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Generation of Human Pluripotent Stem Cell-derived Endothelial Cells and Their Therapeutic Utility

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Abstract

Purpose of Review—Human pluripotent stem cell-derived endothelial cells (hPSC-ECs) emerged as an important source of cells for cardiovascular regeneration. This review summarizes protocols for generating hPSC-ECs and provides an overview of the current state of the research in clinical application of hPSC-derived ECs.

Recent Findings—Various systems were developed for differentiating hPSCs into the EC lineage. Stepwise two-dimensional systems are now well established, in which various growth factors, small molecules, and coating materials are used at specific developmental stages. Moreover, studies made significant advances in clinical applicability of hPSC-ECs by removing undefined components from the differentiation system, improving the differentiation efficiency, and proving their direct vascular incorporating effects, which contrast with adult stem cells and their therapeutic effects in vivo. Finally, by using biomaterial-mediated delivery, investigators improved the survival of hPSC-ECs to more than 10 months in ischemic tissues and described long-term behavior and safety of in vivo transplanted hPSC-ECs at the histological level.

Summary—hPSC-derived ECs can be as a critical source of cells for treating advanced cardiovascular diseases. Over the past two decades, substantial improvement has been made in the differentiation systems and their clinical compatibility. In the near future, establishment of fully defined differentiation systems and proof of the advantages of biomaterial-mediated cell delivery, with some additional pre-clinical studies, will move this therapy into a vital option for treating those diseases that cannot be managed by currently available therapies.

Keywords

Human pluripotent stem cell; Endothelial cell; Cardiovascular disease; Stem cell therapy; Regenerative medicine

Compliance with Ethical Standards

Conflict of Interest Shin-Jeong Lee, Kyung Hee Kim, and Young-sup Yoon declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

Ischemic cardiovascular diseases are the major causes of morbidity and mortality in industrialized countries. Despite significant efforts made over the last several decades, treating patients with ischemic cardiac and vascular disease remains a challenge [1]. Since damage to or loss of blood vessels, of which the main elements are endothelial cells (ECs), are the main pathophysiologic feature of these diseases, targeting ECs and growing blood vessels from them is a rational step for treating these entities. Specifically, stem cell-derived ECs are expected to induce therapeutic neovascularization, proper blood perfusion, and tissue repair [2].

Of available approaches, many earlier studies used adult stem or progenitor cells, such as endothelial progenitor cells, mesenchymal stem cells, or bone marrow mononuclear cells. While animal studies showed very promising outcomes, the overall results of the clinical trials were modest at best. Their inability to make durable vessels in vivo and dependence on paracrine effects are known to be the major reasons [3–6]. Accordingly, more recent studies have attempted using endothelial cells derived from human PSCs (hPSCs), which include human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) for generating ECs in vivo, promoting revascularization, and inducing ischemic tissue repair [7, 8••, 9]. However, hurdles remain in their paths toward clinical use. Particularly, the development of a clinically compatible system for generating ECs and overcoming low survival of transplanted cells in ischemic environments are major concerns. This review will focus on progress of differentiation systems of hPSCs toward ECs from a clinical perspective, their therapeutic potential in vascular regenerative medicine, and strategies to improve survival of the implanted cells.

Differentiation of hPSCs into Endothelial Lineages

Two approaches have been widely known for differentiating hPSCs to ECs: three dimensional (3D) embryoid body (EB)-mediated differentiation and two-dimensional (2D) monolayer-directed differentiation (Table 1). In the EB-mediated system, the first step is to let hPSCs spontaneously form EBs in suspension culture without LIF and FGF2 [10]. These EBs are composed of three germ layers and usually after 2 weeks of culture, cells expressing EC markers and vascular-like structures are observed. In EB-mediated differentiation, since cells are permitted to spontaneously differentiate into multiple lineage cells, the percentage of ECs is low even though various angiogenic factors are used for differentiation (1 to 14%) [11–17]. The 2D culture system, which was developed later to improve differentiation efficiency and consistency, uses a monolayer cell culture of hPSCs with either mouse feeder cells [18, 19] or coating material [8••, 9, 20, 21•, 22, 23]. These systems allow homogeneous exposure of cells to the medium and yield higher and more consistent differentiation efficiency. In the 2D system, coating with extracellular matrix proteins such as Matrigel [9], or collagen [8••] gradually replaced the feeders due to concerns of animal product contamination and difficulties in target cell purification.

The main reason that 2D systems result in higher and consistent efficiency in EC generation is the use of stepwise differentiation methods. By following the embryonic developmental

scheme toward endothelial cells, the differentiation steps are organized into distinct stages and each differentiation step is controlled by using growth factors, cytokines, and small molecules that direct different developmental stages. First, hPSCs are differentiated into mesodermal lineages. Combinations of BMP4, selective small molecule inhibitor GSK-3β, and FGF2 are generally used [7, 8••, 9, 13, 21•, 24]. Markers of mesodermal cells (e.g., T, MIXL, EOMES, or KDR) are commonly used to assess differentiation efficiency. Next, these mesodermal cells are further differentiated into endothelial and vascular progenitor cells. Various growth factors and small molecules are used at this stage. For example, mesodermal-stage cells such as KDR-positive cells are differentiated into EC lineages by BMP4, activin-A, FGF2, and VEGFA [22]. In this study, KDR-expressing cells are further differentiated into more mature PECAM1/CDH5 double-positive ECs [22]. Since the efficiency of these protocols is not high, further refinement was attempted by other groups. Studies demonstrated that in combinations with VEGFA, a small molecular inhibitor of TGF-β (SB431542) or forskolin showed higher expression of CDH5 (VE-Cadherin) in hPSC-ECs [21•, 25]. Even higher expression of CDH5 was achieved when CHIR99021 was combined with DLL4 with a lower concentration of VEGFA (10 ng/ml) [8••]. DLL4, a Notch ligand, has been shown to enhance the efficiency of EC differentiation while inhibiting hematopoietic-lineage differentiation. Usually the final stage is to select EC lineage cells via EC-specific surface markers. KDR and CD34 are selective for progenitor level ECs [9, 22] and PECAM1 [15, 24, 25], CDH5 [8••, 11, 19, 21•], and VWF [7] are used for isolating more mature ECs. In another study, KDR-expressing mesodermal progenitors were differentiated into both ECs and mural cells by VEGF and PDGF-BB [21] While PECAM1 was classically used for purifying ECs, hPSC-derived ECs selected by CDH5 showed excellent EC features [8••, 11, 21•]. CDH5-positive cells express other important EC-specific proteins including PECAM1, KDR, VWF, CD34, CD105, and ANGPT-2 [8••, 11, 21•]. CDH5-expressing hPSC-ECs isolated by magnetic-associated cell sorting (MACS) demonstrated high purity (> 95%) and robust EC characteristics [21•]. Our recent study also demonstrated that CDH5-positive hPSC-ECs are highly enriched in EC proteins: VWF (98.6%), TEK (79.0%), and KDR (66.3%) [8••].

Furthermore, arterial, venous, and lymphatic vascular specification was demonstrated in hPSC-ECs. In certain EC differentiation conditions, lymphatic EC markers such as PDPN and LYVE1 were expressed, suggesting lymphatic lineage differentiation [24]. One study showed differentiation into arterial ECs characterized by Ephrin B2 and Notch1 with a higher concentration of VEGFA, and venous ECs characterized by EphB4 and CoupTFII expression with a lower concentration of VEGFA [24]. In another study, lymphatic ECs were specifically isolated from hPSCs via endothelial differentiation and double-sorting with PDPN and LYVE1 and were shown to improve wound healing by augmenting lymphatic neovascularization [26]. While few studies demonstrated maintenance of EC markers over long-term culture [12, 13], it is usually accepted that an EC phenotype is not well maintained after 2 weeks in culture [16].

Characterization of hPSC-Derived ECs

Four general hallmarks that define ECs are used to prove the identity of hPSC-derived ECs as ECs. These are presence of cobblestone-like morphology, expression of EC-specific

markers at the mRNA and protein levels, in vitro cell biological characteristics of ECs, and EC generation in vivo. ECs differentiated from hPSCs express EC-specific markers such as KDR, TEK/TIE2, CDH5, PECAM1, VWF, and NOS3 at both mRNA and protein levels and show typical cobblestone-like morphology. Immunostaining demonstrates localization of CDH5 and PECAM1 at the plasma membrane, expression of VWF with a typical punctate pattern, and localization of ANGPT-2 in Weibel-Palade bodies [8••, 11, 21•]. Flow cytometric analysis for PECAM1 or CDH5 further confirms their identity as ECs and provides the efficiency of EC generation. More recently, genome-wide transcriptome analysis and metabolic profiling has demonstrated the identity of hPSC-ECs in a more sophisticated manner. In terms of the developmental closeness between hPSC-ECs and adult ECs, studies showed controversial results. While a few studies showed similarity between hPSC-ECs to primary ECs such as HUVECs and human coronary artery endothelial cells [21•], most suggested that hPSC-ECs are less mature than adult ECs. In cell biological assays, hPSC-ECs show specific functional characteristics such as endothelial specific endocytosis and trafficking events. The endocytic capability of hPSC-ECs can be tested by their uptake of acetylated LDL with a punctated pattern [7, 8••, 9, 11, 15, 20, 21•]. P-selectin translocates onto the hPSC-EC surface upon histamine treatment [11]. Nitric oxide (NO) production, a most authentic index representing functional competence of hPSC-ECs, was also demonstrated in a recent study [8••]. Moreover, hPSC-derived ECs formed a tube-like structure within Matrigel gel [8••, 9, 15, 16, 22] or collagen gel [17, 20]. While not a required element for confirming hPSC-ECs, pro-angiogenic potential of hPSC-ECs measured by expression of angiogenic factors and their promotion of tube-formation by HUVECs on Matrigel are frequently demonstrated [8••, 11, 21•]. Finally, EC generation in vivo is the final step to prove the genuine identity and function of hPSC-ECs. For in vivo animal models, hindlimb ischemia models are mostly commonly used because these models can provide therapeutic outcomes at the same, but other models such as skin wound [27] or corneal pocket models are also used to prove in vivo vasculogenic potential [28, 29]. However, not all studies and even animal studies conducted in vivo lacked confocal imaging studies to confirm the exact co-localization of transplanted hPSC-ECs with EC markers and demonstrate their incorporation into the native vasculature. While most of the in vivo studies demonstrated short-term in vivo vasculogenic activities of hPSC-derived ECs, Wang et al. and Lee et al. showed that in vivo transplanted hPSC-derived ECs, when they were delivered with biomaterials, survived and contributed to vessel formation for 5 and 10 months, respectively, suggesting their long-term survival and vasculogenic effects [8••, 20] (Table 1).

Considerations in the Use of hPSC-Derived ECs in Vascular Regenerative Medicine

Studies using hPSC-ECs as a therapeutic agent demonstrated favorable effects for enhancing recovery from ischemia and promoting tissue regeneration [7, 8••, 9]. These results collectively indicate potential therapeutic utility of hPSC-ECs for cardiovascular tissue regeneration. Due to virtually unlimited proliferative capacity, hPSCs have huge advantages as a therapeutic agent for treating cardiovascular disease.

First, hPSCs must be differentiated in conditions that are fully defined and free of xenogeneic components. For example, hPSC-derived ECs generated in presence of animal serum and/ or animal-derived feeder cells will not be compatible for human therapeutic applications due to regulatory issues and graft rejection from exposure to animal-derived pathogens. In recent studies, fully defined differentiation systems that can differentiate hPSCs into ECs were reported [8••, 21•, 25]. Second, the culture system should generate hPSC-ECs with high efficiency and be clinically scalable to lower the costs. Most recently developed systems demonstrated a pre-sorting efficiency between 20 and 40% [8••, 9, 21•, 25] and showed scalability due to the use of 2-D system and defined components. Third, the differentiation protocols should yield similar differentiation efficiency across multiple cell lines [8••, 22]. This is particularly important when considering autologous cell therapy using hiPSC-ECs. Fourth, the ECs derived from hPSCs should be purified at the final stage of EC differentiation from hPSCs to avoid potential side effects caused by the presence of other types of cells. Fifth, vessel-forming or vasculogenic capability must be verified in vivo [7, 8••, 9, 15–17, 21•, 22, 24, 25]. Sixth, therapeutic effects should be confirmed in animal models of ischemic cardiovascular disease [7, 8••, 9]. Seventh, a major risk for therapeutic use of hPSCs is teratoma formation following transplantation [30, 31]. Thus, the tumorigenic potential or adverse effects of implanted cells must be ruled out by long-term follow-up. So far, only one study met all the above criteria and has demonstrated contribution of hPSC-ECs to new vessel formation in vivo [8••].

Use of Biomaterials to Improve Cell Survival In Vivo

A major issue to maximize the clinical utility of hPSC-ECs is their capability for long-term survival. It is well known that regardless of the cell type, low cell survival limits the therapeutic effects of any cell therapy in the cardiovascular field. No or few studies using bare cell transplantation demonstrated meaningful cell survival over more than several months even for hPSC-derived cells. When adult stem cells were injected into ischemic hearts, > 50% of the injected cells disappeared immediately, and virtually, all cells were lost within a month [18, 32]. The survival rate of hESC-derived ECs injected into infarcted hearts was shown to be less than 10% at 1 week, and less than 1% at 8 weeks [33]. Similarly, hPSC-derived cardiomyocytes showed poor survival in ischemic hearts, usually disappearing within a month [34]. Even when cells were injected in a hindlimb ischemia model, the results were not much different. Bioluminescent imaging (BLI) demonstrated that mesenchymal stem cells (MSCs) which were transplanted into ischemic hindlimbs disappeared by day 6 [35] or 20 [36]. Similarly, hPSC-derived ECs showed no detectable bioluminescence signals after 14 days in a hindlimb ischemia model [37]. Intriguingly, our study demonstrated relatively longer (> 3 months) survival of hPSC-ECs when these cells were injected into a hindlimb ischemia model by microscopic examination, although the degree of survival was not robust [8••].

This short-term survival not only limits the therapeutic effects, but also prevents investigation of the cell behavior of hPSC-derived ECs and their genuine capability for vessel formation in ischemic tissues. To overcome low cell survival in vivo, biomaterial-based strategies have been attempted in cardiac models with other cell types [8••, 38–42]. Likewise, endeavors were made for efficient cell delivery and longer-term engraftment to

improve therapeutic effects of hPSC-derived ECs and to determine the in vivo behavior of the transplanted hPSC-ECs [20]. Wang et al. demonstrated that the hESC-derived ECs which were co-implanted with 10T1/2, a mouse mesenchymal precursor cell line in a fibronectin-collagen gel, into cranial windows in severe combined immunodeficient (SCID) mice, contributed to blood vessel formation, integrating into their host circulatory system, and served as blood conduits for 151 days after transplantation [20]. Only two studies are found using hPSC-ECs alone with biomaterials in ischemic animal models. Mulyasasmita et al. demonstrated that in a hindlimb ischemia model, inflammation was reduced and muscle regeneration was promoted by co-delivery of growth factors and hiPSC-derived ECs using injectable MITCH-PEG hydrogels [43]. Lee et al. recently demonstrated that biomaterial-mediated delivery of hPSC-derived ECs could prolong cell survival and improve therapeutic neo-vascularization [8••]. They selected a novel injectable self-assembled peptide amphiphile (PA) nanomatrix gel [41, 44, 45], and generated PA-RGDS by incorporating cell adhesive ligand Arg-Gly-Asp-Ser (RGDS) and a matrix metalloprotease-2 (MMP-2) degradable sequence into the PA. RGDS, a fibronectin-derived cell adhesive ligand, promotes cell adhesion and the MMP-2 degradable sequences permit cell-mediated degradation of the nanomatrix gel and migration of encapsulated cells from the gel into the host tissues. In this study, they demonstrated that hPSC-derived ECs survived for more than 10 months in ischemic tissues, incorporated into the host vessels continuously and dynamically over this period following the remodeling of the vessels, contributed to new vessel formation, and showed better vascular regenerative capacity compared to the bare hPSC-EC injected group.

Summary and Future Perspective

We believe hPSC-ECs will be an important source of cells for ischemic cardiovascular diseases therapy. However, several hurdles remain. Since each of the many studies used its own protocols and cell types, more studies are needed to determine the phenotypic and functional differences between the different types of hPSC-ECs. Also, the successive culturability of established hPSC-ECs was not convincingly demonstrated. This is important because numerous cells are required for clinical therapy even when an autologous approach is employed. While this problem can be somehow addressed by using a large number of hPSCs for differentiation, establishing a protocol for passaging hPSC-ECs while maintaining the phenotype is important biologically as well. In addition, the genomic stability of differentiated hPSC-ECs has not been addressed. Long-term in vivo safety needs to be demonstrated using multiple different hPSC lines. Finally, optimization of biomaterial-mediated cell delivery and tissue engineering with hPSC-ECs will be an important future step in the use of hPSC-ECs.

Other biologically important questions await further studies. The developmental stages or maturity of hPSC-ECs developed by different protocols need to be addressed together with their biological potency. Generation of specific EC type, particularly arterial versus venous ECs, and their phenotypic maintenance has been virtually uninvestigated. Many therapeutic studies still show discrepancies between the degree of functional recovery and the direct vessel-forming effects of hPSC-ECs. Thus, the paracrine effects versus direct vessel-forming effects of various types of hPSC-ECs need to be explored. While there is advancement in the

therapeutic utility of hPSC-ECs, other important applications of stem cells such as disease modeling and drug testing are relatively behind compared to other stem cell-derived cells such as hPSC-derived cardiomyocytes.

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Table 1

Protocols to differentiate human pluripotent stem cells (hPSCs) into endothelial cells (ECs)

Cell	Culture system	Sorting	Efficiency	Animal product	In vitro function	In vivo function	Therapeutic effect	Reference
hESC	3D	PECA− M1+	~ 2%	Serum	Tube formation LDL uptake	Vasculogenesis in polymer scaffolds	–	Levenberg et al. (2002)
hESC	3D	VWF+	Unknown	Sereum	Tube formation LDL uptake	Vasculogenesis in ischemic tissues	Hindlimb ischemia model	Cho et al. (2007)
hESC	3D	CDH5+ KDR+	~ 10%	Sereum	Tube formation	–	–	Goldman et al. (2009)
hESC	3D	PECA− M1+	~ 2%	–	–	–	–	James et al. (2010)
hESC hiPSC	3D	PECA− M1+	~ 6.8%	–	Tube formation LDL uptake	Matrigel plug assay	–	Li et al. (2011)
hiPSC	3D	CDH5+	~ 18%	Serum	Tube formation LDL uptake	–	–	Adams et al. (2013)
hESC	2D	CD34+	~ 10%	Sereum	Tube formation LDL uptake	Vasculogenesis in collagen gel	–	Wang et al. (2007)
hESC hiPSC	2D	PECA− M1+ CD43−	~ 8%	Serum, Feeder	Tube formation	–	–	Choi et al. (2009)
hESC hiPSC	2D	CD34+	~ 20%	–	Tube formation LDL uptake	Matrigel plug assay	Hindlimb ischemia model	Park et al. (2010)
hESC hiPSC	2D	PECA− M1+	~ 20%	–	Tube formation	Zebrafish xenograft	–	Orlova et al. (2014)
hiPSC line	2D	CDH5+	~ 75%	–	Tube formation LDL uptake	Vasculogenesis in fibrinogen	–	Patsch et al. (2015)

Cell	Culture system	Sorting	Efficiency	Animal product	In vitro function	In vivo function	Therapeutic effect	Reference
hESC	2D	CDH5+	~ 35%	–	Tube formation	Vasculogenesis in ischemic tissues	Hindlimb ischemia model	Lee et al. (2017)
hiPSC					LDL uptake			
					NO detection	Matrigel plug assay		

hESC human embryonic stem cell, *hiPSC* human-induced pluripotent stem cell, *LDL* low density lipoprotein, *NO* nitric oxide