and PDGFRβ+ early pericytes) and dispersed them in a synthetic hyaluronic acid-based hydrogel (Kusuma et al., 2013). By day 3, complex vascular networks with patent luminal structures had developed with ECs lining the lumens and pericytes encircling them. Upon subcutaneous implantation in a murine model, the transplanted human vascular network had anastomosed to host vessels, and the hydrogel was mostly degraded by week 2. Samuel et al. also generated endothelial progenitor cells (CD34+ VEGFR2+ NRP1+) from hiPSCs that can form a vascular network and remain stable for 280 days in a mouse cranial model when co-implanted with mouse fibroblasts in collagen (Samuel et al., 2015). These endothelial progenitor cells were also implanted with fibroblasts differentiated from hiPSCs, but the resulting vascular network could only remain stable for about a month, demonstrating the need for further optimization of support-cell differentiation. EC differentiation likely requires additional improvement, considering that the authors' previous study with HUVEC and mouse fibroblast implants lasted almost a year (Koike et al., 2004).

Recent reports have combined the vasculogenesis-driven methods with microfluidics to not only form vascular networks, but also to perfuse these vessels to evaluate their barrier function and interconnectivity as investigated with the vasculature from the top-down methods (Jeon et al., 2014; Kim et al., 2013; Moya et al., 2013; Whisler et al., 2014). In most of these methods, the vasculogenesis process is aided by the presence of added chemical factors (such as VEGF, FGF2, and phorbol ester) and/or supporting cells such as

pericytes or fibroblasts. Belair et al. used commercially available hiPSC-derived ECs in their microfluidics device to demonstrate that these cells can form not only interconnected, but also perfusable vascular networks in vitro when cocultured with primary human lung fibroblasts (Belair et al., 2015).

One of the advantages of using the vasculogenesis-driven bottom-up approach over the angiogenesis-driven approach is its scalability. In vasculogenesis, multiple vessels are formed concurrently all throughout the matrix as opposed to progressing from one region to another. It remains unclear and controversial whether the types of vascular architectures from the bottom-up approaches are beneficial because the resulting vasculature can resemble a dense, “tumor-vasculature-like” structure (Heintz et al., 2016). On the other hand, considering how some organs such as liver are highly vascularized, the bottom-up methods may provide a path to generate high density vasculature at a fraction of the effort used in top-down patterning.

Mechanisms of host-implant anastomosis

Integration of microvessels with the host vasculature poses a challenge distinct from TEBVs in that the vessels are often too small and numerous to be directly sutured into the host. Therefore, most of the microvascular implants have relied on natural anastomoses induced by the host tissue and/or the implanted vasculature.

Effect of cellular & biomolecular composition—In 2002, Levenberg et al. made one of the first observations of inter-vascular connections, when they reported that some of the vessels formed by their human SC-derived ECs seeded within a synthetic scaffold were transporting host blood 14 days post-implantation in a nude mouse (Levenberg et al., 2002). However, anastomosis of implanted vasculature with the host vasculature has mostly been a “black box” with under-investigated mechanisms. Chen et al. suggested that high density of fibroblasts, which are often used to induce endothelial self-assembly, helps accelerate the anastomosis of the co-implanted vasculature, although the precise mechanism of enhancement is still unclear (Chen et al., 2010). Sekine et al. has also reported that addition of basic FGF during the *in vitro* culture of their endothelial vasculature helped the tissue integrate with the host once implanted, although whether this acceleration occurred through the growth factor itself or through enhanced vascularization during *in vitro* culture is unclear (Sekine et al., 2013).

Effect of vascular architecture—Another opportunity anastomosis provides is the mechanistic investigation of the vascular remodeling process in response to physical and/or environmental cues. Hierarchical vascular trees observed in the body has been hypothesized to follow a “minimum energy” principle as described by Murray in 1926 (Kassab, 2006; Murray, 1926). In addition, the luminal fluid flow has proven critical for the vascular plexus to further remodel into an arterial and venous hierarchy (Franco et al., 2015; Lucitti et al., 2007; Sugden et al., 2017). How the vascular remodeling processes occur in the bottom-up engineered vasculature and whether the hierarchical structures can dictate the overall integration and stability of the implanted vasculature are critical topics to investigate.

Using one of the top-down fabrication approaches, we have recently investigated how the pre-defined geometry of the fabricated endothelial arrangement affects the integration of the implant post-surgery (Baranski et al., 2013; Chaturvedi et al., 2015). In a rodent hind limb ischemia model, implants with vessels patterned in a parallel geometry integrated with the host and rescued perfusion better compared with rescue observed with unpatterned implants (Mirabella et al., 2017). Similar results were reported with tissue engineered human liver implants, where implants with parallel-patterned ECs vascularized the implant faster than unpatterned implants, which corresponded with the overall viability of the co-implanted human hepatocytes (Baranski et al., 2013; Stevens et al., 2017). These results suggest the existence of an "optimal" vascular pattern and structure that could be pre-defined to accelerate integration *in vivo*. Further investigation of other structural features, such as vessel diameters, branches, and density, would be informative for designing and fabricating vasculature with the top-down methods.

Insights from vascular biology—Several recent discoveries in the vascular biology field may shed more light on how the vasculature of two different sources connect post-implantation. In a zebrafish model and *in vitro* models using HUVECs and ESC-derived endothelial progenitor cells, anastomosis of two endothelial sprouts or of a sprout and a pre-existing vessel has involved 1) establishment of cell-cell contact via adherin (especially VECad) and tight junctions, 2) polarization of fused tip cells, and 3) blood pressure-driven invagination of apical membranes to form a perfusable vessel (Gebala et al., 2016; Lenard et al., 2013; Sauteur et al., 2014; Szymborska and Gerhardt, 2017). Though the precise mechanism is not identified, anastomoses seems to occur in areas where the target ECs have a low membranous VEGFR1 level (and presumably high VEGFR2 signaling) (Nesmith et al., 2017). Macrophages also interact with ECs to promote anastomosis of two vascular sprouts with mechanisms that still require further investigation (Fantin et al., 2010; Gerri et al., 2017).

Using window chambers at the cranial and dorsal skinfold spaces, Cheng et al. recently demonstrated that self-assembled HUVEC microvessels anastomose with the host vessels via a process termed wrapping-and-tapping, where the implanted ECs first wrap around the preexisting host vessels and replace segments of them to redirect blood flow into the implanted vasculature (Cheng et al., 2011; Samuel et al., 2013). Whether these mechanisms still apply for other implanted tissue-engineered vasculature is unclear, but further investigation could provide potential biomolecular strategies for accelerating *in vivo* integration.

Surgical anastomosis—Some fabrication techniques do allow mechanical anastomosis through sutures. The vascular scaffolds made by Zhang et al. using POMaC polymer (as discussed in a previous section) were surgically integrated with rat femoral vessels using surgical cuffs at the inlet and outlet channels (Zhang et al., 2016). The authors report that most of the vessels inside the scaffold remained clot-free one week after the surgery. However, applications for such implants may be limited to areas with accessible arteries, which may be challenging in certain under-vascularized tissues such as the skin. In addition, such surgical procedures could be more invasive compared to the method described above.

Nonetheless, these results demonstrate that for vascular implants with appropriately sized vessels and strong mechanical properties, direct surgical integration could be an option as well.

Promising Clinical Outlook

Vascular implants containing engineered microvascular beds would be useful for a variety of ischemic conditions arising from microvasculature insufficiencies, such as coronary microvascular disease and diabetic wound healing, and trauma such as burns. In these ischemic instances, the insufficiency is presented microscopically, thus requiring replacement or addition of a vascular bed, as opposed to macroscopic lesions that would require replacement of a diseased large vessel (Mirabella et al., 2017). In addition, such microvasculature could be incorporated into organ-specific tissues engineered *in vitro* to enhance their viability before and after implantation. Compared to TEBVs, engineered microvasculature is still clinically at its infancy where most, if not all, technologies are still at the pre-clinical stage of investigation involving animal models.

ECs derived from stem cells can regenerate and stimulate development of vascular beds in engineered muscle tissue from 3D cultures when mixed with murine myoblasts and embryonic fibroblasts (Levenberg et al., 2005). Once implanted in mice, the constructs continue to differentiate *in vivo* and they permeate host blood vessels. Recently, Chan et al. demonstrated vascular network assembly of endothelial progenitors differentiated from type 1 diabetes mellitus patient iPSCs (Chan et al., 2015). These cells were able to self-assemble into networks in gelatin-based hydrogels *in vitro* (forming denser networks under hypoxia) and incorporate into host vasculature when injected into a zebrafish embryo. Shen et al. then dispersed these cells in hyaluronic acid-based hydrogels to create microvascular implants that could be applied to the wounded region of diabetic mice (Shen et al., 2016). Compared to acellular hydrogels, vascularized hydrogels containing diabetic patient-derived iPSC-ECs showed accelerated vascularization of the granulation tissue and wound closure via re-epithelialization. These results were comparable to implants containing primary human ECs or iPSC-ECs differentiated from healthy donors, suggesting comparable efficacy in wound healing even when the host is diabetic, and the implant's potential for treating other wound healing processes such as burns.

In addition to providing perfusion in ischemic host tissues, microvascular implants can also be used to vascularize engineered organs. Coculture of HUVECs, MSCs, and iPSC-derived hepatocytes enhanced vascular network formation through VEGF-mediated signaling, and when implanted into mice, the engineered, vascularized liver tissue integrated with the host within days and extended the survival of mice after liver injury compared to non-vascularized liver tissue (Camp et al., 2017; Takebe et al., 2014). We also recently showed that after implantation, engineered human liver tissue responds to host liver injury through *in situ* expansion and increased function only when the implanted tissue was engineered with patterned vasculature (Stevens et al., 2017). Similar advantages of pre-vascularization of engineered tissues have been reported in other engineered tissues such as muscle (Sakaguchi et al., 2013; Shandalov et al., 2014; Valarmathi et al., 2017; Zhang et al., 2016) and skin (Abaci et al., 2016; Klar et al., 2014).

Future Directions

Vascular tissue engineering has made great strides since Weinberg and Bell's TEBV. What began as a means of bypassing critical occlusive lesions in the cardiovascular system has progressed towards *ex vivo* microvascular network generation, gradually incorporating the complex tissue's specific cellular interactions. The technological progress towards generation engineered vasculature, however, has not been paralleled with demonstrable improvements in outcomes of CVD. An increasingly ageing population and subsequent rise in advanced chronic illnesses simultaneously intensifies the need for these engineered vessels and makes it harder to improve outcomes in these more challenging populations (Prabhakaran et al., 2017).

The gap between the need for vascular conduits as well as whole organs and the availability of donor material continues to rise (GBD 2015 Mortality and Causes of Death Collaborators, 2016; White et al., 2014). Engineered large vessels have shown promising preclinical and clinical data, suggesting a path forward for the field. Importantly, stem cell technologies may play a key role in improving these approaches. Cytograft's Lifeline vascular graft requires 6 to 9 months of production time prior to implantation, the significant proportion of which is devoted to culturing fibroblast sheets. In comparison, iPSC derived SMCs have been shown to generate abundant collagen within 8–9 weeks of culture time (Gui et al., 2016). The enhanced proliferative capacity demonstrated by both adult stem cells and iPSCs thus may not only reduce production time but also eliminate the need for harvesting cells from patients or donors (Dimitrievska and Niklason, 2017). Vascular engineering is not only important for generating large vessels, but also for generating microvasculature, for example, to support the engineering of organs and tissues *ex vivo*. While complex microvascular networks have been mostly accomplished at the pre-clinical level in animal models, results show promise of integration in the host tissue with no significant immunologic reactions post-implantation (Levenberg et al., 2005; Shen et al., 2016). Again, stem cells appear to offer superior proliferative and synthetic capacity to support vascular development in these engineered tissue contexts (Skardal et al., 2012). Developing a deeper understanding of what advantages and disadvantages such stem cell sources offer, and the molecular basis for those features, will be an important undertaking in the coming years.

As the challenge of scaling up to whole-organs *ex vivo* is undertaken, the biomolecular pathways that control organ specific heterogeneity of ECs and other vascular cells will need to be better defined (Hong et al., 2008; Swift and Weinstein, 2009; You et al., 2005). The phenotypic heterogeneity of ECs extends beyond site and organ-specificity, with extensive zonal heterogeneity observed even within an organ, such as the sinusoidal ECs in liver (Géraud et al., 2017). Similarly, SMCs have known phenotypic and molecular heterogeneity based on their site and respective function in the body (Yoshida and Owens, 2005). Understanding the developmental pathways determining embryologic differentiation to organ-specific cells can suggest potential pathways to engineer stem cells into a diverse array of tissue-specific vascular cell types.

The complexity of host immune response as we progress towards larger vessel conduits or whole organs also remains to be further investigated. Studies have revealed the

immunotolerant potential of stem cells. Haykal et al. demonstrated delayed leukocyte involvement in decellularized tracheal allografts recellularized with autologous MSCs implanted in porcine models (Haykal et al., 2013). Moreover, the phenotypic switch of macrophages from M1 to M2 phase has been implicated in successful scaffold remodeling (Badylak et al., 2008). Further studies into stem cells' impact on macrophage repolarization to site-specific remodeling will be valuable (Wiles et al., 2016). At a broader level, being able to control immune acceptance of cell-based implants remains a critically important challenge for the field.

Conclusions

Despite almost a half-century of focused work and a century of interest in creating synthetic grafts, we are still lacking in definitive cell sources, materials, methods and products. Future work that harnesses continued innovation and discovery in vascular engineering and biology, as well as biomaterial and stem cell engineering, will generate continued motivation and hope for translatable strategies.

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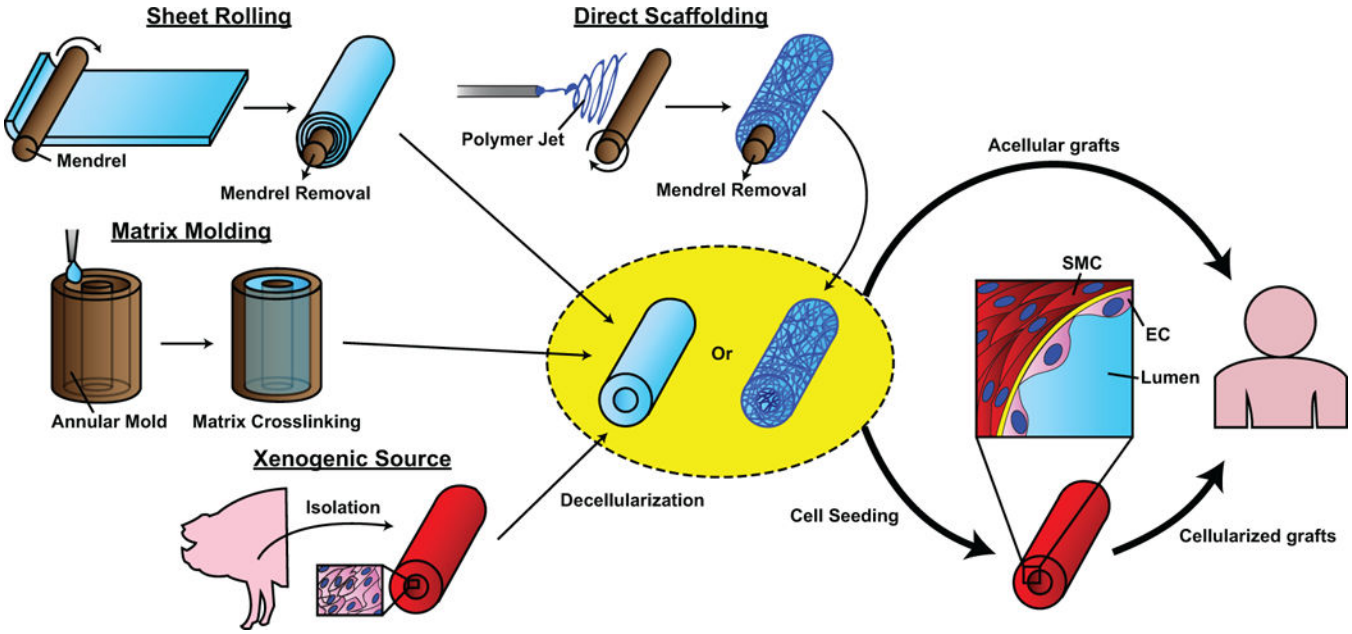

Figure 1. Fabrication Approaches for Engineering Arterial Vessels

Tubular structures that resemble arteries (<6mm in diameter) can be xenogenically sourced or fabricated with biomaterials through sheet rolling, molding, and/or direct scaffolding. Once fabricated, the grafts are seeded with ECs and SMCs and conditioned with biomechanical stimuli (not shown) before implantation. Grafts can also be used without cellularization for applications such as hemodialysis access.

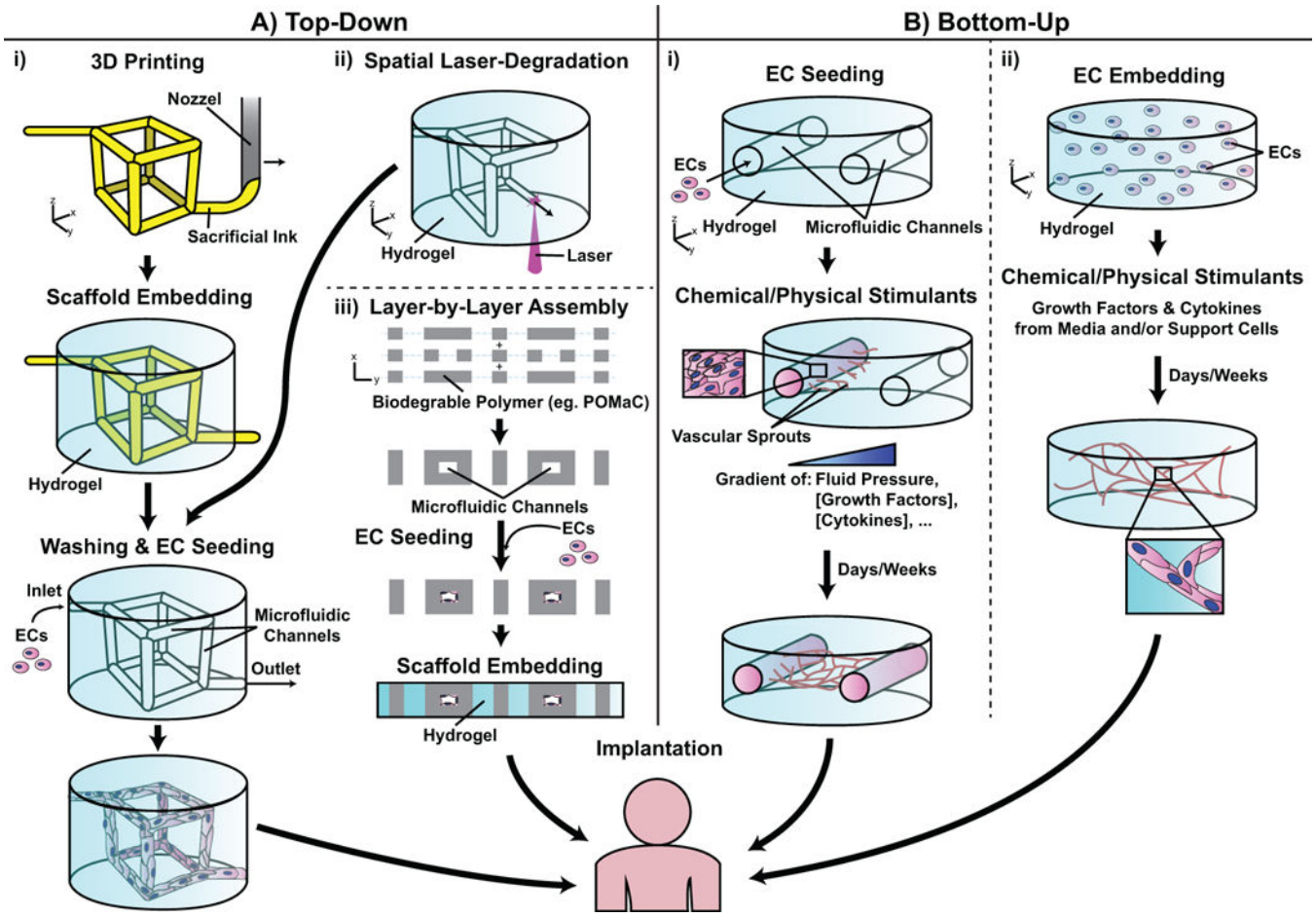

Figure 2. Fabrication Approaches for Engineering Microvessels

Microvessels can be fabricated using either A) top-down or B) bottom-up methods. A) Top-down approaches involve fabrication of pre-designed structures through i) sacrificial 3D printing, ii) spatial laser-degradation, or iii) layer-by-layer assembly (z-axis illustrated normal to figure plane). B) Bottom-up approaches utilize chemical/physical stimulants to induce i) angiogenic sprouting or ii) vasculogenic self-assembly of endothelial cells (ECs) to create a network of interconnected microvessels. Dimensions of fabricated structures can vary but all are sub-millimeter in scale.