Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell–Derived Endothelial Cells

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Abstract—The emergence of induced pluripotent stem cell (iPSC) technology paves the way to generate large numbers of patient-specific endothelial cells (ECs) that can be potentially delivered for regenerative medicine in patients with cardiovascular disease. In the last decade, numerous protocols that differentiate EC from iPSC have been developed by many groups. In this review, we will discuss several common strategies that have been optimized for human iPSC-EC differentiation and subsequent studies that have evaluated the potential of human iPSC-EC as a cell therapy or as a tool in disease modeling. In addition, we will emphasize the importance of using in vivo vessel-forming ability and in vitro clonogenic colony–forming potential as a gold standard with which to evaluate the quality of human iPSC-EC derived from various protocols. *Visual Overview*—An online visual overview is available for this article. (*Arterioscler Thromb Vasc Biol*. 2017;37: 2014-2025. DOI: 10.1161/ATVBAHA.117.309962.)

Key Words: cardiovascular diseases ■ endothelial cells ■ humans ■ induced pluripotent stem cells ■ regenerative medicine

Cardiovascular diseases (CVDs), which are often triggered by endothelial dysfunction, represent one of the leading causes of mortality in the world. In 2015, over 17 million deaths, which represent 31% of global deaths, were caused by CVD.[1] The treatment of CVD through endothelial replacement is hindered by the lack of methods to isolate sufficient numbers of functionally normal autologous endothelial cells (ECs) for transplantation and our limited understanding of how endogenous ECs become dysfunctional during the development of various CVDs. The innovation of human induced pluripotent stem cells (hiPSCs), cells that are reprogrammed from somatic cells to an embryonic stem cell (ESC)–like pluripotent state,[2,3] presents an opportunity for us to generate large numbers of patient-specific EC that can be used for transplantation, drug screening, or studies to probe the mechanisms for endothelial dysfunction in certain disease states. Here, we will retrospectively catalog and survey the methods developed by various groups to differentiate hiPSC to EC. We will focus on several common topics shared by all the papers, including the strategies for hiPSC-EC differentiation, the importance of functional and phenotypic testing of the hiPSC for quality control, and the potential applications of hiPSC-EC as a cell therapy for human subjects. Articles discussing human ESC–derived EC derivation, pluripotent stem cell (PSC)-EC differentiation from nonhuman species, and the development of other cell types through EC intermediates, like hemogenic endothelium, will not be covered here, and discussions on these topics can be found elsewhere.[4–7]

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Strategies for hiPSC-EC Differentiation

Methods to differentiate EC from hiPSC can be divided into 3 general categories: (1) stromal cell coculture, (2) embryoid body (EB) differentiation, and (3) feeder-free monolayer differentiation (Figure 1).

The methods to differentiate EC from hiPSC by coculture with stromal cells, usually murine bone marrow–derived stromal cell lines like OP9 or M10B2, were mainly used in some of the earliest studies in the field.[8–11] This is an undirected differentiation strategy in which the frequency of differentiated EC is generally low and the EC produced were often mixed with other cell types including hematopoietic cells,[8] smooth muscle cells,[9,10] and inevitably murine stromal cells. Thus, this method is not ideal for the generation of a large number of EC for regenerative medicine applications.

EB methods rely on the spontaneous differentiation of aggregated hiPSC in the context of a self-assembled 3-dimensional (3D) structure. The development of many cell lineages in EB seems to recapitulate the progression of early embryonic development. Thus, the differentiation of EB is also not a fully controlled process, and single-step long-term (10–12 days) EB differentiation method usually gives rise to a low EC yield.[12–22] Many researchers differentiate EB for a shorter period (3–6

Nonstandard Abbreviations and Acronyms	
3D	3 dimensional
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9
CVD	cardiovascular disease
EB	embryoid body
EC	endothelial cell
ECFC	endothelial colony–forming cell
ESC	embryonic stem cell
FPAH	familial pulmonary arterial hypertension
hiPSC	human induced pluripotent stem cell
iPSC	induced pluripotent stem cell
NRP1	Neuropilin-1
TALEN	transcription activator–like effector nucleases

days), usually in the presence of added growth factors that promote mesoderm and endothelial lineage specification including BMP4 (bone morphogenetic protein 4), Activin A, bFGF (basic fibroblast growth factor), and VEGF (vascular endothelial growth factor). At this stage, mesoderm and immature ECs are robustly induced. These cells, together with other differentiated cell types from EB,[23–34] or enriched by fluorescence-activated cell sorting or magnetic-activated cell sorting via the use of monoclonal antibodies that identify the expression of mesodermal and EC surface markers like CD309 (KDR [kinase insert domain receptor]),[35–40] CD34,[37] and CD31 (PECAM-1 [platelet endothelial cell adhesion molecule-1]),[35] are then expanded/enriched in monolayer culture conditions that favor the growth of EC. Some protocols developed using this strategy have differentiated functional EC from hiPSC that exhibited the ability to form in vivo vessels.[24,25,29,34,36,38]

The final general strategy is to culture a monolayer of hiPSC on a matrix-coated culture plate and treat them with different molecules or growth factors in a timed fashion to dictate the progressive differentiation from hiPSC to mesoderm and finally toward the EC lineage. To date, the protocols that have led to some of the highest EC yields were developed using this general strategy.[25,41,42] Most protocols using this strategy can be divided into a mesoderm differentiation phase and an endothelial differentiation phase. Some protocols will change the culture medium and growth factors between the mesoderm and endothelial stages, but without disturbing the cultured

Figure 1. General strategies for human induced pluripotent stem cell-endothelial cell (hiPSC-EC) differentiation. 2D indicates 2 dimensional; and EB, embryoid body.

cells,[25,41–62] whereas others purify the mesoderm cells or immature EC through detection of cells expressing KDR,[54,63–65] CD34,[66–68] MESP1 (mesoderm posterior protein 1),[69] or CD31[67] between these 2 phases. Once purified, the cell populations are further optimized to generate homogenous EC preparations.

Manipulating the signaling pathways that specify mesoderm differentiation from the hiPSC is common to these monolayer differentiation systems. Thus, growth factors like Activin A, BMP4, and bFGF are commonly used. GSK-3 (glycogen synthase kinase 3) inhibitors like CHIR99021 and BIO (6-bromoindirubin-3'-oxime) are also frequently added to the medium during this phase to promote the canonical Wnt signaling pathway which stimulates the differentiation of the hiPSC-derived mesoderm toward the endothelial lineage,[25,30,37,43,44,47,48,50,55,56,59,60,62,63,66,67,69] although the effectiveness of GSK-3 inhibition in enhancing EC specification has been questioned.[63] Endothelial lineage cells are expanded in the second phase. VEGF is the most common growth factor that is used during this phase. Inhibition of the TGF-β (transforming growth factor beta) signaling pathway has been reported to be crucial for promoting the endothelial specification from mesoderm cells and the maintenance of EC (to avoid endothelial-to-mesenchymal transition) in some protocols.[30] Thus, SB431542, an inhibitor of TGF-β signaling pathway, has often been included in many protocols to maintain and expand EC in the second phase.[30,43,44,49,51,57,58,61]

To further improve the differentiation efficiency of EC from hiPSC, a deeper understanding of the development of the EC lineage is required. Researchers are trying to discover new signaling pathways or develop novel culture conditions to improved EC differentiation. Park et al[68] have reported that inhibition of the MEK/ERK (mitogen-activated protein kinase kinase / extracellular-signal-regulated kinase) pathway by addition of the inhibitor PD98059 during the mesoderm induction stage increased the production of EC. However, in the study by Lian et al,[66] addition of the MEK inhibitor PD0325901 decreased the production of CD31+CD34+ EC. Sahara et al[25] reported that inhibition of the Notch signaling pathway during the late phase of EC differentiation, either by transcript knockdown or by addition of the γ-secretase inhibitor DAPT, led to a higher efficiency of EC production. Liang et al[51] showed that miR-495 could promote the production of EC from hiPSC and improve the angiogenic potential, as well as the in vivo vessel-forming ability of hiPSC-EC via interacting with vascular endothelial zinc finger 1. Cochrane et al[36] reported that the RNA binding protein Quaking isoform 5 stabilizes STAT3 (signal transducer and activator of transcription 3) and activates the expression of KDR, thus promoting EC differentiation and angiogenesis functions. In addition, cAMP has also been added in the EC phase of differentiation to enhance EC enrichment.[54,64] Zhang et al[70] promoted the differentiation of hiPSC into EC in a 3D porous fibrin scaffold, and 45% of hiPSCs were differentiated to EC. They reasoned that the higher EC differentiation efficiency was because of the 3D surface tension provided by the scaffold.[70] Recently, Zhang et al[69] suspended hiPSC-derived MESP1+ mesodermal cells in a 3D biodegradable gelatin–based scaffold and observed more enrichment of the CD31+ EC population than from hiPSC-derived mesoderm cells grown in 2D cultures.

Because hiPSC-ECs are differentiated in vitro, they are not exposed to the fluid flow, pulsatile pressure, or specific tissue
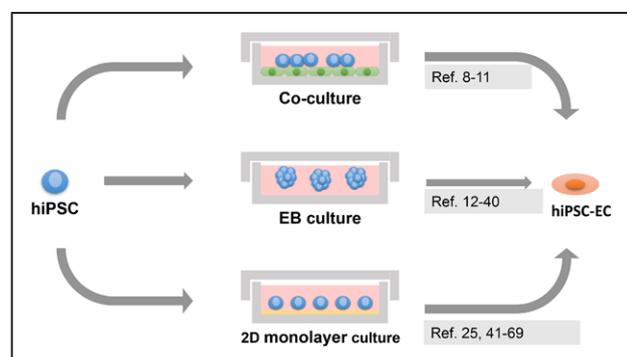
microenvironments that impact endogenous EC differentiation in vivo, and thus, hiPSC-ECs display an indeterminate phenotype; neither committed fully to an arterial or a venous fate.[29] Rufaihah et al[29] discovered that hiPSC-ECs derived using the EB differentiation method possess heterogeneous arterial, venous and lymphatic marker expression and they could be directed to differentiate toward the arterial,[29,31,71] lymphatic,[29,72] or venous[29] EC lineages through the manipulation of culture conditions. HiPSC-ECs have also been induced in various culture conditions that mimic specific tissue microenvironments to induce tissue-specific EC phenotypes. The most extensively studied tissue-specific hiPSC-EC phenotype is brain microvascular EC.[64,73–85] Corneal-like[86] and choroidal-like[87] ECs have also been differentiated from hiPSC. However, at this time, none of these in vitro induced tissue-specific EC has been validated extensively in vivo. Thus, whether these cells genuinely represent their in vivo counterparts and how long can they keep their tissue-specific phenotype in vivo remains unknown.

Quality Controls for hiPSC-EC

Although ECs differentiated from hiPSC through numerous methods exhibit some common traits, including expression of a panel of EC cell surface markers, in vitro 2D capillary-like tube-forming potential, and ability to uptake low-density lipoprotein (Figure 2), these properties alone may be insufficient to assess the function of EC to generate blood vessels in vivo. Indeed, many cell types other than EC, like some myeloid lineage hematopoietic cells, can also express EC markers and exhibit tube formation and low-density lipoprotein binding phenotypes in vitro,[88,89] whereas only bona fide ECs form stable, functional blood vessel networks that inosculate with host vasculature on transplantation.[88] Thus, in vivo vessel-forming potential should be a necessary assay of EC derived from hiPSC (Figure 2). One commonly used in vivo vessel-forming assay is to implant cells within a matrix and deliver the matrix into the subcutaneous space or prepared tissue. In this assay, donor

test ECs are loaded into a 3D matrix comprised collagen,[41,42] Matrigel,[16,19,24,25,29,34,36,38,40,68,90] fibrinogen,[47] or a degradable hydrogel[61] for transplantation. The matrices can be retrieved from the animals at different time points post-implantation to assess the extent of donor-derived vessels, determination of whether or not the donor-derived vessels have inosculated with the host vasculature, and the length of time that the donor cells persist. This method is reproducible (once preliminary studies are completed to optimize the matrix composition, donor cell number, and strain of mice implanted and attention paid to ongoing reagent quality control), straightforward to perform, thus is suitable as a standard test to compare the quality of hiPSC-EC derived from different methods of production. Other more invasive methods to assess endothelial functions of hiPSC-EC have also been tested, including experimental hindlimb ischemia,[22–24,42,68,69] myocardial infarction,[24,25,51] wound healing,[16,65,72] and retinopathy[34,42] models. Though these models are important for testing the therapeutic potential of hiPSC-EC, these interventional models are influenced by a host of variables including animal strain, sex, age, skill of the technicians, and whether or not the host animals are wild type or carry transgenic modifications. Moreover, in these models, the beneficial effect of the donor cell populations can be conveyed via indirect mechanisms in addition to effects derived from the direct formation of donor-derived vessels.[89] For example, cell types like hematopoietic cells and mesenchymal lineage cells, which are common coproducts of many hiPSC-EC differentiation protocols, can also improve blood perfusion by indirect means via secreting angiocrine factors or stabilizing newly formed vessels.[41,89,91] However, these non-EC cell types do not integrate into newly formed blood vessels.[89,92,93] Thus, in their potential clinical applications, whether they can provide long-term protection to the blood vessels to the hosts, especially those who have EC with compromised proliferative potential,[42] is questionable. For this reason, whenever these animal disease models are used for testing the function of hiPSC-EC, it
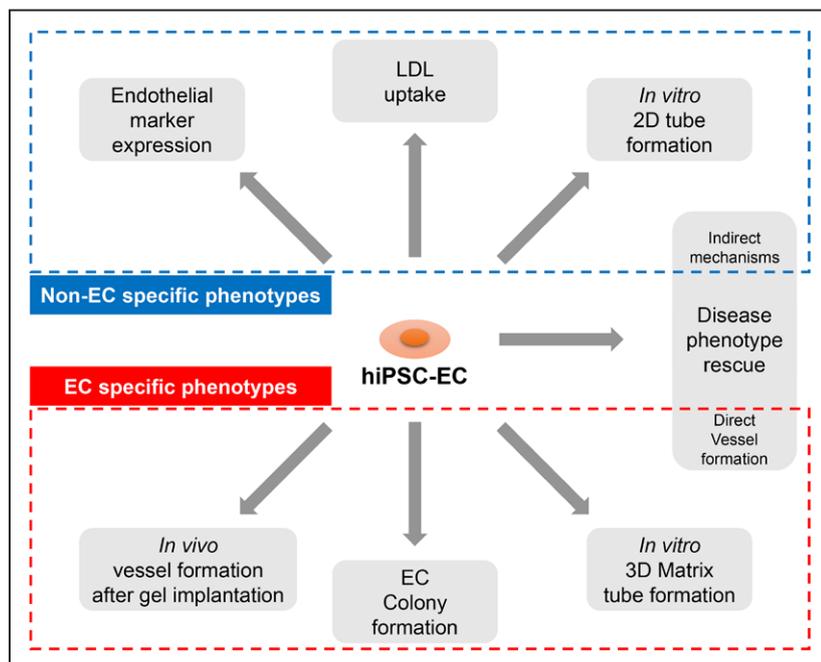

Figure 2. General methods to evaluate the quality of human induced pluripotent stem cell-endothelial cell (hiPSC-EC). 2D indicates 2 dimensional; and LDL, low-density lipoprotein.

is necessary to validate the long-term contribution of donor EC to the host blood vessels. Finally, inclusion of a standard source of primary human EC, such as human umbilical cord venous ECs or umbilical cord blood endothelial colony–forming cells (ECFCs) as a control for comparison to the test hiPSC-EC population in the implant assay, provides important information as to how similar the test EC are functioning in vivo. Recently, in vitro 3D vasculogenesis/angiogenesis models that mimic the vessel-forming process in vivo have been applied to test hiPSC-EC.[34,48,57,61,62,94,95] In these studies, hiPSC-ECs are embedded into 3D matrix made with fibrin,[61] collagen,[34,48] hyaluronic acid hydrogel,[57] or poly(ethylene glycol) hydrogels[62,94,95] under static conditions[34,48,57,61,62,94,95] or flow conditions in microfluidic chips[95] and the hiPSC-EC self-assembled into lumenized blood vessel–like structures.[34,48,57,61,62,94,95] Transcriptome analyses showed that the gene expression pattern of hiPSC-EC cultured in such conditions are more consistent with that of EC during the in vivo vasculogenesis/angiogenesis process compared with cells cultured in vitro under static conditions.[62,95] Thus, in vitro 3D tube-forming assays can be used for prescreening of functional hiPSC-EC before in vivo testing (Figure 2).

Another property of EC that is often neglected in assessing the quality of the EC derived from the various differentiation protocols is a quantitative measure of the proliferative potential of the hiPSC-EC (Figure 2). Evidence from both human and other species has shown that vascular ECs are heterogeneous with respect to proliferative potential. Over the past decade, it has been reported that among the EC derived from blood vessels or within the bloodstream, some ECs display the potential to clonally propagate in vitro, self-renew in vivo, and give rise to more EC during vessel growth or regeneration.[96–103] Those EC identified by their potential to form endothelial colonies in vitro[96–102] have been called outgrowth EC or ECFCs.[98,104] A single high proliferative potential ECFC can give rise to a colony that contains over 10 000 EC progeny, which may include more progenitors with equal or less proliferative potential,[97,98] whereas more mature EC may not be capable of dividing or giving rise to only a few progeny. Indeed, hiPSC-ECs derived from many protocols have displayed restricted proliferative potential and either undergo senescence within 5 to 8 passages or undergo endothelial-to-mesenchymal transition. To date, only a few studies have compared the proliferative potential of different hiPSC-EC fractions and tried to enrich the population of hiPSC-EC with high proliferative potential.[25,41,42] Two studies have compared the colony-forming potential of different EC fractions at a single-cell level.[25,42] Prasain et al[42] reported that after 12 days of hiPSC differentiation, EC could be discriminated into 2 groups based on the expression of NRP1 (Neuropilin-1), a coreceptor of VEGF and CD31. Only NRP1^{+}CD31^{+} hiPSC-ECs gave rise to ECFC that contained high proliferative potential when plated in a single-cell assay and showed overwhelming superior in vivo vessel-forming potential after transplantation than the NRP1^{−}CD31^{+} population.[42] Using this culture strategy, hiPSC-EC derived from this protocol can be propagated for ≤18 passages and the hiPSC:EC ratio reached 1:100 000 000 within a 2- to 3-month period.[42] In the same year, Sahara et al[25] reported that among all hiPSC-EC populations, CD34^{+}CD14^{−} EC displayed the

highest single-cell EC colony–forming potential and in vitro tube-forming ability. EC derived from this population also exhibited in vivo vessel-forming potential in Matrigel plug and myocardial infarction models.[25] In addition, Samuel et al[41] discovered and reported that CD34^{+}NRP1^{+}KDR^{+} EC displayed the highest proliferative potential among all hiPSC-EC tested in a specific protocol. EC from this fraction could be expanded for 15 passages and showed long-term in vivo vessel-forming potential.[41] However, unlike hiPSC-EC derived from the Prasain et al[42] or Sahara et al[25] methods, hiPSC-EC derived from the Samuel et al[41] protocol could not form in vivo vessels alone without the cotransplantation of mesenchymal cells.[41] Thus, whether these cell populations represent a similar cell type among all 3 reports is still elusive. Further efforts to specifically enrich and expand high proliferative ECFC, or an equivalent population, to gain a deeper understanding of the mechanisms of EC self-renewal and proliferation, to develop new culture strategies to maintain high proliferative ECFC fractions, and to compare and contrast specific ECFC populations derived from hiPSC with EC present in the vasculature of human subjects will be important areas for investigation.

Source of Pluripotent Cells and hiPSC-EC Phenotype

Whereas hiPSCs have similar properties and differentiation potential with EC, evidence has emerged that hiPSCs possess slightly different gene expression and epigenetic patterns because of their gain or loss of epigenetic memory from their somatic sourced material during the reprogramming process.[105–108] Thus, to safely apply hiPSC-EC for cell therapy or mechanistic studies, it is crucial to understand the differences among EC derived from hESC and those hiPSC derived from various somatic cell origins.

Many studies that differentiated ECs from hiPSC line have also tested their protocols with ESC lines and some researchers compared the phenotype and function of EC derived from both sources. With a few exceptions,[27] most authors have agreed that hiPSC-ECs generally exhibit comparable EC phenotypes and functions with ESC-derived EC.[19,20,22,24,25,40,42–44,59,65] Three studies compared the transcriptome between multiple ESC-EC and hiPSC-EC lines differentiated using the EB differentiation method and found that gene expression profiles between ESC-EC and hiPSC-EC showed some variance but, overall, were very similar.[19,34,40]

To date, most differentiation protocols have focused on the use of hiPSC derived from fibroblasts, a mature cell type. Some studies compared the EC differentiation potential of hiPSC derived from fibroblasts and other cell sources, include some immature cell populations like cord blood[34] or peripheral blood[109] mononuclear cells, bone marrow mesenchymal stem cells[33] and dental pulp cells,[24] or EC like human umbilical cord vein ECs[17,28] and cord blood ECFC.[43,44] The conclusions from those studies have provided multiple different interpretations of the importance of the source of cells with respect to derivation of hiPSC-EC. Whereas some reports suggest that immature cells or EC-derived iPSCs have a higher tendency to differentiate to EC when compared with fibroblast-derived iPSC,[28,33,34] others showed similar or highly variable differentiation

efficiency between these 2 groups.[24,43,44] Obviously, more studies are needed to answer this question.

Applications of hiPSC-EC

EC derived from patient-specific hiPSC can provide a nearly limitless source of autologous cells for various applications against CVD. The most direct application of hiPSC-EC would be to provide these cells as a therapy to replace injured or dysfunctional EC present in the systemic vasculature. Indeed, in some cases, such as patients suffering critical limb ischemia, evidence has been presented that there is loss of the microvasculature, and within isolated blood vascular EC from these patients, high proliferative ECFC are completely absent.[42] It has been shown that hiPSC-ECs from various protocols form functional blood vessels on transplantation in a 3D gel matrix[16,19,24,25,29,34,36,38,40–42,47,61,68,90] or into zebrafish embryos,[43,44] suggesting that hiPSC-ECs display potential to treat CVD by directly forming vessels and improving blood perfusion. Some other cell types, like mesenchymal and hematopoietic lineage cells, can also promote blood vessel growth and improve blood perfusion through indirect mechanisms in these animal models.[41,88,90] However, these non-EC cell types generally do not integrate into the newly formed blood vessels as long-term components[89,92,93]; thus, as a potential cell therapy, repetitive delivery of these cells might be necessary. Likewise, though some recent evidence shows that extracellular vesicles, like microvesicles and exosomes, from different cell source could also promote angiogenesis,[110,111] their short-half life and low encytosis rate are potential hurdles for their clinical applications.[110] In addition, all indirect proangiogenic effects from these non-EC cell types or extracellular vesicles rely on the communication between these cells/vesicles and local EC in host vessels. Thus, for those patients who had diminished EC proliferative potential, like critical limb ischemia patients,[42] the therapeutic benefits of non-EC agents are unlikely to be effective. In contrast, hiPSC-ECs have the potential to form stabilized functional vessels and thus provide long-lasting support to the patients' blood vessel system. Indeed, although to date, no clinical trial using hiPSC-EC on human CVD patients has been performed, the therapeutic potential of hiPSC-EC has already been tested in many preclinical animal studies including hindlimb ischemia,[22–24,42,68,69] myocardial infarction,[24,25,51] retinopathy,[34,42] carotid artery injury,[26] and dermal wound healing models.[16,65,72] In those studies, in situ administration of hiPSC-EC improved the recovery of model animals after injury, and most reports[16,22–25,34,42,51,68,69,72] highlighted significant levels of integration of donor cells into the host vasculature after transplantation. Thus, patient subjects experiencing peripheral artery disease, delayed wound healing, acute and chronic heart failure, stroke, and certain forms of retinopathy that are associated with endothelial dysfunction and vascular dropout are prospective target groups to benefit from this form of cell therapy (Figure 3).

Other than direct administration of EC suspensions, hiPSC-EC can also be mixed with other cell types in a 3D scaffold to assemble transplantable tissue grafts. Donor EC formed blood vessels can promote the connection of a graft into the host vascular system, thus improving the survival of grafts after transplantation. To date, liver,[46] cardiac,[10,45,53,112] and skin grafts[50] have been developed in vitro and transplanted into injured animals. In all these cases, the engrafted animals showed improved recovery and inosculated donor vessels.[10,45,46,50,53,112] In addition, Lu et al[39] have reported that decellularized murine heart scaffolds could be recellularized with hiPSC-derived multipotent cardiovascular progenitors to engineer heart tissue with contractile function (Figure 3). Other than those tissue grafts, miniaturized organoids that mimics the structure and function of in vivo organs, including blood vessel,[113] kidney,[114,115] liver,[116] cornea,[117] and retina,[118] in 3D culture have also been constructed using hiPSC-EC and other cell types. These organoids can be potentially used for drug screening, disease study, or cell therapy as units of an artificial tissue graft.

Patient-specific hiPSC-EC can also be potentially used for studying disease mechanisms or drug screening to find new molecules to modulate pathways contributing to endothelial dysfunction in a patient- and disease-specific fashion. Compared with animal models, models using patient-derived hiPSC-EC may provide more human-relevant information on disease mechanisms or drug responses (Figure 3). Recently, the pool of CVD patient–specific hiPSC lines is expanding rapidly. Disease-specific hiPSC-ECs have been generated and studied to model the BMPR2 (bone morphogenetic protein receptor type II) mutation that causes pulmonary arterial hypertension,[119–121] Moyamoya disease,[56,58] fibrodysplasia ossificans progressiva,[35] Huntington's disease,[78] Kawasaki disease,[109] type I diabetes mellitus,[41,57,122] atrial or ventricular septal defects, pulmonary valve stenosis, cardiomyopathy,[123] calcified aortic valve disease,[124] and hemophilia A.[11] We have summarized the above-mentioned studies that have used diseased patient–specific hiPSC-derived EC in the Table. More information about patient-derived hiPSC lines is given in International Stem Cell Registry (https://www.umassmed.edu/iscr) or CIRM human pluripotent stem cell registry (https://catalog.coriell.org/1/CIRM) where hundreds of patient-derived lines are available.

Along with the development of advanced gene editing tools such as TALEN (transcription activator–like effector nucleases and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9), more and more new strategies using targeted gene correction to treat or study cardiovascular-related diseases have been developed. To understand the effect of BMPR2 mutations in EC, Gu et al[121] compared EC differentiated from familial pulmonary arterial hypertension (FPAH) patient–derived hiPSC, normal control patient–derived hiPSC, unaffected BMPR2 mutation carrier patient–derived hiPSC, and CRISPR/Cas9 rescued FPAH patient–derived hiPSC. EC differentiated from FPAH patient–derived hiPSC with BMPR2 mutations possessed impaired EC functions and survival, whereas unaffected carrier patient hiPSC or BMPR2 rescued FPAH patient hiPSC-derived EC were relatively normal, suggesting that additional BMPR2 modifiers play a role for the protection of unaffected carriers from FPAH phenotype.[121] Theodoris et al[124] compared gene expression and the epigenetic landscape between EC derived from calcified aortic valve disease patient iPSC with *NOTCH 1* mutation and TALEN
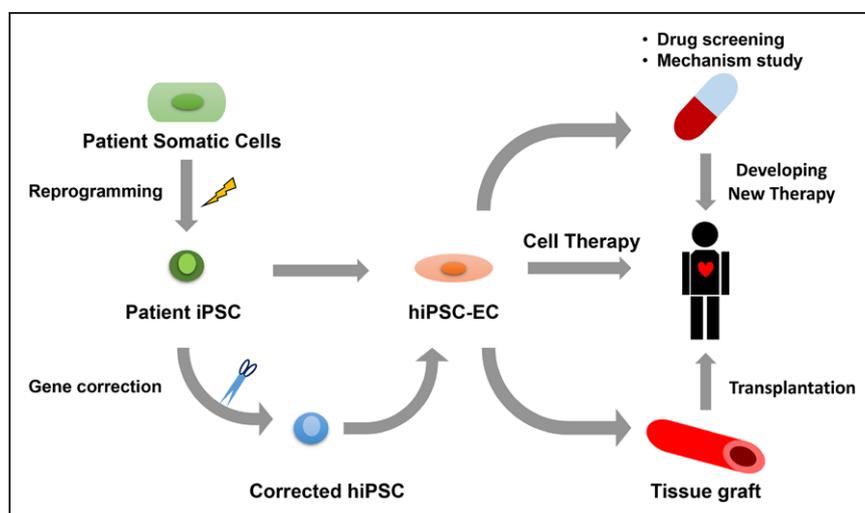
Figure 3. Potential applications of human induced pluripotent stem cell-endothelial cell (hiPSC-EC).

rescued isogenic iPSC and discovered *NOTCH1* haploinsufficiency caused epigenetic changes that lead to the upregulation of osteogenesis gene expression in calcified aortic valve disease patient EC.[124] Yang et al[125] surveyed the genome of patients with bicuspid aortic valve disease and identified *GATA4* (GATA-binding protein 4) as a target of mutation in these patients.[125] ECs differentiated from CRISPR/Cas9 induced *GATA4* targeted mutation hiPSC were then used to confirm that this mutation is associated with impaired endothelial-to-mesenchymal transition.[125] Wu et al[11] have recently generated hiPSC lines from a patient with hemophilia A caused by a factor 8 (FVIII) gene intron 22 inversion and used TALEN to correct this specific mutation. Corrected hemophilia A patient–specific hiPSC-EC restored FVIII transcription and secretion.[11]

As a platform for high-throughput drug screening and toxicity testing, hiPSC-EC can potentially be used to generate personalized therapeutic strategies. Recently, Vazão et al[126] developed a method to screen drugs that could potentially affect embryonic vasculature development and identified 2 drugs, fluphenazine and pyrrolopyrimidine, that could decrease EC viability and disrupted EC network formation. Sharma et al[127] generated hiPSC-EC and cardiomyocytes and fibroblasts from 11 healthy patients and 2 cancer patient to test the cardiotoxicity effect of 21 US Food and Drug Administration-approved anticancer tyrosine kinase inhibitors and developed a cardiac safety index for those drugs. To better mimic in vivo environments in which ECs constantly undergo shear stress from blood flow and often interact with other local cell types, new microfluidic 3D platforms like organ-on-a-chip have been developed and tested for drug screening on various in vitro organ mimics.[128,129] Recently, Wang et al[130] developed an in vitro blood–brain barrier microfluidic model using hiPSC-EC and validated the potential of this approach for screening drugs that affect EC permeability. As more sophisticated in vitro vascularized organ models are developed, it can be expected that preclinical drug development process for CVD can be greatly shortened.

Challenges for the Applications of hiPSC-EC as Cell Therapy

IPSC technology has only been developing as a discipline for a decade. Though hiPSCs have been extensively applied for in vitro mechanistic studies, their application as a cell therapy still faces numerous practical problems, like low reprogramming efficiency,[131] genetic instability,[132–134] and the potential of teratoma formation from undifferentiated iPSC remaining in a potential cell product.[135] To date, only 1 clinical trial has been conducted in human subjects, using hiPSC-derived retinal pigment epithelial cells,[136] and the safety and effectiveness of hiPSC therapy are still under evaluation. In general, application of hiPSC-derived cells for cell therapy for CVD will require a large number of EC to replace the dysfunctional EC present in specific tissues and organs. However, most hiPSC-EC differentiation protocols that have been developed to date generate low EC yields and only produce mature EC with limited proliferative potential. Scaling up the starting hiPSC cell number can partially solve this problem but may not be practical or cost-efficient. Recently, several groups have started to focus on differentiating hiPSC into EC precursors that display high proliferative potential.[25,41,42] It can be predicted that along with a better understanding of the mechanisms of EC lineage specification and proliferation, more efficient proliferative EC differentiation strategies should be developed to get clinical relevant numbers of hiPSC-EC for cell therapies.

The second limitation for current hiPSC-EC studies is the lack of a commonly accepted standard to evaluate the quality of hiPSC-EC derived from various protocols to assess their potential for clinical use. Although the route of delivery and the dose of cells to be used need to be determined case-by-case for each therapy, a basic set of quality control requirements for a hiPSC-EC therapeutic should be discussed and evidence based. However, many in vitro assays that are frequently used to define the quality of the hiPSC-EC, like CD31 expression, low-density lipoprotein binding, or 2D Matrigel network formation, cannot definitively predict the ability of the EC to form vessels in vivo.[88,89] Thus, to evaluate the quality of a hiPSC-EC product, some form of testing to validate in vivo vessel formation in animal models is recommended.

The next challenge for the practical utilization of hiPSC-EC is developing differentiation protocols that mimic the heterogeneous nature and tissue specificity of vascular EC. It is well known that arterial, venous, lymphatic, and capillary ECs show different patterns of gene expression and function.[137–139]

Table. List of Publications Using Patient-Derived Human Induced Pluripotent Stem Cell EC for Disease Study

Disease	Mutation	EC Phenotype	Year	Authors	Reference
Moyamoya disease	RNF213 R4810K polymorphism	In vitro tube formation↓	2013	Hitomi et al	56
		Securin expression↓			
Pulmonary arterial hypertension	BMPR2	Differentiation↓	2014	West et al	119
		LDL uptake↓			
		In vitro tube formation↓			
		Wnt signaling↑			
Type I diabetes mellitus	…	Differentiation no effect	2015	Chan et al	57
Calcified aortic valve disease	NOTCH1 haploinsufficiency	Epigenetic architecture change	2015	Theodoris et al	124
		Pro-osteogenic genes↑			
		Inflammatory genes↑			
Hemophilia A	*FVIII* intron 22 inversion	FVIII secretion↓	2016	Wu et al	11
		FVIII activity↓			
Fibrodysplasia ossificans progressiva	ACVR1 R206H	BMP4 independent differentiation	2016	Barruet et al	35
		Fibroblast-related gene expression↑			
		BMP4-induced SMAD1/5/8 phosphrylation↑			
Moyamoya disease	RNF213 R4810K	In vitro tube formation↓	2016	Hamauchi et al	58
		Proliferation no effect			
		Integrin β3 expression↓			
Intravenous immunoglobulin–resistant Kawasaki disease	…	CXCL12 expression↑	2016	Ikeda et al	109
		Interleukin-6 pathway↑			
Atrial or ventricular septal defects, pulmonary valve stenosis, cardiomyopathy	GATA4 G29S	EC-related gene expression↑	2016	Ang et al	123
Huntington's Disease	CAG repeat expansion in Huntingtin	In vitro tube formation↑	2017	Lim et al	78
		Barrier function↓			
		WNT/β-catenin pathway↑			
Pulmonary arterial hypertension	…	In vitro tube formation↓	2017	Sa et al	120
		Adhesion↓			
		Migration↓			
		Survival↓			
		BMPR2 expression↓			
		Kisspeptin 1 expression↑			
Familial pulmonary arterial hypertension	BMPR2	In vitro tube formation↓	2017	Gu et al	121
		Adhesison↓			
		Migration↓			
		Survival↓			

ACVR1 indicates activin A receptor, type I; CXCL12, C-X-C motif chemokine 12; EC, endothelial cell; FVIII, factor 8; LDL, low-density lipoprotein; RNF213, ring finger protein 213; and SMAD, small mothers against decapentaplegic.

In addition, ECs from each organ vascular bed share some common features but also show unique gene expression signatures.[140] Although most hiPSC-ECs are not fully committed to any specific EC phenotype, several studies have attempted to induce hiPSC-EC to express tissue-specific functions[64,73–87] or arterial/venous/lymphatic EC phenotypes.[29,31,71,72] For example, in vitro cultured ECFCs, which mostly form capillary EC after transplantation, also possess the ability to differentiate into arterial EC in vivo when constitutively activated NOTCH expressing stromal cells are coimplanted.[141] The local tissue microenvironment has been reported to educate murine ESC-derived EC to adopt the gene expression pattern of tissue-specific EC on transplantation.[140] Thus, some ECs possess the capacity to adapt to local environmental cues in

vivo, and further work to develop similar responsive hiPSC-EC are underway.

Finally, although autologous patient–derived hiPSC-ECs are promising for the treatment of chronic CVD, the process of hiPSC induction and EC differentiation can take several months to complete, which makes their potential for treating acute injuries unlikely. Some investigators are directly reprogramming somatic cells into EC without a pluripotent intermediate.[142–144] At present, it is unclear whether the reprogrammed ECs possess high proliferative potential. As an alternative strategy, some investigators are proposing to use banked allogeneic haplotype–matched hiPSC to produce cell products to diminish the potential for immunologic detection and elimination.[145] Other strategies include development of hiPSC lines that are genome edited to make universal donor cells that lack major histocompatibility antigens I and II that should be immunologically well tolerated by the host unmatched patients.[146–148] These strategies will be helpful in overcoming some of the practical issues for production of banks of hiPSC-derived EC for cell therapy.

Conclusions

In the past decade, numerous methods to differentiate EC from hiPSC have been developed, and the potential of applying hiPSC-EC in regenerative medicine approaches has been tested in various animal models of human disease. In addition, many patient-specific hiPSC lines have been generated, and ECs differentiated from these lines have been used for disease mechanism exploration and discovery. The accumulating knowledge on hiPSC-EC differentiation, functional evaluation, and potential application will benefit the development of new strategies against CVD.

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Highlights

- Numerous protocols have been developed to differentiate human induced pluripotent stem cell into endothelial cell using stromal cell coculture, embryoid body (3 dimensional) culture, or 2-dimensional monolayer culture strategies.
- The in vivo blood vessel–forming potential and in vitro clonal proliferative potential should be assayed as a quality control measure before using human induced pluripotent stem cell-endothelial cell for cell therapy.
- Human induced pluripotent stem cell-endothelial cell can be used in patients with cardiovascular disease through direct cell administration, implantation of engineered tissue grafts, or as models for studying the disease mechanisms causing endothelial cell dysfunction, or for drug screening.

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