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Generation of endothelial cells from human pluripotent stem cells: methods, considerations, and applications

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Abstract

Endothelial cells (ECs) are critical for several aspects of cardiovascular disease therapy, including vascular regeneration, personalized drug development, and tissue engineering. Human pluripotent stem cells (hPSCs) afford us with an unprecedented opportunity to produce virtually unlimited quantities of human ECs. In this review, we highlight key developments and outstanding challenges in our ability to derive ECs *de novo* from hPSCs. Furthermore, we consider strategies for recapitulating the vessel and tissue-specific functional heterogeneity of ECs *in vitro*. Finally, we discuss ongoing attempts to utilize hPSC-derived ECs and their progenitors for various therapeutic applications. Continued progress in generating hPSC-derived ECs will profoundly enhance our ability to discover novel drug targets, revascularize ischemic tissues, and engineer clinically relevant tissue constructs.

Keywords

endothelial cell differentiation; stem cell; cardiovascular disease; cell transplantation; tissue engineering

Subject codes:

Stem Cells; Endothelium/Vascular Type/Nitric Oxide; Vascular Disease; Cell Therapy; Vascular Biology

Why do we need endothelial cells?

Endothelial cells (ECs) comprise the innermost linings of blood and lymphatic vessels. ECs regulate many critical physiological processes, including interorgan communication,[1] accessibility of plasma constituents to the underlying parenchyma,[1] tissue perfusion,[2] inflammation,[3] and thrombostasis.[4] Dysfunctional ECs are key drivers of vascular

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pathologies such as hypertension,[5] coronary artery disease,[5] and diabetes.[6] Moreover, it is now increasingly recognized that dysfunctional ECs contribute to “non-vascular” diseases such as neurodegeneration[7] and chronic inflammatory disorders.[8] While it is difficult to precisely estimate the prevalence and burden of endothelial dysfunction, cardiovascular disease and diabetes alone affect 42% and 9% of the US adult population and incur annual costs of $597.2 and $327 billion, respectively.[9–11] Thus, the deleterious effects of EC dysfunction on mortality, morbidity, quality of life, and healthcare costs cannot be overstated.

A readily available source of human ECs has the potential to be an important tool for relieving cardiovascular disease burden. ECs allow us to model vascular pathology, uncover novel drug targets, and identify promising new pharmacotherapies *in vitro*. Furthermore, patient-specific ECs from induced pluripotent stem cells (iPSCs) makes personalized drug development a possibility.[12] ECs are also necessary for the vascularization of engineered tissue grafts or whole organs that may replace dysfunctional tissue in the future.[13] Given the potential importance of these cells to a range of therapeutic modalities, the ability to generate scalable quantities of well-defined ECs may help reveal novel cardiovascular disease treatments.

Methods for generating human endothelial cells *de novo*

Pluripotent stem cells

Primary human ECs obtained by endothelial biopsy[14–16] have provided invaluable insight into the molecular mechanisms of endothelial dysfunction in a number of disease states.[17–20] However, primary human ECs are a suboptimal source of ECs for cell therapy and tissue engineering applications because they are difficult to obtain, have limited replicative potential,[21] and are allogenic unless used in the same individual from which they were obtained. By contrast, human pluripotent stem cells (hPSCs) are capable of unlimited self-renewal and subsequent differentiation into any adult somatic cell type. As such, hPSCs are an attractive cellular source for generating ECs. The majority of hPSC-derived ECs have been generated from either human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs). hESCs were first isolated from the inner cell mass of human blastocysts by Thomson and colleagues.[22] While these cells have been used extensively to generate a variety of adult cell types, there are ethical concerns with using human embryonic tissue. Yamanaka and colleagues overcame this issue when they discovered that human dermal fibroblasts can be re-programmed into hiPSCs by retroviral transduction with 4 pluripotency factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*).[23] hiPSCs are especially valuable because they can be derived from easily accessible cell sources and they allow for the generation of patient-specific hPSCs – essential for the realization of personalized medicine.

While hiPSCs have significant therapeutic and scientific potential, they do have limitations. One important consideration when using hiPSCs, especially early passage hiPSCs, is that they may contain epigenetic imprinting left over from the original cell type.[24,25] Indeed, we have found that when using early-passage hiPSCs, the donor cell source can affect EC differentiation efficiency, gene expression, and identity maintenance.[26] Using later-passage hiPSCs may help to mitigate the confounding effects of leftover epigenetic imprinting.

However, there are also risks with using later passage hiPSCs including accumulation of somatic mutations[27] and chromosomal aberrations.[28,29] Furthermore, we have found that using later-passage hiPSCs can reduce the efficiency of hiPSC-EC differentiation for certain donor cell types.[26] Thus, it is important to match donor cell source and hiPSC passage number when comparing ECs generated from different hiPSCs.

Coculture-based methods

After establishing the first hESC lines, Thomson and colleagues found that CD34+ hematoendothelial precursors (HEPs) can be generated by culturing hESCs on feeder layers of either murine bone marrow stromal cells (M2–10B4 and S17) or yolk sac endothelial cells (C166).[30] Sorting these CD34+ cells and culturing them in endothelial growth media promotes the differentiation of HEPs into ECs (Figure 1 & Table 1).[31,32] Other groups have used similar strategies with hESCs and hiPSCs grown on OP9 (murine bone marrow stromal cells) cells[33,34] and hESCs grown on mouse embryonic fibroblasts (Figure 1).[35] While coculture methods allow for efficient HEP generation,[36] they are not suitable for clinical translation due to the requirement of murine feeder layers. However, elucidating feeder cell-derived cues that promote EC differentiation may offer insight when developing xenogeneic-free protocols for EC generation.

Embryoid body-based methods

Embryoid bodies (EBs) are hPSC aggregates formed by culturing hESCs in suspension and removing pro-pluripotency factors from the media. Spontaneously differentiating EBs can generate all 3 germ layers[37] and are thought to mimic aspects of human embryogenesis. Langer and colleagues first demonstrated that ECs can be isolated from spontaneously differentiating EBs, albeit at a very low yield (~2%).[38] Subsequent studies found that treating EBs with a cocktail of hematopoietic cytokines and bone morphogenic protein-4 (BMP-4) directs hESC differentiation towards a HEP fate.[39] HEPs can then be specified to ECs by culturing them in endothelial growth medium (Table 1).[40–43]

Based on work in murine ESCs,[44–46] Keller and colleagues developed a protocol to generate vascular endothelial growth (VEGF) receptor 2^+ cardiovascular progenitors (CVPs). These cells are distinct from HEPs and can give rise to ECs.[47] This method utilizes sequential treatment of EBs with Activin A, BMP4, and fibroblast growth factor 2 (FGF2), followed by Dickkopf-related protein 1 (DKK1) and VEGF-A to direct differentiation of EBs to a cardiogenic mesodermal fate. Sorted and adherent CVPs can then be treated with VEGF-A, DKK1, and FGF2 to generate cardiomyocytes, ECs, and smooth muscle cells. Directed differentiation of hPSCs through cardiogenic mesoderm and CVP lineages forms the basis for a number of hPSC-EC differentiation protocols now in use (Figure 1, Table 1).[26,48–55]

Other improvements in EC differentiation efficiency have been made using EBs. Namely, treating EBs continuously with VEGF-A increases the notoriously low yield of ECs from EBs.[56] Furthermore, Rafii's group found that chemical inhibition of transforming growth factor β (TGFβ) signaling with SB431542 during EC specification and maintenance also increases the yield of ECs (Figure 1, Table 1).[57] Inhibition of TGFβ signaling appears to

prevent the differentiation of CVPs into smooth muscle cells, thereby preferentially promoting an endothelial fate.

Studies using hESC-based EBs have contributed substantially to our knowledge of EC specification. EBs retain physiological 3D architecture and approximate *in vivo* embryogenesis. Presumably, recapitulating *in vivo* EC development as closely as possible affords the best opportunity for generating hPSC-ECs that most faithfully mimic their *in vivo* counterparts. Furthermore, suspension culture conditions are amenable to scaling EC production for clinical applications. However, EB-based methods have certain limitations, including a generally low yield of ECs, heterogenous cellular exposure to media components and growth factors, complex culture conditions, and the mechanical dissociation required to obtain single cells.

2D monolayer-based methods

Leveraging the directed differentiation protocols developed in EBs, Mummery and colleagues found that ECs can be generated in a monolayer by adding a chemical glycogen synthase kinase-3 inhibitor (CHIR99021 or CHIR) to the cardiogenic mesoderm induction cytokine cocktail.[52] Subsequently, Palecek and colleagues found that treating hiPSCs with CHIR alone, in the absence of cytokines, can generate brachyury+ mesodermal cells. Culturing of these progenitor cells in endothelial growth media generates ECs.[58] Using a single chemical inhibitor to induce cardiogenic mesoderm formation, rather than a cocktail of cytokines, substantially lowers the cost and variability of EC differentiation protocols. Several groups now utilize CHIR to induce cardiogenic mesoderm prior to specifying EC fate (Figure 1, Table 1).[59–61] Interestingly, Patsch et al. observed that adding BMP-4 to CHIR during the mesoderm induction phase greatly enhances the yield of mesodermal cells and, subsequently, ECs from hPSCs.[62] These investigators also discovered that adding forskolin, a protein kinase A activator, and VEGF-A to mesodermal cells promotes EC specification following mesodermal induction.

Improvements in EC yield and lineage specificity have also been realized using monolayer-based protocols. Yoder and colleagues found that sorting Neuropilin-1^{+} cells from CVPs and culturing them in endothelial growth media promotes the formation of endothelial colony-forming cells.[51] Importantly, these cells demonstrate very high proliferation rates, which is important for scalable production of ECs. As alluded to above, ECs have been derived from both HEPs and CVPs. Palpant et al. found that by modulating levels of BMP-4 and Activin A, it is possible to specify hemogenic and cardiogenic mesodermal precursors.[54,63] Both of these progenitors can give rise to definitive ECs. Interestingly, the ECs derived from hemogenic mesoderm also have blood-forming capacity. This suggests that ECs moving through a hemogenic mesodermal intermediate may be similar to ECs derived from HEPs and perhaps to hemogenic endothelial cells.[64] Alternatively, ECs derived from cardiogenic mesoderm may be more similar to the CVP-derived ECs described by Keller and colleagues.[47] Although ECs stemming from these different lineage trajectories share many features, they also have functional and transcriptional differences.[54,65] Thus, it is important to consider the lineage trajectory of hPSC-derived ECs when choosing or developing a

differentiation protocol. Further studies are needed to uncover potential subtle differences among these ECs that may affect their suitability for downstream applications.

Monolayer-based protocols are now the most common method for generating ECs from hPSCs. They offer advantages such as ease of use, clinical translatability, control of cellular exposure to media components, and easy integration with various phenotyping techniques. However, monolayer-based differentiation is not ideal for industrial-scale EC production nor does it necessarily recapitulate embryological EC development. Specifically, EBs more faithfully represent the 3-dimensionality and cellular contact geometry of *in vivo* embryogenesis than do monolayer-based methods. These tradeoffs should be considered in the context of the subsequent application when choosing an hPSC-EC differentiation protocol.

Transdifferentiation

Studies over the last decade have demonstrated that adult somatic cells can be directly reprogrammed into ECs without moving through a pluripotent state. Three main strategies have been used to achieve this goal (Figure 1 and Table 1). Both amniotic cells[66] and fibroblasts[67,68] can be directly converted to ECs by overexpressing transcription factors necessary for EC development.[69] The E-twenty-six (ETS) transcription factor ETV2 appears to be particularly important for this mode of transdifferentiation. Other groups have shown that brief treatment with pluripotency factors can de-differentiate fibroblasts to a multipotent mesodermal state. Subsequent culture of these cells in endothelial growth media completes endothelial reprogramming.[49,70,71] Finally, Sayed et al. partially de-differentiated fibroblasts with a chemical Toll-like receptor 3 agonist (poly I:C) before reprogramming these cells into ECs.[53] While transdifferentiation avoids certain limitations associated with using hiPSCs (e.g., extended culture time and teratoma risk), the EC yield of this approach is limited and it is unclear whether cells are fully reprogrammed.

Over the last 20 years, significant advances have been made in generating ECs from a variety of cellular sources. The methods described above differ in efficiency, differentiation trajectories, clinical translatability, cost, complexity, variability, scalability, and relevance to *in vivo* vascular development. As with most scientific techniques, the proper choice of EC differentiation protocol depends on the downstream application.

Capturing endothelial heterogeneity

The structure and function of ECs vary greatly depending on the specific vessel and tissue bed in which they are located (Figure 2B).[72,73] This heterogeneity arises from variable developmental trajectories as well as vessel and tissue-specific microenvironmental stimuli.[74] These genetic and environmental influences are imprinted epigenetically and transcriptomically as different EC populations have unique molecular signatures.[75–77] Choosing the appropriate EC subtype is an important consideration when generating ECs *de novo*, especially for disease modeling and drug screening. The differentiation strategies discussed thus far typically result in a heterogeneous mixture of ECs.[61,78] However, some groups are now optimizing differentiation protocols to generate ECs with vessel and tissue-specific properties.

Vessel specificity

Several strategies have been developed to generate ECs resembling those of arteries, veins and lymphatic vessels. Given the myriad developmental and environmental cues that govern EC identity *in vivo*, there are a number of different strategies to specify EC identity *in vitro* (Figure 2A). The simplest approach is to identify, isolate, and expand arterial and venous progenitor ECs from a heterogeneous population. Using a protocol optimized for HEP differentiation, Keller and colleagues found that $CD34^{+}CD73^{med}CD184^{+}$ and $CD34^{+}CD73^{+}CD184^{-}$ cells have the propensity to become arterial (AECs) and venous ECs (VECs), respectively.[79] Such progenitors have not yet been identified in cardiogenic mesodermal populations.

A more common strategy for imbuing ECs with vessel-specific characteristics is to modify cellular signaling by adding small molecules and growth factors. Extensive investigation of vascular development in various model systems has identified high levels of VEGF-A and NOTCH signaling as key drivers of arterial specification.[80] Indeed, high levels of VEGF-A promote the formation of AECs from hPSCs.[78,81] In a very elegant study, Zhang et al. found that AECs can be generated by treating CVPs with 5 factors (TGFβ and inositol monophosphatase inhibitors, resveratrol, FGF2, and VEGF-A) while removing insulin from the media.[81] Importantly, they rigorously demonstrated that the resulting AECs functionally phenocopy primary coronary AECs with respect to oxygen consumption, leukocyte adhesion, nitric oxide production, and the response to shear stress. Finally, activating protein kinase A with a cyclic adenosine monophosphate derivative (8-Br-cAMP) also appears to encourage AEC formation from hPSCs.[78]

In addition to biochemically directed differentiation, mimicking *in vivo* biophysical forces is another strategy to specify ECs (Figure 2A). For instance, exposing hPSC-derived ECs to physiological levels of shear stress[82] or hypoxia[83] increases the expression of arterial markers. Furthermore, culturing murine EC progenitors on firmer or softer polydimethylsiloxane substrates promotes AEC and VEC specification, respectively.[84] While extracellular matrix composition and stiffness certainly affect EC differentiation from hPSCs,[85,86] whether these parameters can be tuned to generate vessel-specific ECs remains to be determined.

Most studies thus far have focused on generating AECs from hPSCs. Some groups have made progress identifying soluble factors that promote specification of venous and lymphatic ECs (LECs). As alluded to above, treating hiPSC-derived endothelial progenitors with lower levels of VEGF-A specifies VECs.[78] BMP-4 and insulin also appear to drive VEC specification of mesodermal progenitors.[81] With respect to the lymphatic vasculature, VEGF-C and angiopoietin-1 have been used to produce LECs from hPSCs.[87] One salient observation is that, to the best of our knowledge, no protocols have been developed to specifically generate capillary ECs (CECs). This is most likely due to a dearth of knowledge regarding the developmental trajectory and molecular signature of CECs. CECs mediate fluid and solute exchange between the circulation and parenchyma, and comprise the vast majority of endothelial surface area *in vivo*. As such, they may prove valuable in predicting drug responses and vascularizing engineered tissue constructs.

Tissue specificity

Endothelia from different tissues vary in their response to vasoactive compounds, permeability, transport capacity, and secretomic activity (Figure 2B). To date, blood-brain barrier (BBB) ECs have been the primary target of tissue-specific EC generation. Shusta and Palecek found that treating mesodermal precursors with retinoic acid is key to generating ECs that can form high selectivity, low permeability barriers characteristic of the BBB.[88,89] Alternatively, other groups have derived BBB ECs by culturing cord blood progenitor cells with either pericytes[90] or astrocytes.[91] Soluble factors that promote specification of corneal,[92] choroidal,[93] and outflow tract ECs[94] have also been identified. Finally, Goldman et al. found ECs in a population of hPSC-derived hepatic progenitor cells.[95] These findings suggest that some liver ECs may originate from the endoderm.

Our ability to generate specific subsets of mature ECs is limited, but improving. A deeper understanding of vascular development, parenchyma–EC interactions, and the effects of biophysical forces on EC function will enhance our ability to control EC differentiation *in vitro*. Progress in this area will be especially critical for developing *in vitro* disease modeling and drug screening systems with sufficient predictive power.

Metrics for defining success in generating ECs *in vitro*

The most important aspect of an EC differentiation protocol is whether it generates cells that functionally and molecularly resemble ECs *in vivo*. There are many phenotyping methods available to verify endothelial identity. This subject has recently been covered in great detail[96] and will only be discussed briefly here. The most common methods for confirming EC identity include observation of a cobblestone-like morphology, expression of “EC-specific” genes and proteins (e.g. CD31, vascular endothelial cadherin, von Willebrand factor, and endothelial nitric oxide synthase), endocytosis of fluorescently labeled acetylated low-density lipoproteins (Ac-LDL),[97] and formation of tubular structures in a two-dimensional *in vitro* matrix preparation.[98] These techniques are inexpensive and easy to perform, which make them excellent tools for the initial characterization of ECs. However, these characteristics are not necessarily specific to ECs. For instance, “EC-specific” markers can be expressed by many other cell types, and macrophages can take up Ac-LDL.[99]

A more rigorous validation of endothelial identity requires demonstrating that putative ECs are able to form patent vascular networks *in vivo* using a 3D vasculogenesis assay.[100] In this experiment, putative ECs are suspended in a 3D matrix, typically Matrigel,[101] and implanted subcutaneously in mice. Subsequently, the ability of ECs to form a perfusable patent vascular network and anastomose with the host vasculature is determined. Another increasingly common approach is to use high dimensionality measurements such as RNA-sequencing or metabolomics to compare hPSC-derived ECs to primary human ECs.[62] These techniques afford a huge number of identifiers (i.e., metabolites and transcripts), which can be used for high resolution comparisons between ECs generated *de novo* and primary human ECs. The implicit assumption with this approach, of course, is that gene expression or metabolite levels reflect similarities in cellular function, which is the ultimate metric for confirming endothelial identity. Additional functional assays include measuring nitric oxide production in response to vasoactive compounds,[53] determining metabolic activity through

oxygen consumption measurements,[81] assessing endothelial activation in response to inflammatory stimuli,[102] and observing the phenotype of ECs when exposed to shear stress or flow.[103]

Comparators

As described above, putative ECs can be compared to primary human ECs to verify the success of a differentiation protocol. Given that ECs from different vessel types (e.g., artery, vein, etc.) and tissues exhibit substantial heterogeneity,[72,73] it is important to consider the final application when choosing a comparator cell type. For instance, if the goal is to model EC dysfunction associated with coronary atherosclerosis *in vitro*, differentiation protocols should direct ECs towards a coronary artery EC phenotype. One major caveat to this approach, however, is that commercially available primary ECs are maintained in static culture. ECs rapidly de-differentiate and lose many of their identifying characteristics after a short time in culture.[76] Thus, using fresh, rather than cultured, primary ECs as targets for differentiation protocols will generate ECs that more closely mimic their *in vivo* counterparts.

Therapeutic applications of endothelial cells

Given the prevalence of ischemia and endothelial dysfunction in various diseases, a readily available source of autologous human ECs has enormous therapeutic potential. Below, we consider some ongoing and future applications of ECs generated *de novo*.

Vascular regeneration

Ischemia is a major cause of tissue death in a number of diseases, including coronary and peripheral artery disease, stroke, and diabetes. One potential strategy for restoring perfusion in these conditions is to administer autologous hPSC-derived ECs directly into ischemic tissue (Figure 1). The goal for injected ECs to form new blood vessels in the ischemic region, thus restoring blood flow and tissue function. Thus far, investigators have administered CD34+ bone marrow-derived myeloid angiogenic cells to human patients with various cardiovascular diseases. Treatment with these cells may offer some limited benefit to patients suffering from refractory angina,[104–106] ischemic heart failure,[107] nonischemic dilated cardiomyopathy,[108] and critical limb ischemia.[109] However, CD34+ cells do not appear to improve perfusion or left ventricular function over placebo when they are administered following acute myocardial infarction.[110] As perfusion was not measured in the studies demonstrating CD34+ cell therapy benefit, it is unclear whether the improvement in cardiac function is due to vascular regeneration per se or paracrine effects on parenchymal cells,[111] though it is more likely due to paracrine effects because the extent to which CD34+ cells engraft in ischemic tissue is usually very low.[108]

The therapeutic potential of more mature hPSC-ECs is still being investigated in pre-clinical studies. The data so far are promising. hESC-ECs and hiPSC-ECs administered intramuscularly to ischemic murine hindlimbs increase capillary density and restore perfusion.[48,112–114] hPSC-ECs have also been shown to improve myocardial perfusion and left ventricular function in animal models of myocardial infarction,[115] especially when given

in combination with other cardiovascular cell types.[116] Similar to CD34+ cells, however, retention and engraftment of hPSC-ECs in the infarct zone remain low.[115,117] To overcome these limitations, investigators have begun suspending hPSC-ECs in pro-survival hydrogels prior to injection.[60,118] This approach enhances cellular engraftment and vascular regeneration in mouse models of hindlimb ischemia.

In summary, the use of hPSC-derived ECs has shown promise in restoring function to ischemic tissue in pre-clinical animal models. Optimism for this strategy is currently limited by low cellular survival and engraftment in the target ischemic tissue following administration. Immediate challenges for cell therapy-based vascular regeneration include determining optimally regenerative cell types, enhancing cellular engraftment, and developing strategies for restoring the quality of autologous hPSCs that may be adversely affected by disease.[119,120] Regenerative vascular cells of the future must be able to be delivered systemically, home in on ischemic tissue, survive and proliferate in the harsh ischemic environment for prolonged periods of time, and have sufficient angiogenic and neovascularizing capacity to restore perfusion.

Tissue engineering

An alternative approach to regenerating tissue with cell therapy is to simply replace the dysfunctional tissue with grafts engineered *ex vivo* (Figure 1). Engineered tissue grafts are now available for most organs, including the heart,[121] lung,[122] liver,[123] skeletal muscle,[124] kidney,[125] pancreas,[126] and skin.[127] One limitation of engineered tissue constructs, however, is that as the tissue becomes thicker than 100–200 μm, it becomes increasingly difficult for oxygen and nutrients to reach the cells residing in the core of the tissue. This limitation can be overcome by creating vascularized, perfusable tissue constructs. Having a readily available source of ECs such as hPSC-ECs therefore is critical for engineering tissue to clinically relevant scales. A simple approach to vascularizing tissue constructs is to provide ECs with various chemical, cellular, or biophysical cues and allow them to self-assemble into vascular networks.[55,128–133] While this elegant approach harnesses the power of cell-directed vasculogenesis, the resulting microvasculature is variable and not necessarily physiological, and mostly requires *in vivo* transplantation for perfusion. Alternative strategies for engineering microvascular networks involve fabricating microfluidic channels within biomaterials that can be subsequently endothelialized.[134] To date, investigators have mostly utilized human umbilical vein ECs[103,135,136] to line these microchannels, although hPSC-derived ECs are now being used.[137] An important consideration is the diameter of microchannels used to innervate tissue constructs. Specifically, physiological capillaries are ~5μm whereas engineered microchannels can range from tens to hundreds of microns depending on the fabrication technique. Reducing microchannel diameter will improve the physiological relevance of tissue-engineered microvascular networks.

In addition to engineering microvascular networks for tissue constructs, hPSC-derived ECs may also be useful for generating larger blood vessels. Tissue-engineered large blood vessels have great therapeutic potential in a number of clinical settings, including bypass grafting, hemodialysis access, and congenital cardiovascular defects.[138] Thus far, autologous cellular grafts containing blood mononuclear cells[139] and fibroblasts[140] have been used in humans.

Grafts utilizing hPSC-ECs are now being tested in pre-clinical studies.[141] Whether pre-endothelializing vascular grafts will improve their patency in humans remains to be determined.

Personalized disease modeling and drug development

The holy grail of personalized medicine is to model patient-specific disease processes *in vitro* and subsequently identify drugs that would be most efficacious for that patient. hiPSCs are essential for this goal as they provide an extensive source of patient-specific somatic cells. These cells can be used to elucidate disease mechanisms and directly test potential therapeutics. Perhaps the most tractable diseases to model *in vitro* are those caused by mutations in single genes. hiPSC-ECs have been used to model and identify drug targets in monogenic vascular diseases,[96] such as familial pulmonary arterial hypertension[142] and hemophilia A.[143] The more prevalent diseases of the vasculature (e.g., atherosclerosis) are heterogeneous in etiology and involve complex interactions between environmental and genetic factors as well as multiple cell types. As such, they are challenging to model *in vitro*. Several groups have devised strategies to model aspects of atherosclerosis,[144] diabetes,[145] and hypertension[146] *in vitro*. Ideally, hiPSC-ECs should be combined with these disease models to allow for the prediction of patient-specific disease predisposition and drug responses (Figure 1). As a proof-of-principle, our group has found that doxorubicin is toxic to hiPSC-derived cardiomyocytes from patients who also display doxorubicin-induced cardiotoxicity clinically.[147] With respect to ECs, hiPSC-ECs have been used to assess the toxicity of tyrosine kinase inhibitors[148] and identify bioactive compounds that may affect vascular development.[149]

The power of hiPSC-ECs to predict vascular disease development and drug efficacy, while promising, has not yet been tested explicitly. One inherent limitation on the predictive power of *in vitro* iPSC-based model systems is that they may not capture sufficient complexity to approximate *in vivo* physiology.[150] Improving the physiological relevance of *in vitro* systems is an active area of research. Investigators are now using self-assembled vascular organoids[55] and “vasculature-on-a-chip” platforms[151,152] to study the effects of disease processes and drugs on iPSC-derived vasculature. These systems permit the inclusion of fluid flow, vascular architecture, and interactions between different cell types – all critical contributors to physiological EC function.

The therapeutic applications of hPSC-derived ECs have lagged behind those of other cell types such as cardiomyocytes. This is most likely due to a combination of factors, such as suboptimal differentiation protocols and a focus on parenchymal cell types as drivers of disease. hPSC-ECs have enormous therapeutic potential given the prevalence of diseases with strong ischemic and endothelial dysfunction components. Realization of this potential will require better understanding and control of vascular development as well as finding the appropriate balance of complexity, cost, and usability to model vascular complexity *in vitro*.

Conclusions

Since the establishment of the first hESC lines, scientists and clinicians working in the fields of development, vascular biology, and cardiovascular medicine have endeavored to generate

human ECs *de novo*. Currently, most investigators use 2D monolayer-based protocols which guide hiPSCs through a cardiogenic mesoderm precursor state before EC specification. A better understanding of the developmental and microenvironmental cues that control endothelial phenotypes will allow us to generate hPSC-ECs with improved vessel and tissue-specific properties. Over the last 20 years, hPSC-ECs have provided us a viable system for researchers to investigate vascular development and endothelial function. However, we have only begun to glimpse the full therapeutic potential of these cells. Thus far, CD34+ myeloid angiogenic cell therapy for cardiovascular disease has been largely underwhelming. It remains to be seen whether more mature hPSC-ECs, especially those engineered to engraft and survive in harsh environments, will be more effective at restoring perfusion and function to ischemic tissue. Finally, hPSC-ECs will be crucial for engineering vascularized tissue constructs which can be used for drug screening, disease modeling, and restoration of tissue function. In summary, hPSCs-ECs are a promising source of ECs which, with continued progress, have the potential to produce novel therapies for vascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

Ac-LDL	acetylated low-density liporoptein
AEC	arterial endothelial cell
BBB	blood-brain barrier
BMP-4	bone morphogenic protein 4
CEC	capillary endothelial cells
CVP	cardiovascular progenitor
EB	embryoid body
EC	endothelial cell
FGF2	fibroblast growth factor 2
HEP	hematoendothelial precursor
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell

hPSC	human pluripotent stem cell
LECs	lymphatic endothelial cells
TGFβ	transforming growth factor beta
VECs	venous endothelial cells
VEGF	vascular endothelial growth factor

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Highlights

- Endothelial cells can be generated from human pluripotent stem cells by either co-culture, monolayer, or embryoid body-based differentiation strategies.
- The most common strategy for deriving endothelial cells involves monolayer-based differentiation of human pluripotent stem cells through a cardiogenic mesodermal intermediate prior to endothelial specification.
- Endothelial cells can be imbued with vessel and tissue-specific properties by modulating oxygen tension, exposing endothelial cells to shear stress, altering the biochemical composition of the media, culturing endothelial cells on substrates of different stiffness and co-culturing endothelial cells with other cell types.
- Human pluripotent stem cell-derived endothelial cells have shown promise in treating pre-clinical models of ischemia, vascularization of engineered tissue constructs, modeling of monogenic vascular diseases, and drug screening.

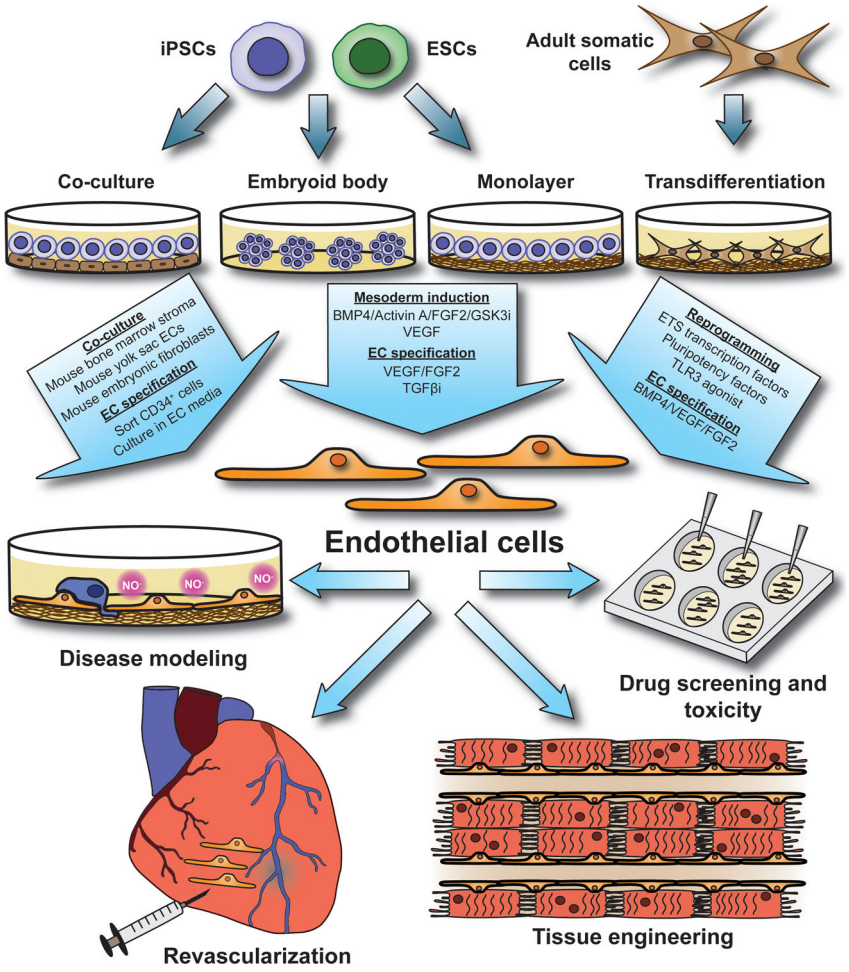

Figure 1. Strategies for generating endothelial cells *de novo* and their subsequent applications.

Induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), and adult somatic cells can be differentiated into endothelial cells (ECs) using co-culture, embryoid body, monolayer or transdifferentiation protocols. The general features of the most commonly used protocols are shown in the thick blue arrows. ECs generated by these various approaches can be used for various therapeutic applications. NO - nitric oxide.

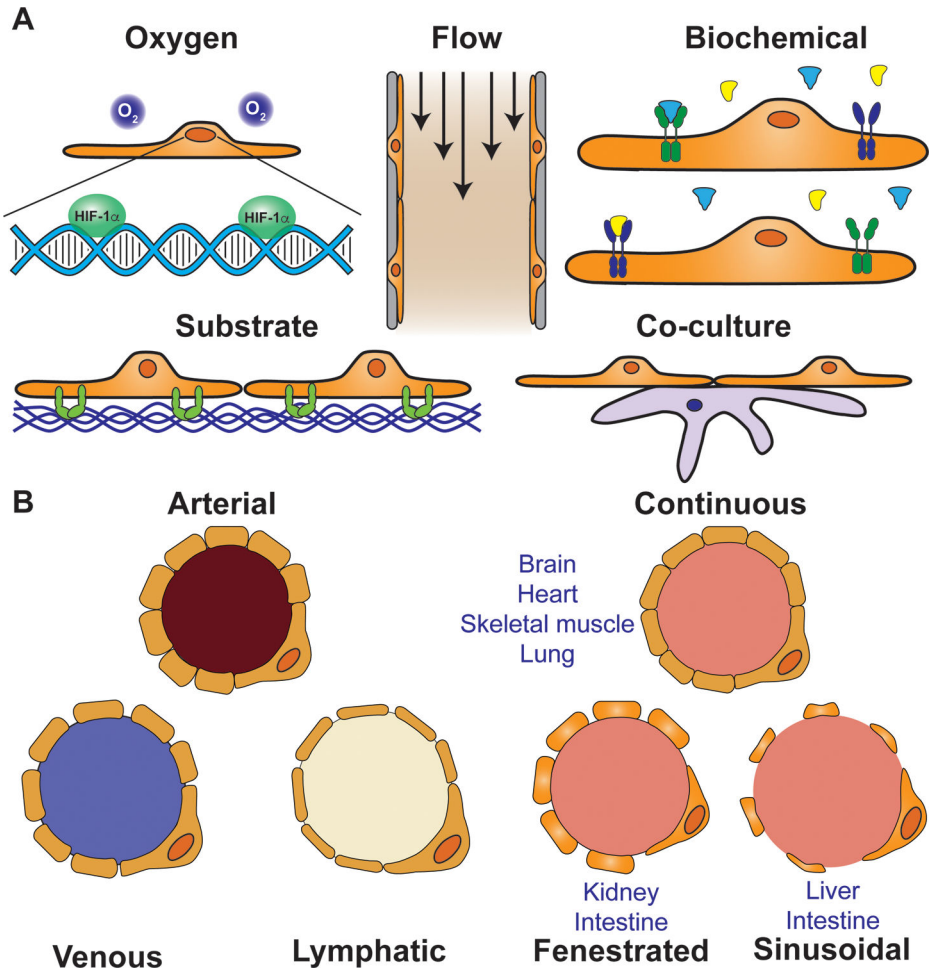

Figure 2. Strategies for recapitulating endothelial heterogeneity *in vitro*.

A) Techniques to mimic *in vivo* endothelial microenvironments include modulating oxygen tension, exposing endothelial cells (ECs) to flow, adding various growth factors and small molecules, culturing ECs on substrates with different properties, and co-culturing ECs with other cell types. **B)** Vessel- and tissue-specific endothelial targets for EC differentiation protocols. Tissue-specific endothelia are grouped by class (i.e., continuous, fenestrated, and sinusoidal). HIF-1α - hypoxia-inducible factor 1-alpha.

Table 1.

Key advances in various techniques for deriving ECs from various cell sources.

	Initial cell type	Feeder cell type	EC differentiation	Key advance	Refs
Co-culture	hESCs	Mouse bone marrow stromal cells (S17 or M2–10B4)	**1** Sort $CD34^+$ cells **2** Culture in EC media	First indication that ECs may be generated from hESC-derived hematoendothelial precursors.	**1** Kaufman PNAS 2001 **2** Hill Exp Hematol. 2010
	Initial cell type	**EC differentiation**		**Key advance**	**Refs**
Embryoid body	hESCs	**1** Sort $CD31^+$ cells from dissociated EBs		First study to show that ECs could be derived from spontaneously differentiating EBs.	Levenberg PNAS 2002
	hESCs	**1** Treat EBs with hematopoetic cytokines and BMP4 **2** Sort VE-Cad^+/$CD31^+$/$VEGFR2^+$/$CD45^-$ cells **3** Culture in EC media		First study to use mesodermal cues to direct differentiation of EBs to hematoendothelial precursor fate.	**1** Chadwick Blood 2003 **2** Wang Immunity 2004
	hESCs	**1** Treat EBs with BMP4/FGF2/Activin A to form primitive streak **2** Treat with VEGF/DKK1 to specify cardiogenic mesoderm **3** Treat with VEGF/DKK1/FGF2 to specify ECs		Showed that ECs can be derived from multipotent cardiovascular progenitor cells by directing differentiation of EBs to cardiogenic mesoderm.	Yang Nature 2008
	hESCs	**1** Treat EBs with BMP4/Activin A/FGF2/VEGF to specify mesoderm and ECs **2** Treat with TGFβ inhibitor to expand ECs		Inhibiting TGFβ can improve EC yield and maintain EC identity.	James Nat Biotechnol. 2010
	Initial cell type	**EC differentiation**		**Key advance**	**Refs**
Monolayer	hESCs/ hiPSCs	**1** Treat with CHIR **2** Sort for $CD34^+$ cells **3** Culture in EC media		Demonstrated that a small molecular GSK3 inhibitor (CHIR), rather than cytokines, can be used to generate cardiovascular progenitor cells.	Lian Stem Cell Reports 2014
	hESCs/ hiPSCs	**1** Treat with Activin A/BMP4/FGF2 to induce mesodermal formation **2** Sort for $NRP1^+CD31^+$ cells **3** Treat with FGF2/BMP4/VEGF to specify ECs		Generated cord-blood endothelial colony-forming cells with high proliferative potential.	Prasain Nat Biotechnol. 2014
	hESCs/ hiPSCs	**1** Treat with GSK3 inhibitor & BMP-4 to induce mesoderm formation **2** Treat with VEGF & PKA activator to specify ECs		Faster and more efficient monolayer-based EC generation.	Patsch Nat Cell Biol. 2015

	hESCs/ hiPSCs	**1** Titrate Activin A/BMP4 and add CHIR to derive either cardiogenic and hemogenic mesoderm **2** Treat with BMP4/FGF2/VEGF to specify ECs **3** Treat with FGF2/VEGF/GSK3 inhibitor to mature ECs		Generated developmentally-distinct ECs by patterning anterior and posterior-like mesoderm.	**1** Palpant Development 2015 **2** Palpant Nat Protoc. 2017
Transdifferentiation	**Initial cell type**	**Reprogramming**	**EC differentiation**	**Key advance**	**Refs**
	Amniotic Cells	ETV2/FLI1/ERG1 lentivirus + TGFβ inhibitor	**1** Culture in endothelial media + TGFβ inhibitor	Forced expression of key EC transcription factors reprograms amniotic cells to ECs.	Ginsberg Cell 2012
	Fibroblasts	Sox2/Oct4/KLF4/ cMyc/LIN28/shP53 episomal vector	**1** Treat with FGF2/ VEGF/ BMP4 to induce mesoderm **2** Culture in EC media to specify ECs	Brief non-integrating expression of pluripotency factors combined with mesodermal induction can be used to generate angioblasts and subsequently, ECs.	Kurian Nat Methods 2013

hESC – human embryonic stem cell, hiPSC – human induced pluripotent stem cell, EC – endothelial cell, EB – embryoid body.