REVIEW

Induced Pluripotent Stem Cell—Derived Endothelial Cells

Overview, Current Advances, Applications, and Future Directions

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Endothelial cells are prevalent in our bodies and serve multiple functions. By lining the vasculature, they provide a barrier to tissues and facilitate the transport of molecules and cells. They also maintain hemostasis and modulate blood flow by reacting to chemokines and releasing signal molecules. Thus, endothelial dysfunction leads to a wide variety of diseases, including atherosclerosis and coronary artery disease. In today's era of stem cell research, induced pluripotent stem cell—derived endothelial cells (iPSC-ECs) have emerged for research and engineering purposes. They are not only tools for studying disease states but are also a crucial part of efforts to engineer vessel and organ grafts. As the techniques in cell culture, microfluidics, and personalized medicine concomitantly improve, the potential for iPSC-ECs is enormous. We review functions of endothelium in our bodies, the development and uses of iPSC-ECs, and the possible avenues to explore in the future. (*Am J Pathol* 2019; 189: 502–512; <https://doi.org/10.1016/j.ajpath.2018.12.004>)

Endothelial cells (ECs) are epithelial cells derived from the mesoderm that line the luminal surfaces of blood and lymphatic vessels. They are dynamically involved in immune, hematological, and transport processes. In the resting state, endothelial cells regulate the transport of oxygen and micronutrients by varying vasomotor activity in reaction to molecules such as nitric oxide (NO), adrenaline, or angiotensin II. They also regulate vesicular transport and modulate barrier function by rearranging intercellular junctions and cytoskeletal proteins. Endothelial cells, which have a polygonal shape at rest, become gradually reoriented and elongated in the direction of flow on exposure to increasing shear stress.¹ This reorientation streamlines the endothelial cells to decrease the effective resistance and dynamically adapt to the shear stress stimulus. Endothelial cells produce antithrombotic molecules, such as NO and prostacyclin, to prevent adhesion of leukocytes and platelets. When

triggered by proinflammatory signals, such as tumor necrosis factor (TNF)- α or IL-6, endothelial cells up-regulate the expression of cell adhesion markers to allow immune cell migration and promote thrombosis by releasing von Willebrand factor and platelet-activating factor. Furthermore, endothelial cells interchange signal molecules with smooth muscle cells of the vasculature, platelets, and leukocytes, and they react to shear stress to minimize resistance and thrombosis. Core endothelial functions are illustrated in

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Figure 1 Schematic shows the important features of endothelial cell function. The left side of the figure highlights thromboregulation, with the left-most endothelial cell in the resting state promoting fibrinolysis and inhibiting the clotting cascade and platelet aggregation. The endothelial cell adjacent is in the active state with release of von Willebrand factor (vWF) from Weibel-Palade body and release of platelet-activating factor (PAF). vWF also initiates the clotting cascade when the subendothelium is exposed. On the right side, the immune function of endothelial cells is illustrated. A leukocyte rolls by binding to the selectin molecules expressed on the surface of endothelial cells, adheres via intercellular adhesion molecule 1 (ICAM-1; and vascular cell adhesion molecule 1, not illustrated), and migrates into the tissue. NO, nitric oxide; PECAM, platelet endothelial cell adhesion molecule 1; PGI₂, prostaglandin I₂; tPA, tissue plasminogen activator.

Figure 1. Further details have been discussed extensively in prior literature.^{2–9}

Although endothelial cells share these core functions, they also exist in great diversity. There are many ways to categorize endothelium into subtypes. For example, they can be divided by their vasculogenesis or angiogenesis formation during development—such as arterial, venous, or lymphatic; by size into macrovascular or microvascular; or in relation to organs. Morphologically, endothelial cells can be characterized as continuous, fenestrated, or sinusoidal. For example, the endothelium in the liver is sinusoidal and discontinuous to allow filtration of fluids, solutes, and macromolecules, and it can play a role in lipoprotein metabolism and atherosclerosis.¹⁰ On the other hand, macrovascular endothelium has tight junctions and maintains an impermeable barrier. Several markers have been found to be associated with these subtypes of endothelial cells. For example, these markers include Notch4, ephrin type-B receptor 4, and Coup-transcription factor II with venous subtypes; EphrinB2 and Notch1 for arterial subtypes; and podoplanin, prospero homeobox protein 1, and lymphatic vessel endothelial hyaluronan receptor 1 with lymphatic subtypes.¹¹ Endothelium also has organ-specific functions, such as angiotensin-converting enzyme production in the lung endothelium and plasma filtration in the spleen and liver via sinusoidal endothelial cells. Because of their diversity, each subtype of endothelium has its unique array of

identifiable markers. However, for research purposes, CD31 (platelet endothelial cell adhesion molecule 1), von Willebrand factor, CD144, and VEGFR2 are widely recognized as endothelial-specific markers shared across all subtypes.^{11–17}

Given their vast presence in our body and the multiple possible pathologies in cardiovascular, hematological, and immunologic systems, endothelial cells have great potential in disease research and regenerative medicine. Currently, human endothelial cells are most commonly harvested from human umbilical veins for laboratory study. However, these cells have limited applications, because most of our vasculature consists of microvasculature.¹⁸ Autologous endothelial cells can also be harvested for therapeutic purposes, but this requires invasive protocols with surgical risks. In this review, we discuss the differentiation of endothelial cells from human-induced pluripotent stem cells (iPSCs), ongoing efforts in disease research and therapeutic applications, as well as current limitations.

Induced Pluripotent Stem Cell–Derived Endothelial Cells

iPSC technology was pioneered by Shinya Yamanaka in 2006 and holds great promise in the field of regenerative medicine.¹³ iPSCs are derived from easily accessible mature

cells, such as dermal fibroblasts, and can be reprogrammed to differentiate into any of the three germ layers. There are already established methods of inducing cells into pluripotent stem cells using retrovirus techniques and ectopic expression of reprogramming factors octamer-binding transcription factor 4, sex determining region Y-box 2, Kruppel like factor 4, and c-Myc.^{13,19–22}

Development and Methods of Induction of iPSC-ECs

iPSC-ECs have been differentiated from multiple different methods and multiple cell lines.^{11,12,14,17,20,23–26} The concept for development of iPSC-ECs is derived from our understanding of vascular development in embryology. Vascular endothelial cells originate from mesoderm-derived angioblasts that proliferate into endothelial cells in the presence of vascular endothelial growth factor (VEGF), mediated by miR-21 and transforming growth factor- β 2.²⁷

Methods to differentiate iPSC-ECs rely mostly on four approaches: co-culture, embryoid body formation, two-dimensional culture with growth factors, and three-dimensional (3D) culture (Table 1).^{11,12,14,28} Earlier efforts to differentiate iPSCs into endothelial cells involved co-culture with stromal cells. Although the local factors from bone marrow stromal cells resulted in differentiation into endothelial cells, the yield was low, with high variability of endothelial differentiation.¹² More recent efforts have been made to further characterize the differentiation pathway and increase the efficiency and yield of iPSC-ECs. Using an embryoid body formation method to differentiate iPSCs in ECs, Rufaihah et al¹¹ showed that bone morphogenetic protein 4 plays a crucial role in differentiating iPSCs into the mesoderm lineage. In addition, high VEGF concentration was found to favor differentiation and specification into arterial subtypes, whereas VEGF-C and angiopoietin 1 were found to favor lymphatic differentiation.¹¹

Although improved from the co-culture method, this embryoid body formation method yielded only 6% to 16% endothelial cells.¹¹ Wang et al¹⁴ further expanded on the differentiation method via two-dimensional Matrigel culture. In the first differentiation stage, a combination of BMP4, activin A, basic fibroblast growth factor-2, and VEGF was used to drive the differentiation of mesoderm cells into cardiovascular cells.¹⁴ Subsequently, these cells were treated with a second combination of factors—Y27632, an inhibitor of ρ -associated protein kinase; SB431542, an inhibitor of transforming growth factor- β receptor; VEGF; and fibroblast growth factor—to induce the differentiation and proliferation of ECs. This direct differentiation method resulted in a decrease in production time from 2 to 3 weeks to 1 week and an increase in yield to 60%.

Furthermore, differentiation of iPSCs into endothelial cells has been shown to be dependent on both the growth factors present in the culture media and the structural support of the culture. Zhang et al²⁸ showed that endothelial cell differentiation is more efficacious when done in a 3D

fibrin scaffold (45% efficiency) versus in a two-dimensional culture in monolayer growth (5% efficiency).

Endothelial cells have been differentiated into arterial, lymphatic, and venous subtypes.¹¹ There is limited literature on iPSC differentiation into organ-specific microvascular endothelial cells, although a few studies do exist. For example, Katt et al²⁹ developed iPSC-derived human brain microvascular endothelial cells that have been shown to express tight junction proteins, transporters, and efflux pumps. Giacomelli et al³⁰ codifferentiated iPSC-ECs and iPSC cardiomyocytes and found that the endothelial cells expressed some cardiac markers present in primary cardiac microvasculature.

iPSC-ECs versus Endogenous Endothelial Cells

iPSC-ECs have been shown to have basic functions in immune, transport, hematological, and mechanical response. Structurally, iPSC-ECs express endothelial markers, such as CD31, CD34, and VEGFR, and are typically isolated on the basis of CD31 or CD144 (vascular endothelial–cadherin). In cultures, they can form a typical cobblestone-like shape, respond to shear stress by aligning, and form 3D networks on Matrigel in the presence of VEGF.^{11–14,28}

Functionally, iPSC-ECs have also been shown to express hematological markers, such as angiopoietin-2, and von Willebrand factor with Weibel-Palade body-like structures seen on electron microscopy.¹⁷ They express endothelial nitric oxide synthase and have the ability to endocytose fluorescent-acetylated low-density lipoprotein.^{17,31}

Immunologically, iPSC-ECs have responses to inflammatory markers that resemble the behavior of primary ECs, suggesting that iPSC-ECs can be used to model and study the inflammatory state. For example, when iPSC-ECs were exposed to proinflammatory stimuli, such as IL-1 β , TNF- α , and lipopolysaccharide, they expressed intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin.^{11,14} Secretion of proinflammatory cytokines, such as monocyte chemotactic protein-1, IL-8, regulated on activation normal T cell expressed and secreted, and interferon- γ –induced protein 10, was seen.¹¹ A barrier study in iPSC-ECs showed that histamine induced a transient increase in permeability and that VEGF induced a sustained increase in permeability in a monolayer of iPSC-ECs. On the other hand, prostaglandin E2 and sphingosine-1 phosphate produced a decrease in permeability.¹⁷

Although iPSC-ECs have been shown to have basic functions in immune, transport, hematological, and mechanical response, the qualitative functionalities of iPSC-ECs compared with primary endothelial cells have not been fully evaluated. Current available data suggest that iPSC-ECs' functionality may not yet be as robust as that of endogenous endothelial cells. iPSC-ECs had decreased capillary sprouting and a decreased number of vessel branch points compared with human umbilical vein

Table 1 Summary of Select iPSC-EC Differentiation Methods for Comparison

First author, year, and pluripotent cell type	Culture setting	Differentiation efficiency	Cell morphology	Cell surface marker and functionality	Response to inflammatory cytokines
iPSC-ECs					
Choi, 2009, ¹² multiple different fibroblast cell lines	OP9 feeders (mouse embryonic fibroblast feeder cell layer)	Variable in seven tested human iPSC lines (range, 2.8%–6%)	<ul style="list-style-type: none"> • Cobblestone morphology on culture plates • Tubular structure formation on Matrigel 	<ul style="list-style-type: none"> • CD31, vWF • VEGFR, VE-cadherin, CD49d, CD105 	Not evaluated
Rufaihah, 2013, ¹¹ adult dermal fibroblasts	Embryoid body formation method Various protocols for arterial, venous, lymphatic, and heterogeneous differentiation	6%–16%	<ul style="list-style-type: none"> • Cobblestone morphology on culture plates • Tubular structure formation on Matrigel 	<ul style="list-style-type: none"> • CD31, vWF • VEGFR, CD144, eNOS • Mixture of markers associated with arterial, venous, or lymphatic ECs • Positive Dil-ac-LDL uptake 	TNF- α stimulation up-regulated ICAM-1
Wang, 2016, ¹⁴ human adult dermal fibroblast	Differentiation using Matrigel culture with BMP4, activin A, bFGF, and VEGF	>60%	<ul style="list-style-type: none"> • Cobblestone morphology on culture plates • Tubular structure formation on Matrigel 	<ul style="list-style-type: none"> • CD31, vWF • ZO-1 • Alignment in longitudinal differential in response to low-flow stress modeling physiological flow 	TNF- α stimulation increased adhesion to human monocyte + disruption of tight junction
Zhang, 2014, ²⁸ human neonatal dermal fibroblasts	Differentiated in monolayer cells or 3D fibrin scaffold	Monolayer: 5% 3D scaffold: 45%	<ul style="list-style-type: none"> • Cobblestone morphology on culture plates • Tubular structure formation on Matrigel 	<ul style="list-style-type: none"> • CD31, vWF • CD144 • Positive Dil-ac-LDL uptake 	Not evaluated
ECs from primary cell origin					
HUVECs	Single-layer culture with collagen or gelatin-coated plates	NA	<ul style="list-style-type: none"> • Cobblestone morphology with large dark nuclei • Forming vessel-like structures with HDF in the presence of VEGF 	<ul style="list-style-type: none"> • CD31, CD105, vWF • Positive Dil-ac-LDL • Angiogenesis 	Variable among donors TNF- α stimulation enhances expression of ICAM-1 or VCAM-1
HAECs	Single-layer culture with collagen or gelatin-coated plates	NA	<ul style="list-style-type: none"> • Cobblestone morphology with large dark nuclei 	<ul style="list-style-type: none"> • CD31, CD105, vWF, DLL1 • Positive Dil-ac-LDL 	Variable among donors TNF- α stimulation enhances expression of ICAM-1 or VCAM-1
HMVECs (from all sources)	Single-layer culture with collagen or gelatin-coated plates	NA	<ul style="list-style-type: none"> • Cobblestone morphology with large dark nuclei • Forming vessel-like structures with HDF in the presence of VEGF 	<ul style="list-style-type: none"> • CD31, CD105, vWF, PECAM, DLL4 • Positive Dil-ac-LDL • Angiogenesis 	Variable among donors TNF- α stimulation enhances expression of ICAM-1 or VCAM-1

3D, three dimensional; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; Dil-ac-LDL, Dil-acetylated-low density lipoprotein; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; HAEC, human aortic EC; HDF, human dermal fibroblasts; HMVEC, human microvascular EC; HUVEC, human umbilical vein EC; ICAM-1, intercellular adhesion molecule 1; iPSC-EC, induced pluripotent stem cell–derived EC; NA, not available; TNF- α , tumor necrosis factor- α ; VCAM-1, vascular cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor, ZO-1, zonula occludens protein 1.

endothelial cells (HUVECs),³² despite another study finding that a proangiogenic regulator, Sox17, was increased in expression in iPSC-ECs.³³ In response to inflammatory signals, most iPSC-ECs expressed E-selectin and intercellular adhesion molecule 1, but only a fraction expressed vascular cell adhesion molecule 1.¹⁷ In addition, compared with HUVECs, iPSC-ECs displayed a lower VEGF-induced permeability and had a slower response to a cAMP analog. In another study, iPSC-ECs did not show a response to histamine and had a decreased response to TNF- α and IL-1 β compared with primary ECs.³³ Endothelial nitric oxide synthase 3 protein was lower in iPSC-ECs compared with human aortic endothelial cells.³¹ Given the heterogeneity of iPSC-ECs and differentiation protocols, it is difficult to generalize these findings for all iPSC-EC cell lines.

Applications

Disease Modeling and Drug Screening

With endothelial cells' wide variety of functions come many sources of dysfunction. Abnormal endothelial physiology or injury to the endothelium lining not only results in widespread systemic disease states, such as atherosclerosis and thrombosis, but also organ-specific disorders, such as preeclampsia, pulmonary hypertension, and retinopathy. In addition, endothelial cells play a critical role in a variety of human disorders, including cardiovascular disease, diabetes, chronic kidney failure, and tumor growth.³⁴

iPSC-ECs provide the advantage of studying endothelial physiology in many variable states of differentiation and diversity in an *in vitro* setting under a controlled environment. iPSC-ECs can also be derived from specific disease populations to study underlying differences in gene expression or response to drugs. Several disease states are expanded on below.

Atherosclerosis and Coronary Artery Disease

Atherosclerosis is a chronic inflammatory condition and a leading cause of mortality.³⁵ Several factors contribute to this disease, including inflammatory markers, mechanical response, lipids, and thrombosis. Tobacco smoking, abnormal circulating lipid levels, diabetes, and family medical history of cardiovascular disease are all risk factors. Endothelial dysfunction occurs before atherosclerotic lesions can be visualized,³⁶ and identifying early markers of endothelial dysfunction and potential targets for therapy is of great interest.

There are several processes involved in endothelial dysfunction. First is an impaired release of NO and subsequent loss of vasodilation, which can lead to decreased blood perfusion of tissues. This function is closely linked to the endothelium's ability to react to mechanical shear stress. Normally, shear stress induces an atheroprotective state by orienting endothelial cells to optimize blood flow with the

least resistance and by inducing NO and other factors to inhibit coagulation and leukocyte migration.⁶ However, in disease states, endothelial cells generate reactive oxygen species rather than producing NO, leading to endothelial release of chemokines and cytokines and up-regulation of adhesion molecules.⁵

iPSC-ECs have been shown to react to multiple factors described above; under atheroprotective flow, iPSC-ECs aligned in the direction of flow, expressed atheroprotection-associated Kruppel-like factors 2 and 4, up-regulated endothelial nitric oxide synthase and argininosuccinate synthase 1, and down-regulated von Willebrand factor.¹⁷ iPSC-ECs were also activated by inflammatory signals, such as TNF- α , IL-1 β , and lipopolysaccharide; they up-regulated E-selectin and intercellular adhesion molecule 1 and secreted cytokines, such as monocyte chemoattractant protein-1, IL-8, and interferon- γ -induced protein 10 to mediate leukocyte migration.¹⁷

Thus far, there have been limited studies examining these endothelial functions in diseased iPSC-ECs. iPSC-ECs have been used to model cadmium-induced atherosclerosis to link the p38 or extracellular signal-regulated kinase signaling pathways to cadmium-induced endothelial apoptosis and dysfunction.³⁷ Other existing studies have examined iPSC-ECs in the context of diabetes mellitus, a disease closely linked to endothelial dysfunction with devastating long-term end-organ damage. In diabetes, hyperinsulinemia leads to endothelin-1 secretion and increased expression of vascular cell adhesion molecule 1 and E-selectin, which promotes vasoconstriction and an inflammatory state.³⁸ Hyperglycemia increases reactive oxygen species, leading to decreased NO secretion.³⁹ In a mouse model study, iPSC-ECs derived from diet-induced obese mice demonstrated a reduced capacity to proliferate, migrate, and form cord-like structures *in vitro*.⁴⁰ When used in a hind limb ischemic experiment—in which endothelial cells are injected into mice with ischemic limbs to examine changes in perfusion—iPSC-ECs from obese mice were inferior to iPSC-ECs from normal mice, with increased muscle atrophy and presence of inflammatory cells.⁴⁰

In an analogous mouse study for coronary artery disease, human arterial or venous iPSC-ECs were injected into mice with a ligated left coronary artery. The survival rate of mice injected with arterial endothelial cells was comparable to those injected with venous endothelial cells. These iPSC-ECs were found to have greater NO production rate, greater protective response to shear stress, and lower TNF- α -induced inflammatory response than HUVECs.⁴¹ Another study administering human iPSC-ECs into immunodeficient mice showed that iPSC-ECs promoted angiogenesis of ischemic hind limb region and improved perfusion by 30% by comparison to vehicle group.⁴² These proof-of-concept studies are encouraging for pursuing iPSC-EC-derived clinical applications in ischemic diseases, such as peripheral artery disease and coronary artery disease.

Response to Pharmacologic Agents

In the age of personalized medicine and pharmacogenomics, iPSC-ECs provide the ability to study the autologous endothelium's response to drugs. Thus far, iPSC-ECs have been studied in lipid transport in the context of HMG-CoA reductase inhibitors. Simvastatin up-regulated kruppel-like factor 2, nitric oxide synthase 3, argininosuccinate synthase 1, thrombomodulin, integrin subunit beta 4, and prostaglandin D2 synthase and down-regulated proinflammatory angiopoietin 2 and endothelin 1 in iPSC-ECs at similar concentrations as primary ECs.¹⁷ In a study of iPSC-ECs derived from obese and normal mice, the iPSC-ECs responded to pravastatin with cell migration, proliferation, inhibition of apoptosis, and an increase in NO levels.⁴⁰ These appropriate pharmaceutical responses suggest that iPSC-ECs are candidates for studying lipid transport in both normal and disease states.

iPSC-ECs have not yet been used in studying other classes of drugs and their pharmacogenetics. Other iPSC-derived cells, such as cardiomyocytes and vascular smooth muscle cells, have been used to study the pharmacogenetics of antihypertensive drugs and may be of use in patients with resistant hypertension.⁴³ As the field of pharmacogenomics progresses, iPSC-derived cells may become more relevant in identifying clinically significant and targetable mutations.

Organ-on-a-Chip

An organ-on-a-chip is a 3D microfluidic cell-culture chip with the aim of converging research in tissue engineering, semiconductor fabrication, and human cell sourcing to generate reproducible and minimalistic organ tissue pathophysiology. The need for this technology has emerged from the current limitations of animal models and the promise of an unlimited supply of high-quality, patient-specific, iPSC-derived cells. Establishing a functional organ unit model could bridge discrepancies between traditional two-dimensional cell cultures and animal model studies with failed clinical trials.^{44,45} The NIH—including the National Center for Advancing Translational Sciences—is funding a complete program dedicated to developing human tissue chips, with the objective to better predict drug safety and efficacy. In this context, some groups have focused their efforts on building a human vascular microphysiological system for *in vitro* drug screening using human cord-blood–derived endothelial progenitor cells⁴⁶ or even iPSC-ECs.⁴⁷ Their results highlight the potential of microphysiological systems to improve the study of human diseases, drug responses, and 3D vascular network engineering.

Graft Engineering

Valvular Grafts

Generating durable and nonimmunogenic valvular grafts is of great interest in cardiac valvulopathies. Currently, homografts, xenografts, and mechanical prosthetic devices are used as short-term solutions for correcting heart defects.⁴⁸

However, patients receiving these grafts often require repeated surgeries to replace prostheses that degenerate over time. In cases of young patients, these grafts also lack the ability to grow with the recipients' bodies and hearts.

Although studies that examine valvular grafts composed of iPSC-ECs still need to be conducted, findings point toward iPSC-ECs being a promising resource for multiple reasons. First, endothelial cells have been shown to improve outcomes in these engineered grafts. Cases of porcine heart valve implantation without reendothelialization have ended in mortality due to valvular rupture or severe degeneration, likely caused by a severe inflammatory response triggered by exposed xenogenic collagen matrices.⁴⁹

Second, autologously sourced cells have better structural integrity because of reduced immunogenicity and inflammation. Valvular grafts generated from autologous and allogenic endothelial sources were compared in sheep models; there was trivial pulmonary regurgitation in autografts, whereas there was moderate regurgitation in allogenic valves, demonstrating the superiority of autografts to allografts in structural integrity.⁵⁰

Last, grafts generated with stem cells or progenitor cells may have the ability to adapt and grow with the recipient patient. A pulmonary valve seeded with autologous endothelial progenitor cells increased in size proportionately with the child and resulted in the resolution of the valvulopathy, suggesting that the tissue-engineered valve had the potential to remodel and grow when seeded with circulating mononuclear hematopoietic progenitor cells.⁵¹

Vascular Grafts

Vascular grafts are applicable predominantly in cardiovascular disease, but also in other areas, such as interventional nephrology for high-flow arteriovenous fistulas required in dialysis for end-stage kidney disease patients.^{52–56} There is a shortage of autologous blood vessels because of the fact that patients who require grafts already have existing systemic endothelial dysfunction and vascular disease. Furthermore, most vascular grafts in the lower extremities fail in the first decade due to progressive endothelial dysfunction and intimal narrowing.⁵⁷ There are commercial polymeric bypass grafts available; however, patency outcomes are poor for small-diameter grafts.⁵⁸ For these reasons, engineering autologous vessel grafts designed to be less prone to atherosclerosis is of great interest.

Efforts have been directed at seeding polymeric vascular grafts with endothelial and smooth muscle cells to generate a functional replacement that is structurally resilient to aneurysm formation and resistant to thrombosis and inflammation. One study used both iPSC-ECs and iPSC-derived smooth muscle cells seeded onto nanofibular scaffolds to generate vascular grafts.⁵⁷ The designed nanofibrils of the scaffolds directed the preferential alignment of the endothelial cells in the direction of flow to decrease inflammatory response and monocyte adhesion. The endothelial-seeded graft had significantly reduced monocyte

adhesion. When compared with primary endothelial cells, iPSC-ECs aligned in a similar manner.

Other engineering approaches have focused on three-dimensional printing technology. The advantages of such technology include an accurate control of the tissue tridimensional architecture and composition, high precision, and reproducibility through automation. There are no articles exploring the use of iPSC-ECs for bioprinting vascular grafts, but it has been proved to be a promising approach using primary human endothelial cells. In rodent models of hind-limb ischemia and myocardial infarction, it was demonstrated that implantation of 3D-printed grafts rescued the perfusion of distal tissues and prevented capillary loss, muscle atrophy, and loss of function.⁵⁹

Supportive Role in Organogenesis

By driving vascularization, iPSC-ECs hold the potential to support organogenesis and improve the functionality of the generated tissue. This has been proved with iPSC-derived liver buds cultured with HUVECS. Within 48 hours of transplantation into immune-deficient mice, these liver buds became vascularized tissues, exhibiting liver-specific maturation features, such as protein production and human-specific drug metabolism.^{60,61} A recent article published by Camp et al⁶² dissected the effect of interlineage communication between endothelial cells and hepatoblasts during liver bud development. The authors used single-cell RNA sequencing to decipher hepatocyte lineage progression during organogenesis and found that hepatic cell maturation was improved in the presence of HUVECS. In addition, using a receptor-ligand pairing analysis and a high-throughput inhibitor assay, they showed that the VEGF pathway potentiates hepatoblast differentiation and is essential to liver bud development. Similarly, recellularization of an intestine graft with human iPSC-derived intestinal epithelium and HUVECS resulted in the maturation of the intestinal epithelium and the development of nutrient absorption functionality after transplantation into rats.⁶³

The supportive role of endothelial cells in organogenesis using iPSC-ECs has also been demonstrated. In an organ-on-chip of the spinal cord, iPSC-derived brain microvascular cells promoted the maturation of iPSC-derived neuronal cells into spinal cord neural tissue.⁶⁴ Recently, a study showed the generation of iPSC-derived cardiac microtissues via simultaneous differentiation of cardiomyocytes and endothelial cells to recapitulate the cardiomyocyte-endothelium cross talk present in native human physiology. This *in vitro* model resulted in beating 3D cardiac microtissues and an increased expression of genes associated with cardiomyocyte maturation.³⁰ Similarly, 3D myocardial tissue constructed with human iPSC-derived cardiomyocytes, vascular mural cells, and ECs demonstrated excellent *in vitro* structural maturation and electromechanical performance. Implantation of this engineered cardiac tissue into *in vivo* rat models of myocardial infarction resulted in regenerated and revascularized

myocardium with improvement in the left ventricular function.⁶⁵ Foster et al⁶⁶ also showed that culturing corneal organoids derived from iPSCs led to the development of a self-organized, multilayered architecture that shared characteristics of epithelial, stromal, and endothelial cells. This study highlights that maturation of iPSC-derived organoids also relies on the formation of self-assembled endothelial cells. The potential role of iPSC-ECs to support maturation and functionality in organogenesis remains to be fully explored, but it promises to be an attractive avenue of investigation for regenerative medicine.

Grafts for Whole-Organ Transplantation

The field of transplant medicine has rapidly grown. Organ transplant is the preferred therapy for eligible patients with end-stage organ failure, but it comes with surgical and medical complication risks, such as organ rejection, and a lifetime course of immunosuppression with increased risk of toxicity, infection, and cancer. In addition, there is a national organ graft shortage with wait lists that are growing over time as more institutions implement transplant services.⁶⁷ In recent years, there have been efforts to engineer organ grafts that are less immunogenic with an adequate functional capacity for transplantation.

One approach to organ engineering is to recellularize extracellular matrices obtained by decellularizing unusable organs. iPSC-ECs are highly relevant to this method of whole-organ graft engineering for two major reasons. First, the iPSC-derivation technology presents the possibility of building an entire organ derived from patients' cells to reduce the risks of rejection and immunosuppression. Second, endothelialized vascular supply is critical in proper transport, hemostasis, immune processes, organ maturation, and organ functionality. Endothelial cells not only protect parenchymal cells from the shear stress of the blood flow, but they also provide barrier protection from the exposed subendothelium of the vasculature. Without reendothelialization, the exposed extracellular matrices are thrombogenic, leading to thrombosis and distal necrosis of the transplanted organ. Bioengineered organs without endothelialized vasculature cannot maintain flow for greater than a few hours because of blood clotting.⁶⁸ When organ matrices are decellularized, they maintain their vascular structure as well as the basement membrane and elastin fibers.⁶⁹ The initial step of preparing the scaffold to maintain structural integrity can be crucial in supporting revascularization; scaffolds that were prepared with 4% sodium deoxycholate had a greater area of endothelial cell coverage compared with those prepared with 8 mmol/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 1% SDS.⁷⁰ Furthermore, the scaffold may play an important role in the further differentiation of endothelial cells. When embryonic stem cells were seeded into a kidney scaffold, they proliferated into glomerular, vascular, and tubular structures with expression of differentiated immunohistochemical markers.⁷¹ In addition, the seeded embryonic stem cells also

remodeled the laminin and collagen of their basement membranes.⁷² In disease studies, the extracellular matrix has been shown to remodel in atheroprone regions with increased fibronectin, suggesting that the endothelium dynamically interacts with its surrounding matrix.²⁹

Reendothelialization has been explored in a variety of organs. In prior literature, human embryonic stem cells were cultured and then transplanted into severe combined immunodeficiency mice, where they formed capillaries along mouse vasculature.²⁵ Rat hearts were recellularized with rat aortic endothelial cells via media perfusion; the endothelial cells implanted onto endocardial surfaces, ventricular cavities, and the vascular tree, and they formed single layers in both larger and smaller coronary vessels.⁷³ Reendothelialization of the rat heart reduced scaffold thrombogenicity and improved left ventricular contractility and vascular tree.⁷⁴ In the lung, organ scaffolds have been repopulated with human umbilical cord endothelial cells, which successfully implanted throughout the scaffold vasculature.⁷⁵ Kidney scaffolds have also been revascularized with human umbilical cord endothelial cells, with successful repopulation of the vasculature.⁷⁶ Vascular resistance was found to be decreased after reendothelialization, although it was still higher than in cadaveric kidneys.

No published work, to our knowledge, has yet described iPSC-ECs repopulating organ scaffolds.

Summary, Limitations, and Future Directions

iPSC-ECs are highly relevant in both disease studies and therapeutic interventions. Multiple protocols have been established for differentiating iPSCs into endothelial cells, with ongoing efforts to further delineate more efficient methods of increasing yield, decreasing time, and further specializing differentiation into arterial, venous, or lymphatic types. Techniques in microfluidics may present opportunities to better control shear stress and cytokine concentration to increase the yield and specificity of cellular products.

There are several limitations of iPSC-ECs. Methods have not been established yet to differentiate iPSC-ECs into various organ-specific subtypes. Such progress would allow researchers to manipulate and study the functionality of organ-specific diseases, such as diabetic retinopathy, coronary artery disease, and pulmonary arterial hypertension. One approach currently proposed for studying pulmonary arterial hypertension is to compare the gene expression changes in iPSC-ECs versus primary ECs obtained from sites of interest to assess methylation changes.⁷⁷ Understanding disease states and genetic alterations in iPSC-ECs could help develop primary prevention treatments targeting endothelial dysfunction.

Furthermore, iPSC-ECs have yet to be thoroughly tested *in vivo* for vascular and valvular grafts. Although stem cells and progenitor cells have been shown to be able to adapt to patients' developmental growth, iPSC-ECs have not been

studied in this context. Moreover, iPSC-ECs will still need to be tested for several safety measures, including their propensity to form teratomas *in vivo* before being used for clinical applications. Therefore, safe, efficient, functional, and scalable protocols to differentiate iPSC-ECs will need to be thoroughly tested before reaching a clinical trial.

In addition, iPSC-derived grafts may not be practical in real-life practice; to generate autologously derived iPSC-derived grafts, foresight is required to construct grafts in time for surgical repair. As such, the use of iPSC-ECs may be less suited for cardiac surgery cases or for congenital heart defects in newborns, and may be more appropriate for nonemergent elective surgeries or in cases in which congenital heart disease is diagnosed *in utero* by ultrasound.⁷⁸ To circumvent this limitation, one option would be to use differentiated and ready-to-go iPSC-ECs for allogeneic transplantations. To this end, several organizations, such as the Global Alliance for iPSC Therapies, are working on a human leukocyte antigen haplobank to provide a repository of information on GMP and clinical-grade iPSC cell lines covering the worldwide population.^{79–81} Other groups are working on generating potential universal iPSC donor cells with minimal allogeneic response by engineering edited iPSC cells that express minimally polymorphic human leukocyte antigen-E molecules.^{82,83}

Future work could include engineering iPSC-ECs to generate vessel grafts with a decreased inflammatory response to reduce risk of atherosclerosis and intimal hyperplasia at the site of surgery due to intraoperative injury and subsequent monocyte adhesion.⁸⁴ In the field of organogenesis, the next steps are to characterize the reendothelialization of scaffolds and to study interactions between the extracellular matrix, surrounding cells, and iPSC-ECs.

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