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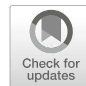

# Human Pluripotent Stem Cells to Engineer Blood Vessels

**Xin Yi Chan, Morgan B. Elliott, Bria Macklin, and Sharon Gerecht**

**Abstract** Development of pluripotent stem cells (PSCs) is a remarkable scientific advancement that allows scientists to harness the power of regenerative medicine for potential treatment of disease using unaffected cells. PSCs provide a unique opportunity to study and combat cardiovascular diseases, which continue to claim the lives of thousands each day. Here, we discuss the differentiation of PSCs into vascular cells, investigation of the functional capabilities of the derived cells, and their utilization to engineer microvascular beds or vascular grafts for clinical application.

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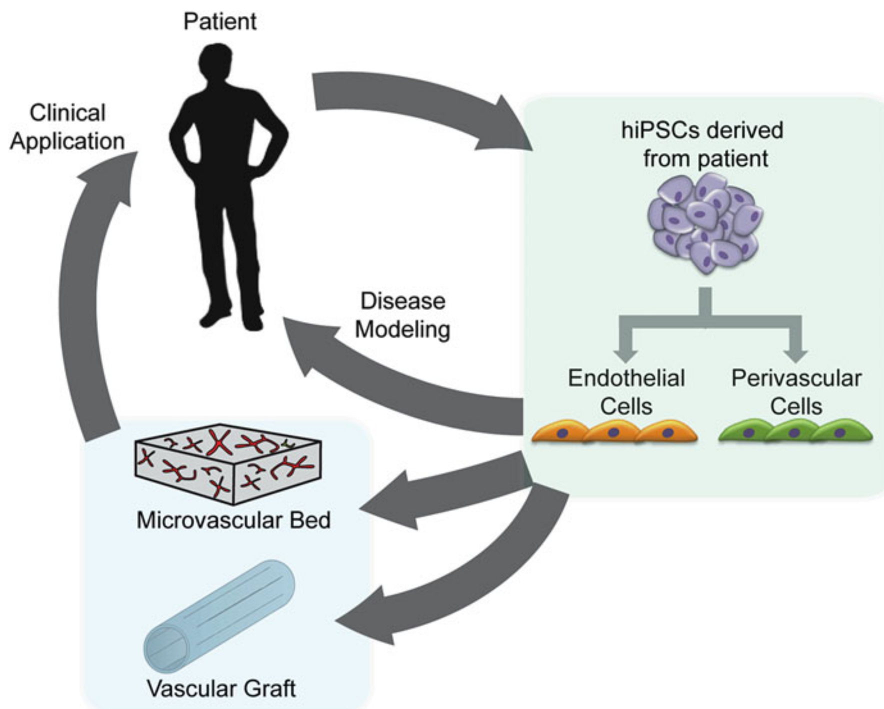


**Graphical Abstract** Human iPSCs generated from patients are differentiated toward ECs and perivascular cells for use in disease modeling, microvascular bed development, or vascular graft fabrication

**Keywords** Human pluripotent stem cells, Small-diameter tissue engineered vascular grafts, Vascular differentiation, Vascular disease modeling, Vascular networks

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## Abbreviations

| | |
|---|---|
| 2D | Two-dimensional |
| 3D | Three-dimensional |
| bFGF | Basic fibroblast growth factor |
| BMPR2 | Bone morphogenetic protein receptor type II |
| BP | Burst pressure |
| CAD | Coronary artery disease |
| CCD | Chronic cardiovascular defects |
| DO | Dissolved oxygen |
| DPI | Diphenyleneiodonium |
| EB | Embryoid body |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| EVC | Early vascular cell |
| FBN1 | Fibrillin1 |
| FPAH | Family members of pulmonary arterial hypertension |
| HA | Hyaluronic acid |
| (h)ESC | (Human) embryonic stem cell |
| HIF | Hypoxia-inducible factors |
| (h)[i]PSC | (Human) [induced] pluripotent stem cell |
| HUVECs | Human umbilical vein endothelial cells |
| ITA | Internal thoracic artery |
| MFS | Marfan syndrome |
| MMP | Matrix metalloproteinase |
| PDGF-BB | Platelet-derived growth factor-BB |
| PEG | Poly(ethylene glycol) |
| PEGDA | PEG-diacrylate |
| PGA | Polyglycolic acid |
| ROS | Reactive oxygen species |
| SMA | Smooth muscle actin |
| SMMHC | Smooth muscle myosin heavy chain |
| SRS | Suture retention strength |
| (s)TEVG | (Small-diameter) tissue engineered vascular graft |
| SV | Saphenous vein |
| TESA | Tissue engineering by self-assembly |
| TGFβ | Transforming growth factor β |
| UMC | Unaffected mutation carrier |
| VEGF(R) | Vascular endothelial growth factor (receptor) |
| vSMC | Vascular smooth muscle cell |

## 1 Introduction

Functional blood vessels are essential for delivering oxygen and nutrients.[1] These vessels are specialized and can be categorized into several classes, including arteries, arterioles, capillaries, venules, and veins. A blood vessel consists of a tube lined with endothelial cells (ECs) in the inner wall and surrounded by support cells such as pericytes and vascular smooth muscle cells (vSMCs), depending on the vascular size (Fig. 1). ECs play an important role as a barrier to pathogens and in many physiological processes such as wound healing, the immuno/inflammatory response, and coagulation [2].

Typically, vascular disease occurs when the cellular makeup of the patient's vasculature changes. Causes of vascular diseases are often linked to genetic disorders such as peripheral arterial hypertension. Historically, transgenic animal models of mice and zebrafish have played an important role in modeling cardiovascular diseases, characterizing the pathology and physiology of the disease, identifying downstream targets, and evaluating therapeutic drugs and treatments. However, following an increased number of promising drug treatment failures in clinical trials, the use of animal models in testing new therapeutic drugs has been criticized for its ineffectiveness. In recent decades, with the development of human induced pluripotent stem cells (hiPSCs) as a source of patient-specific regenerative therapies, hiPSCs have become an ideal alternative to animal models or patient tissue samples as a platform for modeling vascular diseases, because they carry the same genetic abnormalities as the patients from whom the cells were derived. These in vitro hiPSC-generated vascular disease models could possibly advance medical treatment by providing mechanistic insights into vascular diseases and discovering

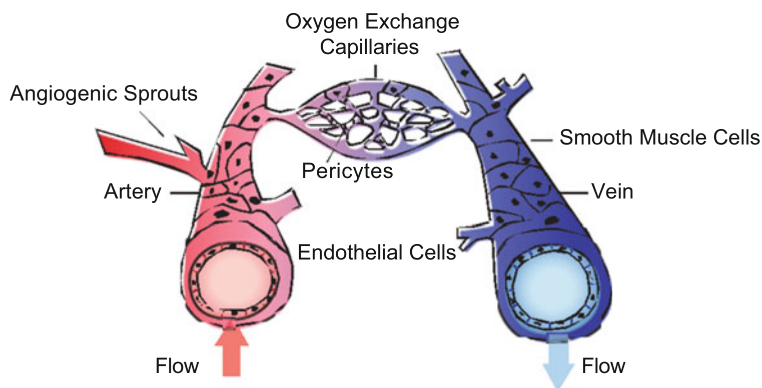


**Fig. 1** Cellular makeup of blood vessels. Taken from [1]

[1]Note: Alterations to the root abbreviation are indicated in parentheses or brackets.

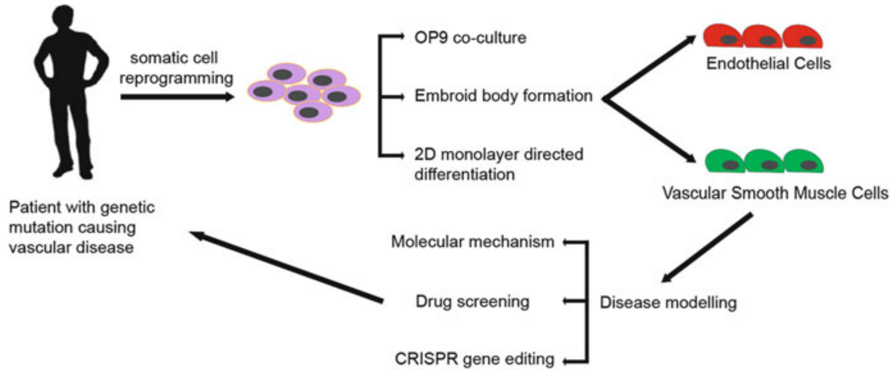


**Fig. 2** Modeling genetic mutations in vascular diseases using hiPSC-derived ECs and vSMCs

new drugs via large-scale drug screens. Here, we discuss the different approaches used to derive vascular cells from healthy hiPSCs and some examples of vascular diseases modeled using hiPSC-derived vascular cells (Fig. 2).

## 2 Derivation of ECs and Perivascular Cells from Healthy and Diseased hiPSCs

### *2.1 EC and vSMC Differentiation from hPSCs*

The first instance of reprogramming human somatic cells into hiPSCs was reported by two independent research groups, those of Yamanaka and Thompson. Yamanaka's group showed that using retroviruses to transfect four factors (OCT4, KLF4, SOX2, and C-MYC) into human fibroblasts is sufficient to reprogram those cells into hiPSCs [3]. On the other hand, Thomson's group demonstrated that reprogramming hiPSCs can be achieved using lentiviral vectors to transfect a different set of factors, including OCT4, NANOG, LIN28, and SOX2 [4]. Generation of hiPSCs from different cell sources without integration of reprogramming factors into the genome can improve the quality of these cells without posing potential risks of a genome-integrating virus vector backbone [5, 6].

Based on published work in vertebrates such as rodents and zebrafish, developmental factors and chemical molecules were utilized to guide hiPSCs to differentiate and mature into functional vascular derivatives. Over the last few decades, multiple protocols have been established to derive vascular cells from hPSCs, including both ECs and vSMCs. In general, the approaches for vascular differentiation described here were first demonstrated with human embryonic stem cells (hESCs) and then similar methods and their improvements were developed for hiPSCs.

### 2.1.1 OP9 Co-culture

The OP9 cell line is a stromal cell line derived from the skullcap of mice with an osteopetrotic mutation in the gene encoding macrophage colony-stimulating factor, a factor that has inhibitory effects on hematopoietic differentiation. Vodyanik et al. demonstrated that hPSCs cultured on an OP9 feeder layer can be directed to differentiate into a subset of $CD34^+$ hematopoietic progenitors and then matured into $CD31^+CD34^+CD43^-$ ECs [7]. They discovered that differentiation of hPSCs on top of the OP9 stromal cells is sufficient to generate a large number or $CD34^+$ cells without adding cytokines. This result indicates the importance of paracrine signaling and cytokines secreted by OP9 cells to direct the differentiation of hematopoietic and endothelial lineages [8]. After improving OP9 co-culture differentiation, another group of researchers found that $CD31^+CD34^+$ vascular progenitor cells differentiated on OP9 feeder cells could be further differentiated and matured into functional ECs and vSMCs separately when cultured in specific media supporting their specific lineage differentiation [9]. The EC derivatives express CD31, CD144, VEGRF2, and CD105, whereas the vSMC derivatives express desmin, α-smooth muscle actin (αSMA), calponin, and SM22$\alpha$.

### 2.1.2 Three-Dimensional Differentiation Via Embryoid Body Formation

Another technique utilized to differentiate ECs and vSMCs is embryoid body (EB) formation. EB aggregates mimic primitive streak formation and induction of all three germ layers during embryonic development by responding to similar cues [10]. Also, because of their nonadherent nature, EB aggregates can be cultured in suspension, which is scalable and thus enables large production of differentiated ECs [11].

*EC differentiation*: Many endothelial differentiation methods typically grow hPSCs into EBs and culture them in suspension in differentiation media for a few days. Differentiation steps typically involve cell sorting (either magnetic or fluorescence-activated cell sorting; FACS) to isolate vascular progenitor cells using markers such as CD34, CD31, and CD144, followed by their culture with specific small molecules and growth factors to promote differentiation and maturation to hematopoietic and endothelial lineages [12, 13]. Different combinations of growth factors have been used in different methods. For example, James et al. added BMP4, activin A, and basic fibroblast growth factor (bFGF) to hPSC-derived EB aggregates to initiate differentiation. Thereafter, they transferred the EBs onto Matrigel and added vascular endothelial growth factor (VEGF)-A and a small molecule inhibitor of the transforming growth factor β (TGFβ) signaling pathway [14], thereby increasing the yield of $CD31^+$ ECs tenfold.

*vSMC differentiation*: Similar to endothelial differentiation, a variety of methods with different combinations of growth factors and extracellular membrane proteins

have been utilized to guide differentiation into vSMCs. The main difference is in the cellular and molecular markers used to validate and assess the successful derivation of vSMCs [15, 16]. In a method developed by Lin et al. [86], hiPSC EBs were treated with growth factors such as VEGF-A and bFGF during early differentiation to direct the differentiation of multipotent cardiovascular progenitor cells. These $KDR^{low}c\text{-}kit^{-}$ progenitor cells were sorted using FACS and subsequently cultured as a monolayer with VEGF and bFGF added to the medium. A second sort was performed to isolate cells that were $CD31^{-}CD166^{-}$ to further direct differentiation into functional vSMCs utilizing a specific smooth muscle growth medium.

### 2.1.3 2D Monolayer Differentiation

Directed differentiation as a two-dimensional (2D) monolayer of hPSCs is another method for guiding vascular differentiation, aiming to overcome limitations such as the relative heterogeneous differentiation of EBs, which could be a result of limited diffusion of chemical cues to the interior of the EBs. In addition, monolayer differentiation methods guide lineage commitment and can increase cell yield and viability after sorting.

*EC differentiation*: Using a 2D culture, scientists can fine tune the chemical cues necessary to induce EC fate directly by adding growth factors and small molecules. Using this approach, differentiation from hPSCs was optimized to a content of 50–70% ECs prior to sorting [17–20]. In our recently published protocols, Kusuma et al. [19] and Chan et al. [20] demonstrated the hiPSC-based derivation of early vascular cells (EVCs), which are characterized by the expression of vascular endothelial cadherin and platelet-derived growth factor receptor β. EVCs can be matured into ECs or pericytes and, when encapsulated in a synthetic hydrogel, can interact with each other, undergo morphogenesis, and self-organize into 3D vascular networks.

*vSMC differentiation*: There are several differentiation protocols demonstrating successful derivation of vSMCs from hiPSCs using growth factors and extracellular matrix (ECM) proteins to guide the differentiation. However, to date, only two protocols reliably differentiate hiPSCs into vSMCs with either synthetic or contractile lineage specification [21, 22]. The synthetic phenotype is characterized by high proliferation, migration, and ECM protein production. The contractile phenotype is characterized by low proliferation, low synthetic activity, and expression of contractile proteins, namely, smooth muscle myosin heavy chain (SMMHC) and elastin. Based on our protocol described by Wanjare et al. [21], hiPSCs were seeded on collagen IV in the first stage of differentiation to derive mesodermal cells and then, following addition of platelet derived growth factor-BB (PDGF-BB) and TGFβ with 10% serum, were derived into synthetic vSMCs. These vSMC derivatives express αSMA, calponin, and SM22a; about 50% also express SMMHC. Continuous culture of these cells in TGFβ and low serum medium can further mature the cells into contractile vSMCs, which express SMMHC and elastin.

## 2.2 Human iPSCs as a Tool to Model Vascular Diseases

The development of iPSC technology has opened up avenues for study of vascular disease by overcoming the challenges of species-specific limitations resulting from animal models. In addition, the difficulty of harvesting sufficient patient vascular tissue samples can be overcome by deriving these tissues from hiPSCs generated from the patient. Both ECs and vSMCs play a crucial role in maintaining vascular function. Genetic mutations affecting development of the vasculature can result in dysfunctional vasculature, leading to vascular diseases such as pulmonary hypertension, Marfan syndrome (MFS), or others (outlined in Sects. 2.2.1 and 2.2.2). The ability to differentiate vascular cell types from hiPSCs enables researchers to study the molecular and pathophysiology aspects of these diseases. In addition, the stages of differentiation of these vascular cells from hiPSCs closely mimic their developmental stages in vivo, presenting a unique opportunity to model and study disease progression in vitro.

### 2.2.1 Human iPSC-EC Disease Modeling

Recently, patient-specific iPSC-ECs have been employed to study pathways involved in pulmonary arterial hypertension (PAH). In PAH, dysfunctional ECs of the pulmonary arteries are the key factor in the initiation and progression of the disease. These dysfunctional ECs in PAH display phenotypes showing features such as decreased cell survival upon injury, impaired adhesion and migration, and disordered angiogenesis. Gu, Shao and colleagues generated patient-specific iPSC-ECs from family members of pulmonary arterial hypertension (FPAH) patients and unaffected mutation carriers (UMC) of bone morphogenetic protein receptor type II (BMPR2) mutation, and compared them with gender-matched controls to investigate the protective modifiers of the BMPR2 mutation [23]. The group demonstrated that EC morphology and BMPR2 expression are similar in FPAH and UMC iPSC-ECs. However, FPAH iPSC-ECs had impaired cell adhesion on multiple ECM substrates and reduced cell survival after serum withdrawal. Elevated BMPR2 activators and reduced BMPR2 inhibitors in UMC iPSC-ECs are responsible for the BMPR2-mediated activation of p-P38 signaling and increased β1-integrin, which improve cell adhesion. Independent of the BMPR2 pathway, the authors also discovered that increased levels of baculoviral IAP repeat-containing 3 (BIRC3) in the UMC iPSC-ECs improved cell survival. Furthermore, correction of the BMPR2 mutation using CRISPR restored the functions of rescued FPAH-ECs to those of control iPSC-ECs. These findings shed light on the importance of protective modifiers for FPAH, which could help in developing potential treatments for FPAH.

#### 2.2.2 Human iPSC-vSMC Disease Modeling

Marfan syndrome (MFS) is a heritable genetic disorder caused by mutations in fibrillin1 (FBN1) that affect the connective tissue of patients due to dysfunctional vSMCs. Patients with this disease often have vSMC defects that affect FBN1 accumulation, ECM degradation, TGFβ signaling, and contraction and apoptosis of the vSMCs. The Sinha group successfully generated MFS-vSMCs from patient-specific MFS-iPSCs [24]. According to the authors, MFS-vSMCs exhibited the same symptoms as in the aortas of Marfan patients. These cells have reduced levels of FBN1 deposition and increased levels of TGFβ and matrix metalloproteinases (MMPs) in the ECM. In addition, MFS-vSMCs showed functional abnormalities, including higher incidence of cell death associated with increased vSMC loss in MFS aortic dilatation, and reduced contractility similar to that observed in MFS aortas. The abnormalities in MFS-vSMCs can be rescued by correction of the FBN1 mutation. Inhibition of TGFβ in MFS-vSMCs was sufficient to rescue the phenotypes of FBN1 reduction and MMP increase, but not to alleviate the high incidence of cell apoptosis, which is regulated by the non-canonical p38 pathway. This particular vSMC disease model provides a platform to study the molecular mechanisms affecting MFS and help develop future therapeutic approaches.

## 3 Harnessing the Extracellular Cues to Engineer Vascular Networks from hiPSCs

### *3.1 Angiogenesis and Vasculogenesis*

Vasculogenesis and angiogenesis are the primary processes that regulate blood vessel formation in all blooded species. Vasculogenesis is the de novo formation of vascular structures, whereas angiogenesis is the formation of vessels from preexisting vasculature. Vasculogenesis occurs in three developmental steps, beginning with cells of the mesoderm. These early mesodermal cells first differentiate into blood islands, which are bicellular aggregates comprising angioblasts on the outer layer and hemopoietic cells internally. Next, in response to an increase in growth factor binding to VEGFR2, VEGFR1, and tie-1, the angioblasts differentiate into ECs. Newly created ECs form the primary vascular plexus, an embryonic structure from which all subsequent vessels form via angiogenesis [25]. Angiogenesis begins with a specific EC, referred to as the “tip cell,” which is activated by cues in the embryonic environment and then leads the sprouting process. Stalk cells, which are in direct contact with the tip cell, begin to proliferate and form laminated structures. Mural cells, including pericytes and vSMCs, are recruited to stabilize the newly formed vasculature by EC-derived ligands (i.e., heparin-binding epidermal growth factor and PDGF-BB) [26].

These complex processes can be mimicked using 3D vascular models in vitro. These models include a natural or synthetic biomaterial-based scaffold to serve as the ECM for the networks, several growth factors that can be added to induce the process, and other physical cues such as oxygen and matrix stiffeners to simulate the surroundings during vasculogenesis and angiogenesis.

## 3.2 Biomaterials

To engineer a viable 3D model, the biomaterial selected must be optimal for the intended purpose. As new vasculature is created, ECs must constantly remodel their ECM via traction forces, proteolytic activity, and cell–matrix adhesion to allow sprouting and lumen formation [27]. Biomaterials for use in vascular network models must allow this remodeling, in addition to being biocompatible and possessing optimal stiffness, structure, and permeability. Hydrogels are materials that have a high water content, yet do not dissolve in water. Hydrogels simulate natural tissues in that they can retain structural integrity in highly aqueous environments and allow easy diffusion of small molecules. Natural, synthetic, and semisynthetic hydrogels are widely used to recapitulate vasculogenesis and angiogenesis.

Commonly used natural hydrogels include collagen, fibrin, and gelatin. These proteins are produced naturally within the body and, thus, the hydrogel derivatives are characteristically biocompatible and biodegradable. Type I collagen gel, for example, allows EC network formation through activation of tubulogenesis pathways (Fig. 3). EC sprouting and migration occur through the creation of "vascular guidance tunnels" via α2β1 integrin binding and MT1-MMP network degradation. When supporting mural cells are introduced, matrix remodeling and ECM

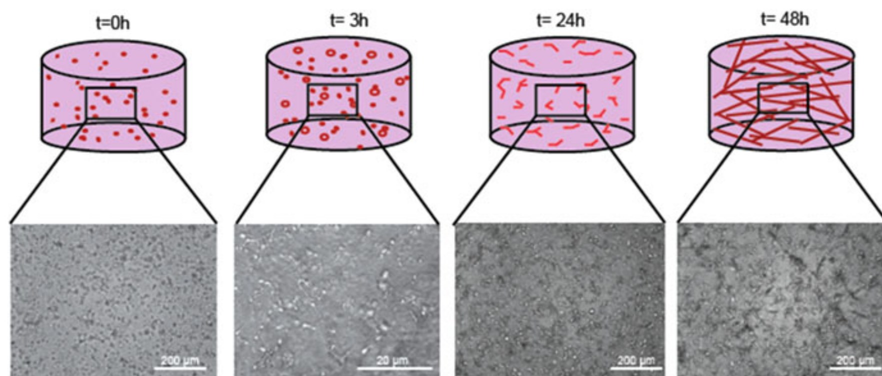


**Fig. 3** Vascular assembly kinetics. Schematic (*upper panel*) and corresponding light microscopy images (*lower panel*) showing the progression of vascular assembly of iPSC-derived ECs encapsulated in collagen gels ($t = 0$ h), including vacuole formation ($t = 3$ h), sprouting events ($t = 24$ h), and network growth ($t = 48$ h). Graphics not drawn to scale. Taken from [28]

production increases, resulting in α5β1, α3β1, α6β1, and α1β1 integrin binding [29]. Collagen gel is also an optimal biomaterial for vascular modeling because of the high concentration of collagen in the body and its natural load-bearing capabilities [30]. Synthetic hydrogels are highly amendable and can be custom-made to the desired structure, stiffness, and degradability. This allows the creation of a much more defined and tunable system. Although natural materials can vary in uniformity from batch to batch, synthetic hydrogels maintain consistency between batches. A commonly used material for synthetic hydrogels is poly(ethylene glycol) (PEG). One major drawback of using synthetic hydrogels such as PEG is that they have little or no cell adhesion or degradation sites. This shortcoming can be resolved by adding functional sites to the polymer, including adhesive sites such as arginine-glycine-aspartic acid (RGD) sequences or MMP-degradable sites, which are routinely utilized to improve cell–material interaction. Semisynthetic hydrogels are a new class of biomaterials that incorporate the advantages of natural materials with the customizability of synthetic polymers. Examples include acrylated hyaluronic acid (HA) and dextran hydrogels [31].

### 3.2.1 Matrix Properties

*Stiffness*: The stiffness of a biomaterial is typically determined by the elastic modulus. The stiffness of natural materials such as collagen and fibrin hydrogels can be modified by increasing the density, which has been shown to affect neovessel growth and sprouting [32, 33]. More specifically, the modulus of hydrogel materials can be altered by increasing the polymer concentration or changing the crosslinking density of the material. We have previously demonstrated the effects of stiffness on endothelial progenitor cells using a semisynthetic HA–gelatin hydrogel with PEG-diacrylate (PEGDA) as crosslinker. By modifying the concentration of PEGDA to 1, 0.4, and 0.1%, three significantly different Young's moduli were generated, creating rigid, firm, and yielding hydrogels, respectively. Physical and biological analyses of the networks affirmed the crucial role of matrix stiffness. Cell cultures in the yielding hydrogel possessed a significantly higher mean tube length, tube area, and tube thickness than cultures in the rigid and stiff hydrogels. Both firm and yielding substrates allowed formation of luminal structures, whereas the stiff hydrogel did not. In response to high concentrations of VEGF, yielding hydrogels showed a decreased expression of MT1-MMP, MMP-1, and MMP-2 [34].

*Degradation*: As mentioned above, the ability of ECs to degrade their ECM is paramount to both angiogenesis and vasculogenesis. Sokic and Papavasiliou utilized a PEGDA-based hydrogel to demonstrate this [35]. Hydrogels were made using MMP-sensitive peptides with either one or three proteolytic cleavage sites and functionalized to PEGDA macromeres with one or multiple MMP-sensitive peptide domains between each crosslink. Hydrogels with only one MMP cleavage site took up to 96 h to degrade completely, depending upon the weight percentage used, whereas hydrogels with three cleavage sites degraded in as little as 1 h. The authors used human umbilical vein endothelial cells (HUVECs) in an invasion assay to show that

hydrogels with more cleavage sites had a greater invasion area and depth of invasion [35]. The ability to control degradation of the hydrogel has also allowed creation of gels that permit controlled release of various growth factors. This is an efficient and directed approach to deliver growth factors. Heprasil™, a hybrid mesh of poly(ε--caprolactone)-collagen blend and HA hydrogel, was dual-loaded with VEGF and PDGF-BB, which were released over 21 days. During the 21 days, HUVECs continued to grow in response to the growth factors [36].

Through optimization of both adhesion and degradation sites, as well as stiffness of acrylated HA hydrogels, we have shown the activation of vasculogenesis pathways of endothelial progenitors [37]. More recently, we have shown that hiPSC-derived EVCs undergo tubulogenesis in these HA hydrogels, resulting in multicellular, functional vascular networks [19].

## *3.3 Oxygen and Hypoxia*

Oxygen tension plays a key role in the regulation of angiogenesis and vasculogenesis, affecting cell viability, differentiation, migration, and ECM remodeling. Hypoxia-inducible factors (HIF) and reactive oxygen species (ROS) govern EC response and adaptation to changes in oxygen levels, allowing increases in crucial growth factors such as VEGF and bFGF [38, 39]. HIF1α controls angiogenesis in hypoxic oxygen levels of less than 1%. We have previously demonstrated the effects of hypoxia on HUVECs in a 3D collagen matrix. Hydrogels encapsulated with HUVECs were allowed to incubate for 48 h while oxygen partial pressure was measured. After 24 h, oxygen within the gel had decreased from ~12% to <5%, whereas gels supplemented with diphenyleneiodonium (DPI), a ROS inhibitor, had oxygen levels that remained at ~20%. Cell viability was also affected after 24 h, with a larger percentage of HUVECs dying in gels not supplemented with DPI. Gel thickness was also shown to regulate oxygen availability at the bottom of the gel. Although inhibition of ROS allowed greater cell viability, the vascular network characteristics (mean tube length, tube thickness, and tube area coverage) significantly decreased compared with untreated gels, providing overwhelming evidence for the importance of hypoxia in angiogenesis [40].

Although it has been shown that dissolved oxygen (DO) levels can also be regulated by adjusting the height of the hydrogel, this is highly uncontrolled and can vary when using natural materials. Our group was the first to synthesize a hydrogel that can regulate DO levels and gradients within its own 3D environment. We were able to show that DO levels can be precisely controlled by modifying reaction kinetics and hydrogel composition. An increase in vascular network characteristics was shown using these hypoxia-inducible hydrogels [41]. More recently, we have shown that EVCs derived from hiPSCs of both healthy and diabetic donors respond to the hypoxic environment, generating extensive vascular networks within the hypoxic hydrogels [20].

# 4 Vascular Graft Fabrication Using hiPSC Derivatives

## *4.1 Clinical Need*

Tissue engineered vascular grafts (TEVGs) are in high demand for replacing harvested autologous vessels used as bypass, endovascular, and interposition grafts [42, 43]. Over 0.5 million patients with coronary artery disease (CAD) undergo coronary artery bypass procedures each year [43–46]. Meanwhile, 1% of children are born with chronic cardiovascular defects (CCD) and require repeated cardiac surgery to reconstruct vascular conduits [47–49]. For single ventricle cardiac anomalies, the most severe CCD, synthetic vascular grafts are the leading cause of complications resulting from their lack of growth during child development [49–53]. For both CAD and pediatric CCD cases, the standard treatment is to replace these small-diameter arteries with autologous tissue grafts [43, 49, 54–56], which have numerous disadvantages. Harvesting the tissue is inconvenient and there may be insufficient tissue available, limiting reconstruction [49, 54, 55]. Repeated surgery, multiple operation sites, limited availability, and sacrificed arteriovenous function to obtain a graft underscore the clinical need for a TEVG with the patency and low thrombogenicity that is characteristic of native vessel grafts.

Synthetic TEVGs are commonly used for procedures that require a graft larger than 6 mm in diameter [16]. However, for small-diameter TEVGs of under 6 mm in diameter (sTEVGs), synthetic materials have not shown clinical effectiveness and are inferior to autologous tissue grafts [49, 54, 55, 57]. Currently available artificial grafts have low durability because of atherosclerosis and stenosis and may catastrophically fail after 8–12 years [45]. Although several efforts using mature or progenitor cell lines have been successful in developing sTEVGs [58–62], a functional graft has remained elusive because of post-implantation challenges, including thrombogenicity, decreased elasticity, decreased compliance, aneurysmal failure, and intimal hyperplasia [58, 62, 63]. Significant improvement is required in order to provide CAD and pediatric CCD patients with an ideal sTEVG to replace the autologous graft gold standard.

### 4.1.1 The Ideal sTEVG

Patient-derived vessels should be matched to the size specifications of the patient and be able to grow with pediatric patients. The ideal engineered structure is nonimmunogenic, capable of scale-up with a clinically relevant shelf-life, has low thrombogenicity, and exhibits long-term patency [43, 60, 63]. Additionally, the sTEVG should have mechanical properties similar to those of native vessels such as the internal thoracic artery (ITA) and saphenous vein (SV), commonly used as autologous grafts [64]. The mechanical properties used to compare grafts with native tissue are burst pressure (BP), suture retention strength (SRS), and compliance. SV parameters have values of 2,134 mmHg, 1.92 N, and 25.6%/100 mmHg, respectively, and those for ITA are 3,073 mmHg, 1.72 N, and 11.5%/100 mmHg [64]. A fully biomimetic graft that recapitulates arterial properties is ideal and difficult to achieve [64].

The development of functional cellularized sTEVGs requires complex interactions and specific organization among ECs, vSMCs, and several ECM proteins. Crucially for sTEVG applications, ECs reduce platelet activation and adhesion, have antimicrobial properties, aid in fibrinolysis, and prevent intimal hyperplasia and leukocyte adhesion [65–67]. Meanwhile, vSMCs provide mechanical strength, vasoreactivity, and improved stability of TEVGs [68–70]. Although each cell type plays a unique role in vascular function, cellular crosstalk affects vessel function and further complicates graft fabrication [69, 71, 72]. Debate on the ideal cell source for vascular engineering is ongoing, but hiPSCs may be the answer for clinically relevant, patient-specific grafts [73–76]. The ability to derive ECs that can generate patient-specific blood vessels from type I diabetic patient-derived hiPSCs [20], a population with associated vascular diseases for which autologous vascular grafts may be difficult to obtain [73], shows the clinical relevance of the cell source and potential for relevant in vitro disease models. Design of a robust sTEVG seeded with hPSCs would provide a substantial benefit for patients.

## *4.2 Current Efforts to Develop sTEVGs*

Within the field of vascular tissue engineering, there are three classes of techniques for developing sTEVGs: scaffold-based methods, tissue engineering by self-assembly (TESA), and decellularized matrices (Fig. 4) [64, 76]. Scaffold-based

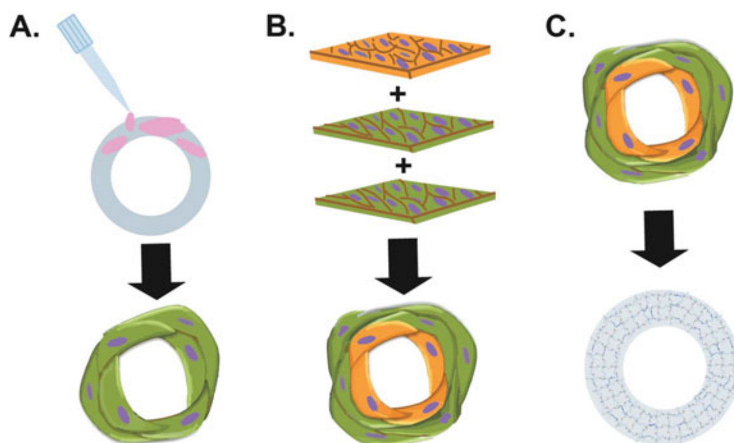


**Fig. 4** Three techniques for fabricating sTEVGs. (**a**) A commonly used scaffold-based method is drip seeding a cell solution onto a graft-like structure that is typically made of synthetic material and vSMCs (*green*). (**b**) An efficient TESA method is culturing several types of cell sheets and concentrically rolling them to attain a multilayered graft structure. The cell sheets may be ECs (*orange*), vSMCs (*green*), or fibroblasts (not shown). (**c**) Native vessels or hPSC-derived engineered vessels can be decellularized to make an acellular natural matrix that can be used as an off-the-shelf vascular graft

methods focus on using a natural or synthetic matrix as a base, onto which cells are seeded [64]. TESA techniques do not utilize a scaffold or matrix [64]. The decellularization methods remove cells from vessels to fabricate an off-the-shelf, nonimmunogenic graft [64]. Using PSCs with these methods only started to gain momentum in 2012, but noteworthy progress has been made.

### 4.2.1 Scaffold-Based Methods

The Niklason laboratory pioneered scaffold-based methods for fabricating sTEVGs, originally beginning with mature vascular cells and recently adding hPSC-based approaches. The group's first attempt used hESC-derived mesenchymal stem cells that could be further differentiated using TGFβ1 into contractile calponin-positive vSMCs seeded onto a 1-mm diameter polyglycolic acid (PGA) scaffold [44]. After 8 weeks and applying pulsatile flow during graft culture, a collagen-rich cell wall that positively stained for αSMA was achieved [44]. The lack of graft calponin staining was unsurprising given the use of 20% serum for maintaining the synthetic vSMC phenotype and encouraging cell proliferation [44]. Subsequent reduction in serum concentration could lead to a contractile vSMC phenotype and increase calponin expression on the graft. The importance of the growth factor cocktail was highlighted by the combination of TGFβ1 for differentiation and bFGF for enhanced proliferation, which together with nutrient availability may have contributed to the expression of osteo- and chondrogenic markers near the lumen [44]. This hESC-derived attempt raises serious concerns over the vascular cell fate stability and plasticity on sTEVGs.

Niklson's group next focused on iPSCs drip-seeded on PGA scaffolds, splitting cultures into distinct proliferation (20% serum, PDGF-BB) and differentiation (10% serum, no growth factors) culture stages, each lasting 4 weeks [77]. The final stage incorporated mechanical stimulation to enhance differentiation [77]. Eliminating TGFβ1 and using only PDGF-BB reduced unwanted differentiation into osteo- and chondrogenic lineages [77]. This resulted in vSMCs positive for αSMA, SM22α, and calponin, but not the mature, contractile SMMHC marker [77]. It was again found that cells closer to the lumen were less differentiated, indicating that diffusion of nutrients or propagation of mechanical stimuli through the 250-μm thick wall, similar to the SV, may have had an effect [77]. The structure was highly collagenous, containing glycosaminoglycans and fibronectin, but no elastin [77], which is crucial for a biomimetic, mechanically responsive sTEVG. However, a BP of 700 mmHg and SRS of 30 g were measured for one graft [77]. Interestingly, karyotypically abnormal iPSCs led to high calcification and a senescent phenotype not seen with karyotypically normal cells [77]. Later, Gui et al. reverted to using both TGFβ1 and PDGF-BB with αSMA- and calponin-positive hiPSC-derived vSMCs to create a highly collagenous, SMMHC-positive sTEVG after being cultured for 9 weeks in vitro without mechanical stimulation [78]. Mature elastic fibers were still absent, despite a BP of 500 mmHg and SRS of 70 g in one graft [78]. Furthermore, the graft was implanted for 2 weeks as an abdominal aorta interposition graft in rats [78]. The graft did not rupture, remained

patent, recruited host cells, and did not result in teratomas [78]. A longer in vivo study is necessary because maximum thrombus formation occurs over the first 4 weeks, teratomas may take 4–6 weeks to form, and the slight dilatation that occurred could increase over time [67, 78, 79]. These sTEVGs are some of the most mechanically robust, but also take the longest to develop, a significant barrier to clinical relevance.

Mechanical stimulation shortens the required culture period, as shown for hiPSC-derived ECs seeded in a bioreactor and stimulated with a shear stress of 5–10 dyn/cm$^2$, resulting in arterial-like mature cells within 24 h [80]. The latter scaffold-based case can be classified as a "pre-sTEVG" because the cylindrical poly (L-lactic acid) construct measuring 5 mm in diameter and seeded with iPSC-derived synthetic vSMCs would be a perfusable sTEVG if the center was punctured out to create tissue rings [76]. Using 5% FBS, Wang et al. were able to induce the contractile SMC phenotype [76]. A nonfunctional, 2-week in vivo study was performed 24 h after cell seeding, which resulted in collagenous matrix formation and maintenance of the SMC phenotype [76]. Although the mechanical properties of the pre-sTEVGs are not known, these two cases indicate that a more clinically relevant timeline could be attained for achieving graft functionality, especially with the combination of mechanical stimulation and decreased serum concentration.

### 4.2.2 Tissue Engineering by Self-Assembly

The first PSC self-assembly method used Matrigel encapsulation of mouse ESCs to form a cell layer on a four-well Labtek Chamber-Slide culture system and yielded varied displacement of the gel with shear stress [42]. Abilez et al. suggested that differentiation into ECs, SMCs, and fibroblasts could be used to create an autologous TEVG [42]. However, the group did not demonstrate the ability to differentiate ESCs and assemble a vessel from individual cell layers. The next attempt used iPSC-derived vascular cell sheets cultured on a temperature-responsive surface that were subsequently wrapped around a 0.8-mm diameter PGA–L-lactide and poly(L-lactide-*co*-ε-caprolactone) scaffold [73]. This seeding method increased cellularization efficiency by 80% relative to the drip seeding method used in scaffold-based methods [73]. Upon implantation in an inferior vena cava interposition model, the graft showed no thrombus or aneurysm formation, graft rupture, or calcification [73]. An abdominal aorta interposition model would be more rigorous because of increased pressure and shear stress; however, host cells had replaced the implanted cells by 10 weeks [73]. Alarmingly, iPSC-derived cells did not colocalize with vWF or αSMA, suggesting de-differentiation. Furthermore, 25% of mice had teratomas [73]. Improved cell lineage commitment, complete differentiation, and cellular purification are needed before implanting PSC-seeded sTEVGs.

In a unique self-assembly method, a ring shaped agarose well containing culture medium including 20% FBS, PDGF-BB, and TGFβ1 was used to form highly cellularized, uniformly thick vascular conduits from hiPSC-derived vSMCs [81]. After 14 days of culture, tissue rings of 2 mm inner diameter and robust, contractile, and highly collagenous walls of 0.84 mm thickness were attained

[81]. Although αSMA, SM22$\alpha$, calponin, SMMHC, and elastin markers were present after differentiation, the presence of elastin and mature elastic fibers within the rings was not examined [81]. In combination with a 21-day differentiation protocol [81], this was one of the most clinically relevant timelines for facile production of robust sTEVGs. The group also modeled supravalvular stenosis syndrome by producing rings with decreased contractility, decreased SMMHC expression, and increased proliferation [81]. This study yielded both healthy and diseased physiologically relevant sTEVGs.

#### 4.2.3 Decellularized Matrices

Combining this sTEVG fabrication method with PSCs has been minimally investigated, but any of the mentioned efforts could be included by adding a decellularization step. Carefully karyotyped and characterized iPSC-derived cells could yield sTEVGs with uniform biological and mechanical properties after decellularization [77]. Although the risks of immunogenicity and teratomas could be eliminated using acellular grafts of decellularized matrix, the exclusion of iPSC-derived vSMCs would decrease the mechanical properties of the graft, already below those of native vessels. Future efforts should investigate decellularized matrices from hPSC and hPSC derivatives as implantable, off-the-shelf sTEVGs.

### *4.3 Remaining Challenges*

Opportunities for improving hiPSC-derived, patient-specific sTEVGs remain. Most sTEVGs would benefit from increased elastin content, better mechanical properties, lower cell lineage variability, reduced tumorigenesis, and clinically applicable production timelines. Culture of mature vSMCs and fibroblasts under pulsatile perfusion can yield mature elastic fibers within 30 days [82]. Both increased culture time and mechanical stimulation can increase elastin content of engineered vessels [82]. Similarly, increased ECM production can resolve the limited mechanical properties of PSC-derived sTEVGs because vessel mechanical properties are mostly provided by the ECM [82]. Co-culture of vSMCs with fibroblasts, which produce ECM significantly faster than vSMCs, might yield sTEVGs suitable for implantation [82]. Improved mechanical properties would help prevent dehiscence, rupture along the surgical anastomosis site, and compliance mismatch [76, 82].

The formation of teratomas shows the pressing need for the selectivity and maintenance of cell lineage commitment and fastidious purification of hPSC derivatives [44, 73]. However, hiPSCs from both healthy and type I diabetic patients can be reliably differentiated into vascular cells [83, 84]. A pure hPSC-derived population must be balanced with production of sTEVGs on a clinically applicable timeline, from cell isolation to graft cellularization. Samuel et al. found that 2D differentiation is more efficient than 3D differentiation and can be

accomplished within 2 weeks [83]. Emergency situations could require the use of banked, patient-matched iPSC-derived sTEVGs or decellularized matrices [77], whereas more extended fabrication of patient-specific grafts would be possible for chronic cases. Producing xenogeneic-free sTEVGs using human serum may also be beneficial for clinical trials, because culture with 20% FBS can result in up to 30 mg of bovine serum proteins in cells, which could cause an immune reaction or zoonosis [85].

Most groups have only used PSC-derived vSMCs to fabricate sTEVGs, showing the infancy of the application of hPSCs to sTEVG construction. Co-culture of vSMCs with fibroblasts or ECs has been shown to be synergistic by increasing collagen production or reducing thrombus formation, respectively [76, 82]. Co-culture could yield a fully biomimetic sTEVG that can be used to treat CAD and pediatric CCD patients and provide opportunities to model human vascular diseases for investigation and drug testing, which may help prevent the failure of expensive clinical trials [81]. Ultimately, hPSC-derived vascular cells could provide the means to achieve the ideal, patient-specific sTEVG with low thrombogenicity, long-term patency, and mechanical properties similar to those of native vessels.

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