

Methodological Reviews discuss methods that are of broad interest to the community of cardiovascular investigators and that enable a better understanding of cardiovascular biology, particularly recent technologies in which the methods are still in flux and/or not widely known. It is hoped that these articles, written by recognized experts, will be useful to all investigators, but especially to early-career investigators.

Endothelial Cells Derived From Nuclear Reprogramming

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Abstract: The endothelium plays a pivotal role in vascular homeostasis, regulating the tone of the vascular wall, and its interaction with circulating blood elements. Alterations in endothelial functions facilitate the infiltration of inflammatory cells and permit vascular smooth muscle proliferation and platelet aggregation. Therefore, endothelial dysfunction is an early event in disease processes including atherosclerosis, and because of its critical role in vascular health, the endothelium is worthy of the intense focus it has received. However, there are limitations to studying human endothelial function *in vivo*, or human vascular segments *ex vivo*. Thus, methods for endothelial cell (EC) culture have been developed and refined. Recently, methods to derive ECs from pluripotent cells have extended the scientific range of human EC studies. Pluripotent stem cells may be generated, expanded, and then differentiated into ECs for *in vitro* studies. Constructs for molecular imaging can also be employed to facilitate tracking these cells *in vivo*. Furthermore, one can generate patient-specific ECs to study the effects of genetic or epigenetic alterations on endothelial behavior. Finally, there is the opportunity to apply these cells for vascular therapy. This review focuses on the generation of ECs from stem cells; their characterization by genetic, histological, and functional studies; and their translational applications. (*Circ Res.* 2012;111:1363-1375.)

Key Words: endothelial cell ■ nuclear reprogramming ■ pluripotent stem cell ■ therapeutics development
■ vascular regeneration

The endothelium is a delicate monolayer of cells lining the lumen of vessels. Aposposed between the circulating blood elements and the vessel wall, it exerts substantial control over the cardiovascular system. The endothelium regulates immune cell entry into the vessels and the surrounding parenchyma by its expression of adhesion molecules and chemokines. Vasoactive factors secreted by the endothelium modulate vessel tone and blood flow. The proliferation and migration of vascular smooth muscle cells is also tightly regulated by the endothelium. Thus, the endothelium plays a critical role in vascular homeostasis. Metabolic, hemodynamic, inflammatory, or age-related derangements of endothelial function initiate vascular disease, and permit its progression toward cardiovascular morbidity and mortality. Accordingly, there has been intense interest in understanding endothelial biology, ever since the recognition that it was more than a mere cellophane wrapper lining the vessel wall.^{1,2}

Studies of human endothelial cell (EC) function in living human subjects are limited by the difficulty in directly accessing

or imaging the cells. Nevertheless, useful biological and prognostic information has been obtained by innovative clinical investigators. For example, the endothelium synthesizes vasodilator factors, which are known to be released by increases in shear stress, to induce flow-mediated vasodilation.^{3,4} Flow-mediated vasodilation can be visualized by ultrasonography or angiography;^{5,6} is impaired by hypercholesterolemia and other metabolic perturbations;^{7,8} and is predictive of cardiovascular morbidity and mortality.^{9,10} Alternatively, one may measure biomarkers of EC function in the circulating blood (such as soluble adhesion molecules, which are increased with vascular inflammation and endothelial activation). Circulating ECs and endothelial progenitor cells (EPCs) may be detected by fluorescent activated cell sorting (FACS).^{11,12} The number of EPCs is reduced in patients with cardiovascular risk factors, and seems to be an independent predictor for mortality.^{13,14} Recently, direct endothelial biopsy has permitted immunohistochemical and genetic techniques for single cell analyses.¹⁵ However, these approaches have severe limitations with respect to gaining molecular insights into endothelial biology,

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Non-standard Abbreviations and Acronyms

bFGF	basic fibroblast growth factor
EBs	embryoid bodies
ECs	endothelial cells
EPCs	endothelial progenitor cells
ESCs	embryonic stem cells
FACS	fluorescent activated cell sorting
Flk1	fetal liver kinase-1
iPSCs	induced pluripotent stem cells
VEGF	vascular endothelial growth factor

therefore methods for culturing ECs in vitro have been developed and refined.

The development of EC culture 30 years ago dramatically accelerated our understanding of endothelial biology. This methodology facilitated molecular insights into the response of the endothelium to hemodynamic and humoral factors,^{4,16} as well as its activation by inflammatory cytokines and lipids.^{17,18} Cell culture studies have revealed important insights into the endothelial regulation of vascular tone and structure, blood fluidity, vascular permeability, and angiogenesis. Recently, the availability of methods to derive ECs from human pluripotent cells has extended the scientific range of human EC studies. One may genetically engineer the parental pluripotent stem cells, that is, either embryonic stem cells (ESC) or induced pluripotent stem cells (iPSCs), expand them, and differentiate them to ECs for in vitro studies (ie, ESC-ECs- or iPSC-ECs). Constructs for molecular imaging can be employed to facilitate tracking the cells in vivo. Furthermore, one can also generate patient-specific ECs to study the effects of genetic or epigenetic alterations on endothelial behavior. Ultimately, these cells may be used for therapeutic cell delivery applications. This review focuses on the generation of ECs from stem cells, their characterization by genetic, histological and functional studies, and their possible applications.

EC Culture Methodology

The quality of the science depends on the quality of the cells. Accordingly, one must adhere meticulously to good cell culture practice. The details of good cell culture practice are discussed in depth elsewhere,¹⁹ but the general tenets as they apply to EC culture are worthy of brief review.

Authenticity

It is essential to regularly confirm the authenticity, as well as the stability, of the EC lines. ECs should be regularly interrogated for the expression of characteristic functions, surface markers, and gene expression (described below). Careful recording of these features, and recording of growth conditions and population doublings, should be used to establish the optimal passage numbers and growth conditions for experimental protocols.

The derivation of the cells should be known, and their phenotypic and genotypic characteristics confirmed. It is of particular importance in the generation of new EC lines from pluripotent cells that the parental cells be well characterized. For those cells derived from patient materials, one should

record key patient characteristics (eg, age, sex, disorders, medications) and the tissue origin of the cells. Furthermore, the methods by which the parental cells were reprogrammed and differentiated to ECs should be recorded in detail.

Standardizing Culture Conditions

The media and other reagents used in the cell culture should be well characterized, and standardized so as to reduce variability. However, although serum-free medias are available (eg, Cell Applications, Genlantis) endothelial media usually contains 10% fetal calf serum. Because of lot-to-lot variability and incomplete characterization of serum and other animal products, it is important to evaluate each new batch in parallel with the existing batch. Additionally, commercial endothelial growth media are typically supplemented with defined growth factors to promote proliferation and maintenance of phenotype (ie, vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF], insulin-like growth factor-1) or undefined growth factors derived from bovine neural tissue. The media may also contain other chemical factors (eg, ascorbic acid, heparin, hydrocortisone).

Passaging ECs

Even under pristine conditions, cells undergo phenotypic and genotypic changes in culture. Primary cell lines have a limited number of population doublings (usually no >50–60 doublings but often less), a phenomenon known as the Hayflick limit. At the Hayflick limit, cells enter a senescence phase where the cells have lost the ability to divide because their telomeres have shortened to a critical length. However, the signs of senescence are often present before this limit is reached. ECs during senescence will lose their cobblestone morphology and assume a fried egg morphology, replicate more slowly, have greater expression of oxidant-sensitive genes such as adhesion molecules and chemokines, and have reduced endothelial function (ie, reduced expression of endothelial NO synthase).²⁰ The investigator must be familiar with these changes over time, and restrict their studies to ECs in a range of passage numbers (more specifically, population doublings) where the experimental protocol will be unaffected by senescence.

The phenotypic alterations occurring over time in cultured cells are in part because of the artificiality of the culture system. Cells grown on polystyrene (even that coated with a thin layer of matrix proteins) experience a substrate rigidity that is many orders greater than that of tissue (ie, polystyrene has a Young's Modulus of 10⁶ kPa, in comparison with physiological soft tissues which are 2–50 kPa). Culturing cells on soft hydrogels, which possess biomimetic physicochemical properties, for example, muscle stem cells retain their ability for asymmetric division and replication, and undergo less cell death.²¹ Preliminary studies from our laboratory indicate that more tissue-like substrate rigidity can favorably influence the maintenance of endothelial phenotype.

Immortalized cell lines can be subcultured almost indefinitely. However, such cells are often derived from malignancies, from viral transformation, from treatment with mutagens, or by genetic modification (eg, with SV40 large T antigen or

telomerase). For example, the human microvascular endothelial cell (HMEC-1) line is a line of human microvascular dermal ECs that was immortalized by transfection with a region of the simian virus 40 gene product, large T antigen. These cells can be passaged for <95 passages without undergoing senescence, where as normal microvascular dermal ECs enter senescence after only 8 to 10 passages.²² However, because of their genetic and epigenetic alterations, the behavior of immortalized cell lines may be less representative of cells *in vivo*, and new scientific insights using these cells should be confirmed with primary cell lines.

Care and Handling of ECs

Standard operating procedures for cell culture should be established. Care should be taken not to expose the cells or tissues to inappropriate conditions (eg, excessive time out of the incubator). The optimal culture temperature and oxygenation should be established. Lower temperatures (<34°C) may slow growth, but higher temperatures (>38°C) may favor apoptosis. Traditionally, EC cultures have been carried out in 95% O₂/5% CO₂, but a more appropriate oxygen tension may be in the physiologic range of 5% to 20%.²³ The optimal pH range (≈pH 7.4) for EC culture is narrow.

To obtain single cell suspensions of ECs we have found that the use of TrypLE Express (Invitrogen), a recombinant fungal trypsin-like protease that is temperature-stable, induces less cell death than other approaches.

Cryopreservation permits EC lines to be stored for prolonged periods, but care must be taken during the processes of freezing, storage, and recovery. We have good results when we harvest cells for storage during exponential growth stage, and use 10% v/v DMSO in 10% fetal bovine serum as a cryoprotectant. Commercial freezing media contains proprietary components but are designed to increase the recovery of cells upon thawing. Cells should be gradually exposed to the freezing media to minimize osmotic shock. Upon reconstitution in freezing media, the cells should be cooled at 1° per minute in cell freezing containers stored at -80°C. After 4 to 24 hours,

the vials can be transferred to liquid nitrogen. During transfer, care should be taken to avoid exposing the frozen vial to room temperature, as the vial's temperature may change rapidly even within only 1 minute.

The cells should be free of biological contamination (other cells, pathogens such as mycoplasma, or endotoxins). Commercially purchased cells are commonly prescreened for biological contamination, but primary isolated cells are at greater risk for biological contamination. Endotoxin levels can be quantified using a limulus amoebocyte lysate colorimetric assay. Mycoplasma levels can be quantified using commercial kits that detect mycoplasma RNA or DNA. Mycoplasma is a common contaminant. This bacterium from the class of *Mollicutes* competes with eukaryotic cells for nutrients. Mycoplasma contamination can alter cellular function such as proliferation rates, and NO signaling, and can introduce genetic aberrations.²⁴ Once mycoplasma is detected, it can be eliminated by treatment of anti-mycoplasma reagents or antibiotics. However, it may be more prudent to simply discard the infected cells, and carefully decontaminate the incubator and other equipment that has been in contact with the cells.

Genetic, Histological, and Functional Confirmation of Endothelial Lineage

Immunohistochemical and Genetic Characterization

EC Characterization by Immunocytochemistry. To document endothelial lineage, no one immunohistochemical marker is definitive. Rather, one should use a set of endothelial markers, such as platelet-endothelial cell adhesion molecule-1 (CD31, Figure 1), VE-cadherin (CD144), endothelial NO synthase, von Willebrand factor, and fetal liver kinase-1 (Flk1, also known as kinase insert domain receptor or VEGF receptor 2). Briefly, the cells are fixed with paraformaldehyde (4%), permeabilized with Triton X-100 (0.1%), and blocked with either normal goat or donkey serum (1%) for 30 minutes, followed by overnight incubation with the primary antibodies at 4°C. The cells are washed with 1× PBS and incubated with secondary antibodies (ie, Alexa Fluor-488 or -594) for 1 hour at room temperature.

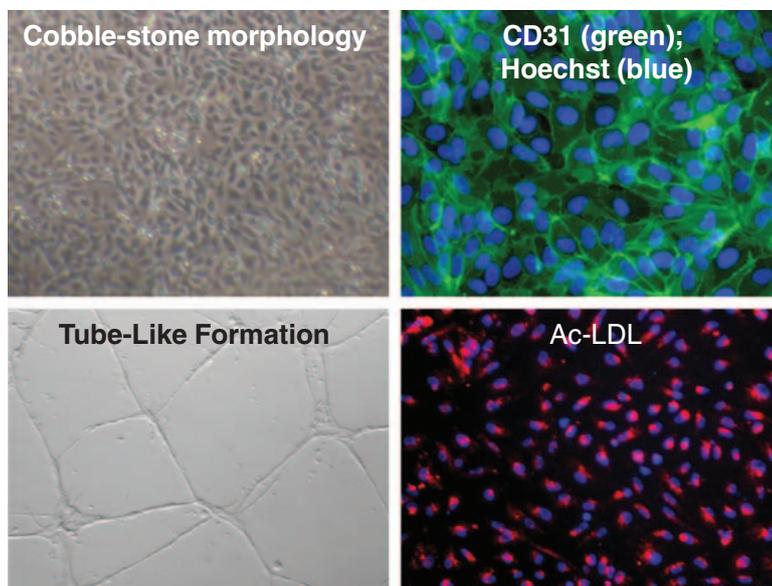


Figure 1. Endothelial cell characteristics. Human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) have a characteristic endothelial cell cobblestone morphology, express the endothelial marker CD31, form tube-like structures in Matrigel and incorporate acetylated low-density lipoprotein (ac-LDL).

Nuclei may be stained with Hoechst 33342 dye and the cells examined by fluorescent microscopy. However, because none of the histological markers alone are specific to ECs, functional assays are required to confirm EC lineage.

Functional Assays

ECs Synthesize and Release NO

ECs regulate blood flow and blood pressure by releasing the endothelium-derived relaxing factors including NO and prostacyclin. These endothelium-derived factors are critical for maintaining vascular homeostasis.²⁵ The detection of NO radical in biological samples has been done using a porphyrin electrode, or by spin trapping of NO combined with electron paramagnetic resonance.²⁶ These are technically difficult because of the short half-life and low concentration of these radicals. The oxidized degradation products, nitrite and nitrate, are stable and can be measured by chemiluminescence after reacting the sample in a reduction chamber to regenerate NO and react it with ozone.^{26,27} However, the most popular and simple method, albeit less sensitive, is to use the fluorimetric Griess reaction assay to measure total nitrates and nitrites.^{28,29} In addition, there are NO fluorescence dye indicators including the 4,5-diaminofluorescein dye, but these are not satisfactory because of nonspecific reactions, including with ascorbyl radical in the culture medium.^{30–32} Improved NO probes for intracellular detection are under development, but are not yet commercially available.³³ As an alternative indicator of bioactive NO production, the amount of cGMP, a secondary messenger of NO, may be measured.

EC Activation

ECs are activated by inflammatory cytokines, such as tumor necrosis factor- α in vitro, which induces the transcription of cell adhesion molecules (such as intercellular adhesion molecule 1) involved in the recruitment of leukocytes.^{34,35} This activation process may also be involved in the homing of highly proliferative EPCs and thus is important in both neo-vascularization in regions of ischemia or reendothelialization of areas where the endothelium is impaired.^{34,35} A monocyte-EC adhesion assay can be used to quantify EC activation. Briefly, ECs are washed with Hanks balanced salt solution containing 2 mmol/L Ca²⁺, 2 mmol/L Mg²⁺, and 20 mmol/L HEPES. Human acute monocytic leukemia cell line (THP-1) are washed and added to the EC monolayer at a final concentration of 2×10^6 cells/mL. The dish with ECs and monocytes is placed on a rocking platform and rotated frequently to ensure even distribution of monocytes. After 30 minutes, the medium is removed and ECs are washed with fresh Hanks balanced salt solution to remove nonadherent monocytes. Cells are fixed (2% glutaraldehyde in Hanks balanced salt solution), stained (CD14 antibody is used to visualize the monocytes), and adherent monocytes are counted by microscopy.^{20,36}

Acetylated LDL Uptake by EC

Normal ECs can take up acetylated low-density lipoprotein (LDL) (Figure 1). The uptake of acetylated LDL serves as a commonly used marker for the identification of EC. For acetylated LDL uptake assay, cells are incubated with Dil-labeled acetylated LDL (10 μ g/mL; Invitrogen) for 4 hours at 37°C.

After incubation, the cells are visualized and photographed under a fluorescence microscope.

Angiogenesis and Vasculogenesis Assays

New blood vessels arise by 2 general mechanisms during development, vasculogenesis, and angiogenesis. Vasculogenesis involves the generation of new vascular networks by incorporation of circulating progenitor cells. Angiogenesis is the formation of new vessels by sprouting from existing vessels. Both angiogenesis and vasculogenesis require EC proliferation and migration. The ability of EC to form tube-like networks in vitro has been employed widely to identify ECs, and to assess vasculogenic and angiogenic potential (Figure 1). Notably, other cell types can form networks in Matrigel, but only ECs are capable of forming tubes with lumens. For the in vitro Matrigel assay, cells (2.5×10^5) are seeded on 24-well plates precoated with growth factor-reduced Matrigel and incubated for 24 hours at 37°C to induced tubular network formation. To determine whether these ECs form functional blood vessels in vivo, a Matrigel plug assay is performed using immunodeficient NOD SCID mice, if using human ECs. Matrigel is mixed with bFGF (50 ng/mL) and ECs (5×10^5). The mixture is subcutaneously injected into the mice. After 14 days, the Matrigel plugs are removed, paraffin-embedded, sectioned, and stained with CD31. Capillaries can be quantified using fluorescent microscopy; if human ECs were used, human and mouse specific EC antibodies help to determine the contribution of the human ECs in the Matrigel network.

Furthermore, we also assess the functionality of pluripotent stem cell-derived ECs (ESC-ECs and iPSC-ECs) using the mouse hindlimb ischemia model (Figure 2). Unilateral hindlimb ischemia is induced by ligating the femoral artery of (4–6 months) male NOD SCID mice.^{34,35} The cells are then delivered, for example, by intramuscular injections into the ischemic limb, and perfusion of the ischemic and nonischemic hindlimb is assessed over the next 4 weeks using laser Doppler.^{34,35}

EC Gene Expression Profiles

There is limited information about the gene expression profiles of the ECs derived from stem cells and the native ECs. Li et al³⁷ compared the gene profiles of human iPSC-ECs or ESC-ECs, and human umbilical vein ECs using whole genome microarray. The results suggested that gene expression variation of iPSC-ECs and ESC-ECs may contribute to biological differences between iPSC-ECs and ESC-ECs as compared with human umbilical vein ECs. For iPSC-ECs, it is important to note that somatic memory does exist^{38–40} which may be useful to explain why iPSC-ECs have slower growth rate and faster loss of endothelial phenotype than ESC-ECs or native ECs. Nevertheless, a better understanding of the gene expression profiles that guide the development and differentiation of iPSC would facilitate methods to improve the derivation of high-fidelity iPSC-ECs.

Deriving ECs From Pluripotent Stem Cells

Rationale

Successful derivation of ECs from human pluripotent stem cells has opened up new opportunities for understanding

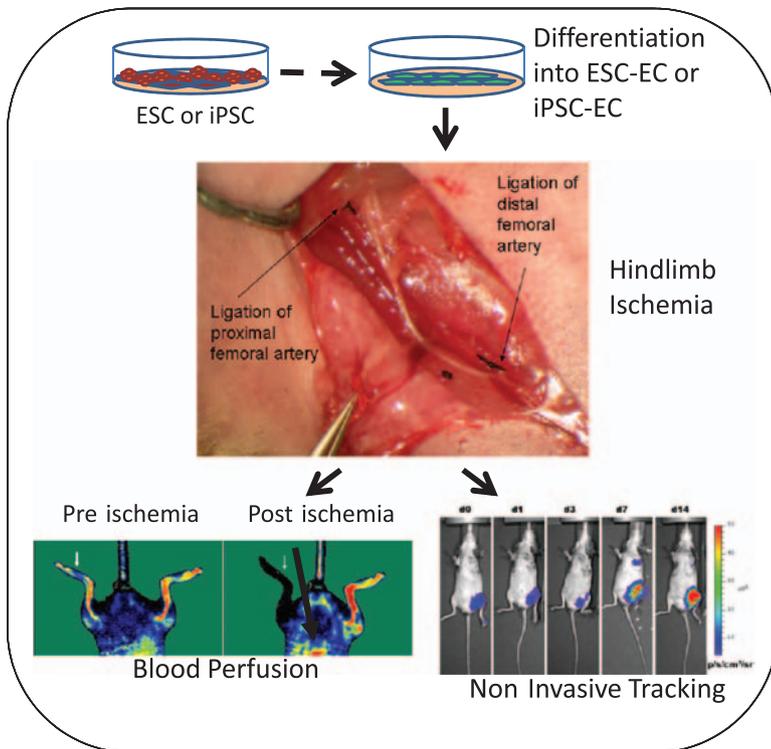


Figure 2. Assessing the functionality of pluripotent stem cell-derived endothelial cells using the mouse hindlimb ischemia model.

Unilateral hindlimb ischemia is induced by ligating the femoral artery. Cells are injected intramuscularly, and perfusion in the ischemic and nonischemic hindlimb is assessed using laser Doppler. Additionally, noninvasive tracking may be used to investigate homing of the cells to the ischemic tissue. (Images are reproduced with permission from Niiyama et al¹²⁶). ESC indicates embryonic stem cell; iPSC, induced pluripotent stem cell; EC, endothelial cell.

endothelial biology. Researchers are now able to genetically engineer the parental cells into pluripotent stem cells, expand them, and differentiate them to ECs for in vitro studies. These cells can be tracked during in vivo studies by employing constructs for molecular imaging. Similarly, patient-specific ECs can be generated to study the effects of genetic or epigenetic alterations on endothelial behavior. More importantly, the ability to derive large numbers of ECs from parental pluripotential stem cells increases the feasibility of cell therapy applications.

Types of Stem Cells

Stem cells are capable of self-renewal and directed differentiation. Before the introduction of iPSCs in 2006, 2 broad categories of stem cells had been classified: ESCs and adult stem cells. ESCs are pluripotent stem cells derived from the inner cell mass of the fetal blastocysts. They are able to differentiate into all derivatives of the 3 germ layers: ectoderm, endoderm, and mesoderm. In contrast to ESCs, adult stem cells are already partially committed to a lineage, and can thus produce only a limited number of cell types. For example, hematopoietic stem cells are adult stem cells that are multipotent and can give rise to all types of blood cells but are not able to produce cells of endodermal or ectodermal lineage. However, by comparison to adult differentiated cells, adult stem cells have greater capacity for proliferation and ability to repopulate or repair tissue.

Deriving ECs From ESCs

ESCs are pluripotent cells derived from the inner cell mass of the blastocyst. Human and murine ESCs characteristically express pluripotency markers, such as Oct3/4 and Nanog transcriptional factors. However, human and murine ESCs express species-specific markers as well. For example,

murine ESCs express epithelial cadherin and stage-specific extracellular antigen-1, whereas human ESCs express stage-specific extracellular antigen-3/4 and TRA-1-60. Human stem cell lines were first established by Thomson et al.⁴¹ Since then, intensive efforts have been made at methods for directed differentiation and isolation to obtain pure cell populations without contamination of undifferentiated cells, which can give rise to teratomas or tumors consisting of tissue derived from the ectoderm, endoderm, and mesoderm germ layers.

General Considerations of Microenvironmental Conditions

Soluble factors represent the most conventional method to induce endothelial differentiation of pluripotent stem cells. Among the variety of cytokines and growth factors, VEGF seems to play a critical role during the differentiation of ESCs into ECs. VEGF at 50 ng/mL greatly increases the yield of CD31⁺ cells from mouse ESC-derived embryoid bodies (EBs).⁴² VEGF is required for the proliferation of mouse ESC-derived VEGF receptor 2 positive progenitor cells.⁴³ VEGF also dose-dependently enhances the formation of EC colonies and plays a critical role in the determination of arterial and venous fate of the ECs.^{43,44} During the differentiation of Flk1⁺ sorted cells, high VEGF concentrations can promote upregulation of arterial markers whereas lower concentrations of VEGF induce venous marker expression.⁴⁴

Apart from growth factor- or cytokine-mediated endothelial differentiation, extracellular matrix plays a key role in angiogenesis and vasculogenesis through supporting adhesion, migration, proliferation, and survival of ECs.⁴⁵ Among those extracellular matrix molecules, gelatin, fibronectin, and collagen type I and IV have been demonstrated to support ESC differentiation into mesodermal Flk1⁺ cells which have

the potential to become hematopoietic cells and ECs.⁴⁶ These Flk1⁺ sorted cells were able to give rise to ECs that could generate endothelial type tube-like formation.⁴⁷ In addition, laminar shear stress can influence the differentiation of ESC into ECs through the Phosphoinositide 3-kinase-Akt pathway.⁴⁸ Stimulation of murine ESCs with laminar shear stress (15 dyn/cm²) enhanced their differentiation into Flk1 expressing cell.⁴⁹ Furthermore, exposure of Flk1⁺ cells to shear stress (5 dyn/cm²) promotes differentiation into CD31⁺ ECs.⁵⁰

Murine ESCs to ECs Differentiation

Mouse ESCs spontaneously differentiate into endodermal, mesodermal, and ectodermal lineage on removal of leukemia inhibitory factor from culture.⁵¹ Additionally, mouse ESCs can spontaneously differentiate to form 3-dimensional clusters of cells called EBs when they are cultured in suspension. The hemangioblasts are a precursor population of cells within the EBs that can give rise to hematopoietic cells and ECs.^{52–54} Thus, the ESC can be directed to differentiate into vascular ECs (ESC-derived ECs).^{55–57}

EB Approach to Generating ECs

We have previously demonstrated that murine ESCs can be differentiated into endothelial lineage by dissociating the ESC colonies into single cells using TryPLE Express and then plating the cells onto ultra-low adhesion dishes in a differentiation media consisting of α minimum Eagle's media, 10% serum, and β -mercaptoethanol.³⁵ In suspension culture, the murine ESCs form floating aggregates known as EBs. After 4 days of suspension culture, the EBs are then allowed to attach onto 0.2% gelatin-coated dishes and cultured for another 10 days in differentiation media. Flk1 is expressed at day 3 of differentiation whereas late-stage endothelial markers VE-cadherin and tyrosine kinase receptor 1 are detected after day 5 of differentiation.⁴² Once the EBs are seeded on the 2-dimensional tissue culture plates, EC formation can be observed at the borders of the EB colonies.⁵³ After 3 weeks of differentiation, the murine ESC-derived ECs can be purified by FACS using their expression of vascular endothelial cadherin (VE-cadherin). We prefer to use VE-cadherin rather than CD31 for the purification of murine ECs, because murine ESCs are known to express low levels of platelet-EC adhesion molecules (CD31) even in the pluripotent state.⁵⁸

2-Dimensional Culture of murine ESCs to Derive ECs

ECs can be differentiated in the absence of EB formation by using 2-dimensional culture systems. Yamashita reported the differentiation of murine ESCs into ECs using VEGF and collagen IV-coated dishes.⁴⁷ Murine ESCs were differentiated in differentiation media consisting of α -minimum Eagle's medium, fetal bovine serum (10%), and β -mercaptoethanol (0.05 mmol/L) on collagen IV-coated dishes for 4 days before purification of Flk1⁺ and epithelial cadherin⁺ mesodermal progenitors. The sorted cells were then grown on collagen IV-coated dishes in differentiation media supplemented by VEGF. Using the same differentiation media, Narazaki et al⁵⁹ differentiated murine iPSCs on collagen IV-coated dishes for 4 days before FACS-sorting vascular progenitor cells using Flk1⁺. Upon purification, the Flk1⁺ cells were then cultured on collagen IV-dishes in differentiation media supplemented with VEGF (100 ng/mL)

and 8-bromoadenosine-3':5'-cyclic monophosphate (8-Br-cAMP, 0.5 mmol/L) for an additional 3 days before further characterizing for endothelial phenotypic markers. McCloskey et al⁶⁰ reported a modification of this procedure in which murine ESCs were first differentiated on collagen IV-coated dishes in the same differentiation media. After 4 days, the cells were purified for Flk1⁺ expression by FACS and plated onto collagen IV-coated dishes in differentiation media supplemented by VEGF for 1 week, producing ECs with cobblestone morphology. However, in our experience, a differentiation period of 3 weeks yielded higher efficiency of ECs when compared with shorter time periods of differentiation.

An important consideration when designing such differentiation protocol is that cell density seems to influence EC differentiation. For instance, when Flk1⁺ progenitor cells from mouse ESCs were isolated and plated at a low density, the cells failed to differentiate into ECs.⁴³ We have also observed that EC differentiation is dependent on the cell density, suggesting that cell-cell interaction plays an important role in differentiation. On the basis of these findings, a defined cell seeding density of 10 000 cells/cm² is used to induce endothelial differentiation after the selection of Flk1⁺ progenitor cells from mouse ESCs.⁴⁷

Differentiation to EC in Semisolid Culture or on Stromal Cells

Another differentiation approach involves semisolid culture. Using this method, murine ESCs are cultured in differentiation media composed of Iscove modified Dulbecco medium, 1% methylcellulose, 15% fetal bovine serum, 2 mmol/l glutamine, 100 U/mL penicillin G, 100 μ g/mL streptomycin, 100 μ mol/L monothioglycerol, 1 \times BSA, insulin and transferrin (BIT). When cultured in this semisolid environment for 11 days, the ESCs can form aggregates that can be directly transferred into collagen gels to facilitate sprouting of vascular networks. Furthermore, the aggregates can be dissociated for purification by FACS, or studies by conventional quantitative polymerase chain reaction or immunocytochemical staining.

Co-culture of murine ESCs with OP9 stromal cells have also been shown to promote endothelial differentiation. OP9 stromal cells are derived from murine bone marrow. These cells have been immunophenotyped to be positive for antigens associated with mesenchymal stem cells, such as CD29 and CD44, although being negative in hematopoietic markers CD34 and CD45.⁶¹ When murine ESCs or iPSCs are co-cultured with OP9 in the presence of an angiopoietin-1 variant, the pluripotent cells efficiently differentiate into Flk1⁺ mesodermal precursors.⁶²

Differentiation Efficiencies

The reported efficiency of differentiation protocols varies considerably. However, it is difficult to compare the efficiencies of the various differentiation protocols because of the fact that different starting cells and markers are employed at different time points using different technologies. That being said, the reported efficiencies for yielding ECs with the EB formation method are 16% in murine ESCs,³⁵ and range from 10% to 50% in human ESCs.^{34,63,64} The OP9 co-culture method yields 35% ECs from murine ESCs,⁶² 7 to 13% from human ESCs,^{65,66} and 16%

from human iPSCs.⁶⁶ The collagen IV method yielded 15% to 39% in murine ESCs,^{47,59} and 14% in murine iPSC-ECs.⁵⁹

Characterization of ESC-Derived ECs

These ESC-derived ECs (ESC-ECs) are validated to be of EC lineage based on the multiple surface markers, gene expression, and functional studies as described earlier.⁴² Subsequently, the cells can be further characterized *in vivo*, by their ability to incorporate into the vasculature of the ischemic limb or myocardium.^{67–69} We tracked the fate and function of transplanted ESC-ECs in the ischemic murine myocardium.^{35,70} Murine ESCs were first transduced with a construct that encoded luciferase (for bioluminescence imaging) and red fluorescent protein (for histological tracking). After the ESCs differentiated into ECs, the ESC-ECs or vehicle were injected into the ischemic area of the left ventricle after ligation of the left anterior descending coronary artery. Bioluminescence imaging showed that the ESC-ECs survived to 8 weeks, and echocardiography revealed improved systolic function in the hearts injected with ESC-ECs. Histological studies revealed increased myocardial capillary density in the hearts treated with cell therapy. Additionally, we have shown the ESC-ECs engraft into ischemic hindlimbs (a murine model for peripheral artery disease, Figure 2) and restore perfusion in the leg.³⁵ These studies suggest that ESC-EC may have a therapeutic effect in the treatment of ischemic vascular diseases.

Human ESCs to ECs Differentiation

Human ESCs exhibit a number of differences from murine ESCs, such as their independence from leukemia inhibitory factor and dependency on bFGF for maintenance of self-renewal. However, Levenberg et al⁷¹ reported that human ESCs can be differentiated spontaneously through EB formation in the absence of bFGF. After 10 to 15 days of differentiation, these human EBs gave rise to various lineages including CD31⁺ ECs. When the CD31⁺ cells were purified from the EBs by FACS, ~80% purity as achieved.⁷¹ As a modification of this protocol, human ESCs were grown in suspension as EBs in the presence of Iscove's modified Dulbecco's medium, 20% defined fetal bovine serum and 450 mmol/l monothio-glycerol.⁶³ After 4 days, the EBs were reattached to gelatin-coated dishes in the presence of EC growth medium-2 that contained numerous growth factors including VEGF and epidermal growth factor. After 12 days of differentiation, the EB outgrowths were FACS purified based on CD31 expression. This method yielded 12% CD31⁺ cells, and on expansion and repurification, the cells were 97% CD31⁺.

Using a more directed approach mimicking the growth factor induction signals during cardiovascular development, Yang et al⁶⁴ demonstrated a stepwise differentiation protocol yielding endothelial, cardiac, and smooth muscle lineages from the same precursor stem cell. The human ESCs were differentiated in the presence of bone morphogenetic protein 4 on day 0; bone morphogenetic protein 4, bFGF, and activin during days 1 to 4; Wnt inhibitor Dickkopf-related protein 1 (DKK1) and VEGF from days 4 to 8, and VEGF, DKK1, and bFGF thereafter.

The derivation of ECs from human ESCs via different culture conditions include human ESC induced EB formation grown on gelatin-coated plates,^{34,64,71–74} human ESC grown on bone marrow stromal OP9 co-culture,^{65,66,75} human ESC grown

on collagen IV-coated plates,⁷⁶ and human ESC-induced EBs grown on methylcellulose.⁷⁷ Although the differentiation of human ESCs to ECs have been achieved using a variety of differentiation protocols, methodologies involving cytokines/growth factors and extracellular matrix environments need to be further explored and refined.

iPSCs as a Source for ECs

Generation of iPSC

In 2006, Yamanaka and colleagues⁷⁸ demonstrated that mouse fibroblasts could be reprogrammed to iPSCs by introduction of 4 transcriptional factors including octamer-binding transcription factor-3/4, SRY-related high-mobility-group-box protein-2, Krüppel-like factor 4, and c-Myc.^{78,79} The induced expression of these 4 transcriptional factors activates a network of genes required for pluripotency, and gradually transforms the somatic cells into pluripotential stem cells. These cells exhibit similar morphological and growth patterns compared with ESCs and they have the potential to differentiate into lineages of the 3 germ layers.^{80–83} In 2007, Thomson et al⁸⁴ used octamer-binding transcription factor-3/4 and SRY-related high-mobility-group-box protein-2 together with Nanog and Lin28 as reprogramming factors to generate iPSCs. The development of iPSCs has opened a new avenue for cardiac and vascular regeneration, as well as the opportunity to understand the differentiation of ECs from pluripotent stem cells.^{13,85,86}

Differentiating iPSCs to ECs

iPSCs are capable of differentiating into all cardiovascular cells including ECs, vascular mural cells, smooth muscle cells,⁸⁷ and cardiomyocytes.^{55,59} Generally, approaches for differentiation of human or murine ESCs can be also applied in the differentiation of human iPSCs (Figure 3). We differentiate human iPSCs after dissociating the colonies (with type IV collagenase for 10 minutes) and then transferring iPSCs to ultra-low attachment dishes containing differentiation media for 4 days to form EBs. The differentiation media consisted of α -minimum Eagle's medium, fetal bovine serum (20%), β -mercaptoethanol (0.05 mmol/L), nonessential amino acids (1%), bone morphogenetic protein 4 (50 ng/mL), and VEGF-A. The 4-day EBs were then seeded on 0.2% gelatin-coated dishes and cultured for another 10 days in differentiation media in the absence of bone morphogenetic protein 4. Differentiation media was changed every 2 days. To purify the pluripotent stem cell-derived ECs, single cell suspensions were obtained using accutase for 20 minutes at 37°C to dissociate differentiated cells, which were then washed with 1× PBS containing 5% BSA, passed through a 70- μ m cell strainer, and incubated with phycoerythrin (PE)-conjugated anti-human CD31 antibody and anti-VE-cadherin antibody for 30 minutes. Isotype-matched antibody served as negative control and 1% propidium iodide was used to stain the nonviable cells. Then FACS purified ECs were expanded in EGM-2MV. Currently, our methodology yields 10 to 20% VE-cadherin⁺/CD31⁺ cells, which can be purified to 75% to 90% with a second FACS.³⁴

Direct Reprogramming as a Source for ECs

The ability to generate iPSCs has already had practical applications in the development of disease-specific cells for

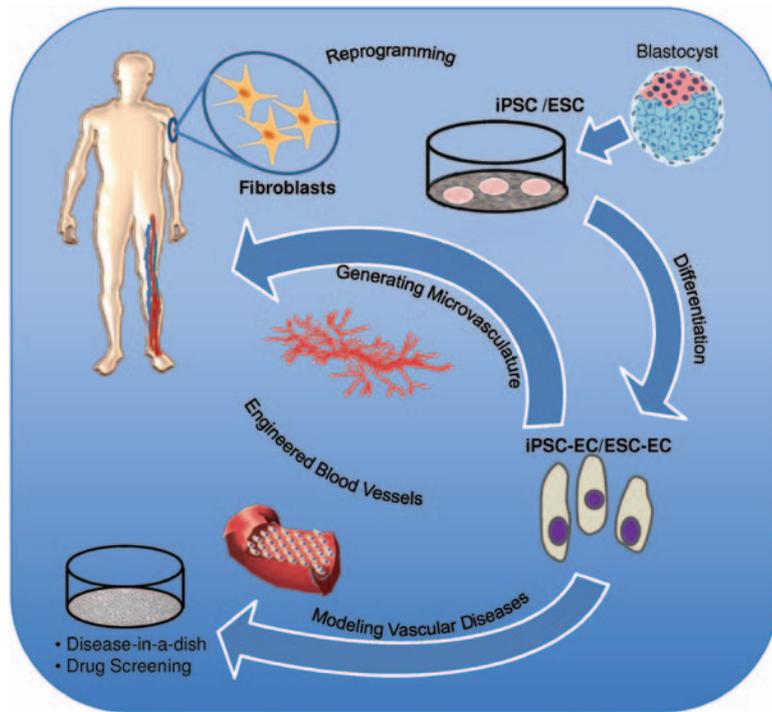


Figure 3. Potential therapeutic applications of endothelial cell derivation. An immediate application of induced pluripotent stem cell (iPSC) technology is to derive vascular cells from patients with vascular disease, which may be useful for drug screening for vascular disease therapeutics. Endothelial cells (ECs) derived from pluripotent stem cells may be used in the development of vascular grafts and engineered blood vessels. Additionally, in the future iPSC-ECs may be used for vascular regeneration through the injection of these cells directly into ischemic tissue. ESC indicates embryonic stem cell.

understanding mechanisms of disease, and in the production of iPSC-derived cell lines for drug screening. Although there is promise for the application of iPSC-derived cells for therapy, there are major limitations, including the risk of teratoma, the difficulty in differentiating high-fidelity therapeutic cells, and the prolonged process which currently takes months. Direct reprogramming, from one somatic cell type to another desired somatic cell type, is thus an attractive alternative approach. For example, fibroblasts have been converted to neurons,⁸⁸ and to cardiomyocytes.⁸⁹ Typically, a handful of genes, known to be involved in development of the desired cell type, are overexpressed in the fibroblast for transdifferentiation. A recent article by Margariti et al⁹⁰ converted fibroblasts to ECs using the Yamanaka factors and culture conditions favoring EC growth.

Heterogeneity of the ECs Derived From Nuclear Reprogramming

There is heterogeneity of endothelial structure and function in vivo, that is not generally appreciated, nor fully maintained in cell culture. In vivo, the phenotype of the EC is influenced by the developmental program (eg, lymphatic, venous, or arterial), the surrounding parenchyma and its metabolic activity (eg, the elaboration of angiogenic cytokines by tumor cells), as well as hemodynamic and humoral factors. Endothelial heterogeneity is necessary to support specialized tissue function. For example, the endothelium of the cerebral microvasculature forms tight cell junctions and is far less permeable than the lymphatic endothelium which is fenestrated. In the former case, the endothelium protects the brain from shifts in interstitial fluid that could adversely effect intracranial pressure, whereas in the latter case, the increased permeability of the lymphatic endothelium subserves transport of interstitial protein back to the systemic circulation. One manifestation of endothelial heterogeneity is the elaboration of different surface

proteins, that have been termed vascular zip codes. The remarkable heterogeneity of endothelial surface markers have been well demonstrated by phage display studies.⁹¹ The functional relevance of this heterogeneity is illustrated by the expression of specific adhesion molecules that facilitate monocyte entry at sites of disturbed flow in the aorta, whereas different adhesion molecules regulate the trafficking of immune cells through the venous endothelium of the lymph nodes.^{92,93}

The meticulous investigator is cognizant of endothelial heterogeneity. At the very least, one should confirm by immunohistochemistry or FACS that the ECs one is using experimentally are of venous (Ephrin B), arterial (Ephrin B2), or lymphatic (podoplanin or Lyve 1) subtype. We have found that our iPSC-derived ECs are heterogenous, with all 3 subtypes represented. Furthermore, the culture conditions can influence the percentage of each subtype. Higher doses of VEGF-A (50 ng/mL) and 8-Br-cAMP in the differentiation medium increase the prevalence of the arterial phenotype, for example (unpublished results). One should be aware that in the absence of their normal milieu, ECs may lose some of their in vivo characteristics, or acquire new properties, which could affect experimental results, and the relevance of the cell culture model.

MiRNAs and Endothelial Lineage

Recent work has highlighted the role of miRNA (miR) in regulation of EC development and phenotype. Pivotal studies by Yang et al⁹⁴ showed that knockdown of miRNA processing enzyme, Dicer, disrupted angiogenesis. Since then, a score of miRs has been identified to modulate EC genes including miR-126, miR-19a, and miR-21.^{95,96} However, little is known about the role of miRNA in the EC differentiation. In a recent article, Kane et al⁹⁷ found that overexpression of miR-99b, -181a, and -181b levels increased the expression of

EC markers and function. In addition, long noncoding RNA regulates vascular development. Alterations in the ratio of tie-1 mRNA to the tie-1 antisense long noncoding RNA, has been implicated in human vascular anomalies.⁹⁸ It is likely that with a greater understanding of the regulation of their roles in endothelial development, miRNA and long noncoding RNA may be manipulated for the purpose of EC differentiation or directed reprogramming.

Potential Therapeutic Applications of EC Derivation

Modeling Vascular Disease

An immediate application of iPSC technology is to derive vascular cells from patients with vascular diseases. In particular, one would focus on vascular diseases that are believed to have a strong genetic component; the pathophysiology of which is not well understood; and do not have good animal models. Fibroblasts may be harvested from a patient with such a vascular disease. The fibroblasts can be induced to the pluripotent state using reprogramming factors, and then induced to form endothelial or vascular smooth muscle cells. Once their vascular phenotype has been confirmed by the characterization studies described above, the iPSC-derived vascular cell may be further studied to characterize abnormal cell functions (Figure 3). For example, patient-specific iPSC-derived cardiomyocytes recapitulated the morphological and functional phenotypes for familial dilated cardiomyopathy and these cells may serve as a useful model to explore disease mechanisms and therapeutics.⁹⁹ Similarly, once the disease-in-a-dish model is created, and the model is shown to faithfully recapitulate the vascular disease of the patient, then it may also be a useful tool for drug or small molecule screening for vascular disease. Such an approach may uncover therapeutic avenues that may merit further development.

ECs in Engineered Blood Vessels

Vascular regeneration refers to the restoration of normal vascular function and structure, the reversal of vascular senescence, and the development of vascular networks. ECs are essential components in vascular regeneration, and in many different tissue engineering applications such as vascular grafts. Vascular tissue engineering may involve the in vitro engineering of biological conduits for revascularization of various tissues (Figure 3). An early report described a vascular graft made with ECs, vascular smooth muscle cells, fibroblast, and collagen gel medium on a Dacron mesh.¹⁰⁰ Since then, a variety of tissue-engineered grafts have used different scaffolds to enhance cell viability, maintain vascular phenotype, and provide for the mechanical strength to withstand hemodynamic forces. Vascular cells can be seeded to various materials including collagen,¹⁰⁰ biodegradable scaffolds,¹⁰¹ or vessels made of decellularized matrices.¹⁰² ECs and SMCs may be obtained from the patient's own vein segments, then cultured in vitro to get sufficient cell number for a graft. The ECs can form a barrier layer and are able to secrete von Willebrand factor and prostacyclin in in vitro condition.

The major disadvantages of using adult cells from blood vessels are their limited proliferation capacity and susceptibility to cellular senescence.¹⁰³ Furthermore, patient-specific deficiencies of endothelial function, or even tissue-specific

differences in endothelial function, may reduce the applicability of autologous adult ECs.¹⁰⁴ However, the use of stem or progenitor cells to derive ECs may surmount these difficulties.

So-called EPCs may be isolated from the patient's blood or bone marrow (using FACS and surface markers such as CD34, Flk1, and VEGF receptor 2), and expanded in ex vivo culture.^{11,12} Parenthetically, the widely used term EPC is a misnomer. The surface markers that are commonly used for identification of human EPCs include markers that are not specific for endothelial lineage, such as CD133 and kinase insert domain receptor.¹² In addition, methods for harvesting, purifying, and culturing EPCs are not standardized. Thus, semantic confusion is compounded by methodological variation. Casual readers of the literature may not recognize that EPCs are a mixed population of progenitor cells of different lineages. Within this population of cells, there are true endothelial progenitors that can incorporate into the vascular network, whereas hematopoietic progenitors may contribute by secreting angiogenic cytokines. In cell culture, EPCs may form early-outgrowth and late-outgrowth colonies. Cells derived from the former colonies are clearly not of endothelial origin, expressing markers of hematopoietic lineage, and are morphologically distinct from late-outgrowth cells, which grow in a cobblestone pattern reminiscent of ECs. At present, there are no surface markers that clearly distinguish early endothelial progenitors; the best approach currently is to define endothelial lineage morphologically (ie, with tubulogenesis assays). The formation of a tubular network in Matrigel is a defining feature of endothelial progenitors, which can also incorporate into existing tubular networks formed by differentiated ECs. Kaushal and colleagues demonstrated that EPC-seeded decellularized porcine iliac arteries implanted into sheep were structurally, functionally, and morphologically similar to a native vessel with a high flow patency.¹⁰⁵ EPCs have also been grown on a decellularized vessel, and successfully transplanted in an interposition graft in the carotid artery.¹⁰⁶ However, this approach may generate an endothelial monolayer with some differences from in vivo ECs, with lower expressions of NO synthase, prostacyclin, and tissue plasminogen activator.¹⁰⁷ It should be noted also that EPC decrease in number with aging, which might complicate the use of these cells for clinical conditions.¹⁰⁸

Pluripotent stem cell-derived ECs may have several advantages over ECs from patient vein segments or EPCs, including that they can potentially generate larger quantities of ECs. In one study, mouse ESCs were added to a microporous tube made of polyurethane, and differentiated into ECs and SMCs in vitro.¹⁰⁹ ECs may also be genetically engineered, for example, to reduce senescence. ECs derived from mouse ESCs were transduced with human telomerase reverse transcriptase to immortalize the ECs.¹¹⁰ These genetically modified ECs were used to endothelialize hybrid grafts made of a SMC layer on polyglycolic acid scaffolds which were then subcutaneously implanted into hairless SCID (nude) mice. A typical blood vessel structure with EC-lining and a vessel wall composed of SMC and collagen was generated using this approach.

ECs for Generating the Microvasculature

ECs have the ability to form capillary-like networks in vitro.^{111–113} Human umbilical vein ECs, keratinocytes, and fibroblasts were seeded together in a collagen sponge to form spontaneous capillary-like structures in vitro, followed by implantation in mice.¹¹⁴ The capillary-like tubes formed within the engineered skin tissue connected with the host vessels within 4 days of implantation. To produce larger and more complex tissue constructs, homogeneous distribution of microvessels throughout the tissue needs to be achieved for transport of nutrients and oxygen.¹¹⁵ Also, grafts seeded with CD34⁺ bone marrow derived EPCs showed significantly improved endothelialization and increased microvessels within the neointima when these grafts were implanted into the thoracic aortae of adult dogs.^{116–118} In human, vascular regeneration has been attempted with autologous BMNCs or granulocyte colony-stimulating factor–expanded peripheral blood mononuclear cells in patients with coronary as well as peripheral arterial disease. Phase I and II trials have been completed in patients with acute myocardial infarction,¹¹⁹ as well as in subjects with chronic ischemic heart disease.^{120–122} Thus far, the majority of these early trials have suggested that modest improvements may be gained in some cardiac end points such as global and regional contractility with little risk for these cell-based therapies.¹¹⁹ Additionally, since the first study in 2002 there have been at least 30 reported therapeutic cell trials in patients with peripheral arterial disease, some of which have shown promise in improving perfusion, healing ulcers, and increasing walking distance.^{123,124} However, most of these trials have been small observational studies, and need confirmation in larger randomized clinical trials (Figure 3).

Concluding Remarks

One is only as old as one's endothelium.¹²⁵ Cardiovascular health begins with restoration of endothelial homeostasis. Accordingly, studies of endothelial biology will continue to lead to new insights of clinical relevance and therapeutic promise. The generation of disease-specific iPSC-derived vascular cells provides an opportunity to delve deeper into the mechanisms of disease. Such cells can be used to understand the early events in the pathogenesis of diseases that are now only recognized in their late stages. Furthermore, adult and pluripotential stem cells are under investigation to determine their utility for ischemic syndromes affecting the coronary and peripheral circulations. Although it remains to be seen whether adult stem cells promote vascular regeneration and relieve ischemic syndromes, early studies have been promising, albeit the effect sizes modest. Because they have greater proliferative capacity, pluripotential stem cells seem to hold greater promise for the future of vascular regeneration. The fidelity and safety of iPSC/ESC-derived ECs needs to be confirmed before clinical trials. Furthermore, methods for their derivation need to be improved. Still to be determined are important clinical issues, such as dosing, duration, and methods of delivery of therapeutic cells. Under development are bioengineering solutions, in which the cells are administered within a decellularized or synthetic matrix. These exciting developments in translational endothelial biology will open new vistas for exploration and novel therapeutic avenues for development.

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Disclosures

None.

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