Endothelial Cells Derived From Human iPSCs Increase Capillary Density and Improve Perfusion in a Mouse Model of Peripheral Arterial Disease

Abdul Jalil Rufaihah, Ngan F. Huang, Sina Jame, Jerry Lee, Ha N. Nguyen, Blake Byers, Abhijit De, Janet Okogbaa, Mark Rollins, Renee Reijo-Pera, Sanjiv S. Gambhir, John P. Cooke

Objective—Stem cell therapy for angiogenesis and vascular regeneration has been investigated using adult or embryonic stem cells. In the present study, we investigated the potential of endothelial cells (ECs) derived from human induced pluripotent stem cells (hiPSCs) to promote the perfusion of ischemic tissue in a murine model of peripheral arterial disease.

Methods and Results—Endothelial differentiation was initiated by culturing hiPSCs for 14 days in differentiation media supplemented with BMP-4 and vascular endothelial growth factor. The hiPSC-ECs exhibited endothelial characteristics by forming capillary-like structures in matrigel and incorporating acetylated-LDL. They stained positively for EC markers such as KDR, CD31, CD144, and eNOS. In vitro exposure of hiPSC-ECs to hypoxia resulted in increased expression of various angiogenic related cytokines and growth factors. hiPSC-ECs were stably transduced with a double fusion construct encoded by the ubiquitin promoter, firefly luciferase for bioluminescence imaging and green fluorescence protein for fluorescent detection. The hiPSC-ECs (5×10^5) were delivered by intramuscular injection into the ischemic hindlimb of SCID mice at day 0 and again on day 7 after femoral artery ligation (n=8). Bioluminescence imaging showed that hiPSC-ECs survived in the ischemic limb for at least 2 weeks. In addition, laser Doppler imaging showed that the ratio of blood perfusion was increased by hiPSC-EC treatment by comparison to the saline-treated group (0.58±0.12 versus 0.44±0.04; P=0.005). The total number of capillaries in the ischemic limb of mice receiving hiPSC-EC injections was greater than those in the saline-treated group (1284±155 versus 797±206 capillaries/mm²) (P<0.002).

Conclusion—This study is a first step toward development of a regenerative strategy for peripheral arterial disease based on the use of ECs derived from hiPSCs.

Key Words: angiogenesis ■ endothelium ■ differentiation ■ induced pluripotent stem cells ■ peripheral vascular disease

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tem cell therapy for angiogenesis and vascular regeneration has been investigated previously using diverse cell types, including adult mesenchymal stem cells, endothelial progenitor cells, embryonic stem cells (ESCs), and recently with human induced pluripotent stem cells (hiPSCs). The hiPSCs share many similar characteristics with human ESCs. In addition to having similar capacity for self-renewal, they are capable of differentiating into cells of all three germ layers. However, unlike hESCs, hiPSCs are derived from reprogramming of somatic cells and therefore represent a source of autologous pluripotent stem cells that may obviate the immunologic concerns of hESCs. Furthermore, hiPSCs can be experimentally derived from easily accessible and plentiful human tissue sources such as skin or fat. Consequently, hiPSCs provide a suitable platform for modeling diseases in vitro and also serve as a potential source of cells for regenerative medicine.

Previously, hiPSCs have been shown to differentiate into endothelial cells (ECs). However, the therapeutic potential of hiPSC-derived ECs (hiPSC-ECs) for the treatment of ischemic diseases has not been reported. In this study, we describe the differentiation of hiPSCs into ECs and characterize their histological and functional properties in vitro. In addition, using molecular imaging and laser Doppler perfusion studies, we observe evidence of cell localization and survival in the ischemic limb in association with improved blood flow in a murine model of peripheral arterial disease. Immunohistochemical and proteomic studies indicate that
hiPSCs increase microvessel density and secrete angiogenic cytokines. Our results demonstrate that fibroblast-derived hiPSCs have the potential to promote vascular regeneration in ischemic tissue.

Methods (Expanded Methods Section is Available Online at http://atvb.ahajournals.org)

Cell Lines and In Vitro Studies

Derivation and Differentiation of Human Induced Pluripotential Stem Cells
The human induced pluripotential stem cells (hiPSCs) were derived from human foreskin fibroblasts using retroviral constructs encoding the Yamanaka factors as previously described.7 Their complete characterization is described elsewhere (B. Byers, unpublished data, 2010); but in addition, we performed alkaline phosphatase staining, immunohistochemistry for pluripotency markers, and teratoma assay (see supplemental files available online at http://atvb.ahajournals.org). To initiate differentiation, confluent cultures of hiPSCs were transferred to ultra-low attachment dishes containing differentiation media for 4 days to form embryoid bodies. The 4-day embryoid bodies were then seeded on 0.2% gelatin-coated dishes and cultured for another 10 days in differentiation media. To purify the hiPSC-ECs, we incubated single cell suspensions with PE-conjugated anti-human CD31 antibody (Ab). Flow cytometry was then performed to obtain purified hiPSC-ECs.

Characterization of hiPSC-ECs
The hiPSC-ECs were stained with Abs against endothelial markers such as PECAM-1, vascular endothelial-cadherin, endothelial nitric oxide synthase, and von Willebrand factor (vWF). Uptake of acetylated LDL was assessed by incubating the cells with Dil-labeled ac-LDL. For the tube formation assay, cells were seeded on 24-well plates precoated with growth factor-reduced Matrigel and incubated for 24 hours. Human antibody arrays were used to assess the various cytokines secreted by the hiPSC-ECs in normoxic and hypoxic conditions.

In Vivo Studies
For in vivo Matrigel injection, Matrigel was mixed with hFGF and hiPSC-ECs (5×10⁵). The mixture was subcutaneously injected into SCID mice. After 14 days, the Matrigel plugs were removed, paraffin-embedded, sectioned, and stained with CD31 Ab. Subsequently, the Matrigel plugs were also placed in matrigel, and injected subcutaneously into SCID mice. After 14 days, the Matrigel plugs were removed, paraffin-embedded, sectioned, and stained with CD31 Ab.

Isolation and Characterization of hiPSC-ECs
The hiPSC-ECs were isolated by FACS after 2 weeks of differentiation and then expanded for further characterization. The typical yield of ECs generated from the HUF5 hiPSC line ranged between 5% to 20% (Figure 1A). The expanded hiPSC-ECs formed a “cobblestone” monolayer, and immunofluorescence staining revealed that these cells were positive for endothelial markers such as CD31, CD144, endothelial nitric oxide synthase, and vWF (Figure 1B–1E). In addition, they were able to incorporate acetylated LDL and form networks of tubular structures after 24 hours of culture on matrigel (Figure 1F–G). Furthermore, when hiPSC-ECs were placed in matrigel, and injected subcutaneously in immunodeficient mice, they formed capillaries (Figure 1H). These capillaries were continuous with the murine systemic circulation as they contained blood cells (Insert, Figure 1H).

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Derivation and Characterization of Transduced hiPSC-ECs
The hiPSC-ECs were stably transduced with the double fusion reporter construct (Supplemental Figure II, available online at http://atvb.ahajournals.org) and FACS sorted for dual expression of GFP and CD31. Less than 20% of the hiPSC-ECs expressed both GFP and CD31 after transduction; but after several passages, about 75% were GFP and CD31 positive before cell transplantation (Figure 2A). They maintained the cobblestone morphology in culture and manifested mRNA and protein expression of CD31 and CD34 (Figure 2B–D). There was a strong correlation between the Fluc activity and cell number (R²=0.99; Figure 2E).

Survival of hiPSC-ECs in the Ischemic Hindlimb
In initial studies, we injected single dosages of 5×10⁵ hiPSC-ECs to the ischemic limb only on day 0 and tracked the survival of the cells over 14 days. We observed a decline in BLI that became undetectable by day 11 (Supplemental Figure IX, available online at http://atvb.ahajournals.org). For this reason, for the remainder of the studies, we administered cells on day 0 as well as day 7.

On transplantation into the ischemic limb, the hiPSC-ECs could be detected in the ischemic limb noninvasively by BLI (Figure 3). After each of the injections on day 0 and on day 7, there was an increase in bioluminescence intensity, followed by a gradual decrease over several days (Figure 3B).

All animal studies were approved by our Administrative Panel on Laboratory Animal Care.

Results

Characterization of HUF5 iPSC Line
The hiPSC line was generated by retrovirus-mediated transduction of Oct-4, Sox-2, Klf-4, and c-Myc using primary human adult dermal fibroblasts obtained from a healthy 46-year old female. The hiPSC colonies expressed pluripotency markers such as SSEA3/4, TRA-1 to -81, TRA-I-60, Nanog and alkaline phosphatase (Supplemental Figure IA, available online at http://atvb.ahajournals.org) and formed teratomas in vivo (Supplemental Figure IB).

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Figure 1. Derivation and characterization of human induced pluripotent stem cells-endothelial cells (hiPSC-ECs). A, [Left panel] Isotype control. [Right panel] FACS plot of data obtained using CD31+ antibody to quantitate ECs derived from hiPSCs. B–E, Immunofluorescent staining of the hiPSC-ECs for endothelial markers CD31, CD144, eNOS, and von Willebrand factor. F, hiPSC-ECs form a capillary-like network on Matrigel 24 hours after seeding the cells. G, hiPSC-ECs take up acetylated LDL. Scale bar: 50 μm. H, In vivo formation of capillary structures in matrigel plugs after subcutaneous transplantation in SCID mice, stained using an antibody specific for human CD31.
Additional experiments were carried out in which ischemic hindlimbs of SCID mice were injected on days 0 and 7 with human fibroblasts that were transduced by the same lentiviral vector for Fluc expression. As shown in Supplemental Figure III (available online at http://atvb.ahajournals.org), the fibroblast bioluminescence also correlated strongly with cell number. Like hiPSC-ECs, there was a decline in bioluminescence signal during the first 4 days, followed by an increase after the second dose of cell injection on day 7, and then a gradual decrease with time. In contrast to hiPSC-ECs, the fibroblasts were detected for up to 4 weeks in the ischemic limb (Supplemental Figure IV—VI, available online at http://atvb.ahajournals.org).

**Figure 2.** Characterization of human induced pluripotent stem cells-endothelial cells (hiPSC-ECs) transduced with lentiviral construct for bioluminescence imaging and histological detection. **A,** hiPSC-ECs previously transduced with a construct encoding luciferase and Fluc were sorted by FACS using green fluorescence protein (GFP) and CD31 expression. **B,** Transduction with GFP construct does not affect expression of EC markers. **C, D,** Immunofluorescence staining of GFP, CD31, and CD144 in the purified transduced hiPSC-ECs. **E,** Quantification of the correlation between the cell number and the bioluminescence imaging signal (R²=0.99). Scale bar: 50 μm.

**Improvement of Blood Perfusion in Ischemic Hindlimbs by hiPSC-EC Transplantation**

Laser Doppler perfusion imaging was performed to determine the effects of localized transplantation of hiPSC-EC on the ischemic hindlimb at days 0, 4, 7, and 14 post-treatment. The hindlimb perfusion ratio (ischemic/control hindlimb) was significantly improved in the hiPSC-EC treated mice by comparison to the saline-treated mice (P<0.005; Figure 4A–B).

To assess the survival and effect of hiPSC-ECs with a longer time course, and in comparison to treatment with fibroblasts, additional experiments were carried out to 4 weeks postinjection. The cells in this experiment were not transduced with Fluc to preclude the possible effects of lentiviral vectors on cell behavior. As shown in Supplemental Figure VI, the mean perfusion ratio of the hiPSC-EC-treated group (0.53±0.10) was significantly greater after 4 weeks, than that of the saline- or fibroblast-treated groups (0.37±0.04 and 0.42±0.07; P=0.009 and P=0.003, respectively). This data suggests that hiPSC-ECs were therapeutic cells that enhanced limb perfusion for up to 4 weeks postdelivery.

**Improved Blood Capillary Density on hiPSC-EC Transplantation**

To verify the laser Doppler data, we quantified capillary density by immunofluorescence staining. Murine capillary density in the 2-week cell-treated group was greater than that of the saline-treated group (Figure 5A–B). Dual staining for both GFP and vWF demonstrated the persistence of hiPSC-ECs in the ischemic limb at low frequency (Figure 6A). Human-derived vascular endothelial-cadherin positive cells in capillaries were also occasionally observed in the tissue sections (Supplemental Figure VII, available online at http://atvb.ahajournals.org). These results suggest that the therapeutic effect of hiPSC-ECs may be related to its ability to incorporate into and/or enhance expansion of the endogenous microvasculature.
Angiogenic Cytokines Secreted by the Transplanted hiPSC-ECs in the Ischemic Hindlimbs

To further understand the mechanism by which hiPSC-ECs enhanced angiogenesis in the ischemic limbs, we assessed the cells in vitro for the expression of angiogenic cytokines under hypoxic and normoxic conditions. Indeed, hiPSC-ECs expressed a majority of the angiogenic cytokines, in some cases at higher levels of expression than primary HMDECs. These cytokines include Angiopoietin-1, vascular endothelial growth factor-A and -C, and platelet-derived growth factor-AA (Figure 6B). The results for the entire angiogenic protein array of 55 cytokines and growth factors are shown in Supplemental Figure VIII (available online at http://atvb.ahajournals.org). This upregulation of angiogenic cytokines by the hiPSC-ECs in hypoxia is consistent with the enhanced blood perfusion and capillary density in the ischemic limbs that received hiPSC-ECs.

Discussion

Somatic cells may be reprogrammed into iPSCs using induced expression of transcriptional factors involved in maintaining pluripotency.7,12 Expression of Oct3/4, Sox2, Klf4, and c-Myc in somatic cells reprograms these cells as evidenced by the lengthening of the telomeres13,14 and the acquisition of pluripotency similar to that of embryonic stem cells.15 Thus, hiPSCs represent a promising cell source for regenerative medicine. These cells are particularly attractive because they can be used to generate patient specific pluripotent cells that do not face an immunologic barrier. However, the therapeutic potential of hiPSCs remains largely untested. Recently, hiPSC-derived neurons, cardiomyocytes, and mesenchymal stem cells have shown therapeutic promise in preclinical studies.16–18 The present study is the first to assess the potential of hiPSC-derived endothelial cells for ischemic vascular disease. We find that hiPSCs can be differentiated into ECs as indicated by typical cobblestone monolayer morphology, expression of EC surface markers and cytoplasmic factors, and manifestation of characteristic endothelial functions (acetylated LDL uptake and capillary-like network formation in matrigel).

When these cells were delivered to the ischemic limb of the mouse by intramuscular injection, they increased capillary density and limb perfusion. In contrast, injection of fibroblasts did not improve limb perfusion by comparison to vehicle. We observed some hiPSC-ECs that appeared to incorporate into the microvasculature and others that were in close proximity. However, the observed increase in capillary density could not be accounted for simply by the incorporation of hiPSC-EC into the existing vasculature. Instead, it is likely that the injected cells had a paracrine effect. Indeed, we find that the hiPSC-ECs secrete angiogenic cytokines and

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Localization and survival of human induced pluripotent stem cells-endothelial cells (hiPSC-ECs) in the ischemic limb. A, hiPSC-ECs were delivered by intramuscular injection of the ischemic limb and were tracked noninvasively by bioluminescence imaging. B, Quantification of BLI signals in the ischemic limb of mice after cell injection. Dashed line refers to the threshold to discriminate background noise from a positive bioluminescence imaging signal.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Improvement in blood perfusion in the ischemic hindlimb after human induced pluripotent stem cells-endothelial cells (hiPSC-EC) transplantation. A, Images of laser doppler perfusion imaging at day 14 after treatment. A greater increase in perfusion is observed in the ischemic limb (arrow) of the mouse that received hiPSC-EC transplantation by comparison to the saline-treated animal. B, Perfusion ratio of ischemic limbs at day 14 after treatment. The perfusion ratio (value of the ischemic limb divided by that of the nonischemic limb) was greater in mice that received hiPSC-EC transplantation compared to those that received saline (n = 8 each group, *P* < 0.05).
growth factors in the presence of hypoxia, as well or better than primary human endothelial cells.

One concern in using iPSC-derived cells is the possibility of contamination of the therapeutic cells with parental iPSCs, which could lead to teratoma formation. We did not observe any tumor formation in any of our animals receiving hiPSC-ECs. However, longer term studies with greater numbers of mice, and/or more sensitive probes to detect undifferentiated cells, are necessary to provide greater assurance that the differentiation process is complete. Also, further study is needed to resolve concerns regarding the possible adverse effects of abnormal imprinting, copy number variation, or other somatic mutations arising from the reprogramming or differentiation process.

Using molecular imaging, we observed a reduction in cell number over time in the ischemic limb. Bioluminescence imaging is a sensitive and accurate method for tracking cells in vivo with as few as 500 cells. We found that the number of transplanted cells began to decrease within 24 hours post-transplantation. In order to improve the efficacy of our hiPSC-EC transplantation, we did a second injection at day 7 postsurgery. The transplanted cells face the serious challenge of an unfriendly environment characterized by reduced oxygen and nutrient supply and the presence of various cytotoxic and inflammatory products in the ischemic limb. In addition, although we are using an immunodeficient mouse model, the human cells may stimulate a mild immune reaction and/or may not be receiving appropriate signaling required for their efficient integration into the mouse vasculature.

Adult stem cells (such as those derived from the bone marrow, circulating mononuclear cells, or mesenchymal stem cells) have been used in small clinical trials of patients with myocardial ischemia or peripheral arterial disease. Only a few of these trials have been randomized, with large enough numbers of subjects, followed for sufficient period of time, to draw conclusions. These early data have shown proof of concept that cell therapy can provide some benefit in the setting of ischemic syndromes. However, adult stem cells such as endothelial progenitor cells have limited replicative capacity, and are few in number and dysfunctional, in elderly patients with cardiovascular risk factors. By contrast hESCs have unlimited capacity to replicate, can be differentiated into ECs, and in preclinical studies have shown beneficial effects in the setting of myocardial or limb ischemia. Previously we have observed that ESC-derived ECs can home to sites of peripheral ischemia, incorporate into the microvasculature, increase capillary density, and improve limb perfusion. However, there is an immunogenic barrier for ESC-derivatives and this can pose a challenge in clinical application.

By contrast, hiPSCs can be used to generate autologous therapies, minimizing or eliminating the need for immune suppression after cell transplantation. However, before hiPSCs can be considered for clinical applications, a number of...
technical issues need to be addressed. Currently most hiPSCs are generated using integrating viral vectors. However, integration of foreign DNA could inadvertently silence dispensable genes, or generate an oncogenic phenotype. Thus, clinical grade therapeutic cells might be derived using non-integrating episomal vector systems, cell permeant peptides, RNA-based and/or small molecules or RNA-based approaches. In addition, the efficiency and speed of hiPSC generation should be increased, and the fidelity of reprogramming (as assessed by epigenetic, genomic, proteomic, and functional studies) must be assured. In this regard, we and others note heterogeneity between hiPSC clones in differentiation potential. These differences are more exaggerated in hiPSC as compared to hESC clones. The reasons for the reduced tendency to differentiate down some lineages in some hiPSC lines are not fully known but may include factors such as persistent “epigenetic memory” as recently described. Differentiation to functional lineages must be assured, and the risk reduced of transplanting pluripotential or suboptimally differentiated cells. Finally, it is unclear how iPSC-ECs compare in therapeutic efficacy to other clinically relevant cell types such as bone marrow mononuclear cells, endothelial progenitor cells, and mesenchymal stem cells, which each enhance blood flow recovery and improve angiogenesis in the murine hindlimb ischemia model (44–47).

In conclusion, this is the first study to demonstrate that functional human ECs may be differentiated from hiPSCs, as evidenced by in vitro characterization and in vivo application in a murine model of peripheral arterial disease. This is another step forward toward developing hiPSC-derived cell therapy for vascular regeneration.

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None.

References


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Supplemental Materials

Expanded Methods

Maintenance of cell lines

The hiPSCs were derived from human skin fibroblasts using retroviral constructs encoding the Yamanaka factors as previously described (7), and their complete characterization is described elsewhere (Byers B, BS, unpublished data, 2010). The undifferentiated hiPSCs were cultured as described previously on mitomycin-inactivated CF1 mouse embryonic fibroblasts which served as a feeder cell layer (8). They were grown using hESC media consisting of DMEM:F-12 supplemented with knockout serum replacement (20% v/v), L-glutamine (2mM), β-mercaptoethanol (0.1mM), non-essential amino acid stock(1%) and basic fibroblast growth factor (bFGF; 10ng/ml). All cultures were routinely passaged every 5-7 days after disaggregation with collagenase type IV (1mg/ml) and the media was changed daily. The hiPSCs were passaged every 5 to 6 days using collagenase IV and transferred to dishes with a feeder cell layer. The human dermal microvascular endothelial cells (HMDVECs) were grown under standard conditions in EGM-2MV growth medium (each from Lonza, Walkersville, MD). Human foreskin fibroblasts (BJ; ATCC, Manassas, VA) were cultured in fetal bovine serum (10%) and penicillin/streptomycin(1%).

Alkaline Phosphatase and Immunofluorescence Staining

Alkaline Phosphatase (AP) staining was performed for 30 min at room temperature in the dark using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA). For immunofluorescence staining of pluripotency markers, the cells were stained for SSEA3, SSEA4, Tra-1-60, Tra-1-81 (all from Millipore, Bedford MA) and Nanog (Abcam) as previously described (9). Secondary antibodies used consisted of Alexa 594-conjugated goat
anti-rat IgM or anti-rabbit IgG, Alexa 488-conjugated goat anti-rat IgM, anti-mouse IgM or anti-mouse IgG (all from Invitrogen).

**Teratoma Assay**

The hiPSCs were manually harvested, washed and resuspended in hESC medium (~10⁶ cells/300 µl) and then injected subcutaneously into female NOD SCID mice (Charles River Laboratories International, Inc., Wilmington, MA). Visible tumors at 4–8 weeks post-transplantation were dissected and fixed overnight with 4% paraformaldehyde/PBS solution. The tissues were then paraffin embedded, sectioned, stained with hematoxylin and eosin, and examined by a pathologist for the presence of representative cells of all three germ layers.

**hiPSC-EC differentiation and purification**

To initiate differentiation, confluent cultures of hiPSCs were incubated with type IV collagenase for 10 minutes and transferred to ultra low attachment dishes containing differentiation media for 4 days to form embryoid bodies (EBs). The differentiation media consisted of α-Minimum Eagle’s Medium, FBS (20%), β-mercaptoethanol (0.05mmol/L), non-essential amino acids (1%), bone morphogenetic protein-4 (BMP-4; 50ng/ml) and vascular endothelial growth factor-A (VEGF-A) (both from Peprotech, Rocky Hill, NJ). 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 BMP-4. Differentiation media was changed every 2 days. To purify the hiPSC-ECs, single cell suspensions were obtained using Accutase (Sigma, St Louis, MO) for 20 minutes at 37°C to dissociate differentiated cells, which were then washed with 1x PBS containing 5% BSA, passed through a 70-µm cell strainer (BD Biosciences, Bedford, MA), and incubated with PE-conjugated anti-human CD31 antibody (eBioscience, San Diego, CA) for 30 minutes. Isotype-
matched antibody served as negative control and 1% propidium iodide was used to stain the non-viable cells. Flow cytometry was then performed using BD Digital Vantage cell sorter (BD Biosciences, San Jose, CA). The purified hiPSC-ECs were expanded in EGM-2MV.

**hiPSC-EC characterization**

To characterize the phenotype of hiPSC-ECs, the purified cells were stained with antibodies against endothelial markers such as PECAM-1 (CD31, R&D Systems, Minneapolis, MN), VE-cadherin (CD144; R&D Systems), endothelial nitric oxide synthase (eNOS; BD Pharmingen, San Diego, CA, USA) and von Willebrand factor (vWF; Abcam, Cambridge MA). Briefly, the cells were 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 were washed with 1x PBS and incubated with Alexa Fluor-488 or -594 secondary antibodies for 1 hour at room temperature. Cells were washed with 1x PBS and the nuclei were stained with Hoechst 33342 dye (Invitrogen, Carlsbad, CA, USA). Uptake of acetylated LDL was assessed by incubating the cells with Dil-labeled ac-LDL (10µg/ml; Invitrogen) for 4 hours at 37°C. After incubation, they were washed with 1x PBS before being visualized and photographed under fluorescent microscope. For Matrigel tube formation, cells (2.5 x 10^5) were seeded on 24-well plates pre-coated with growth factor-reduced Matrigel (BD Discovery Labware, Bedford, MA) and incubated for 24 hours in 37°C. Images were taken using a light microscope. For in vivo Matrigel injection, Matrigel was mixed with bFGF (50ng/ml; Peprotech, Rocky Hill, NJ) and hiPSC-ECs (5x10^5). The mixture was subcutaneously injected into SCID mice. After fourteen days, the animals were euthanized and dissected to remove the Matrigel plugs. Paraffin-embedded matrigel sections were stained with CD31.
Assay for angiogenic cytokines

Human Angiogenesis Proteome Profiler™ antibody arrays (R&D Systems) were used to assess the various cytokines secreted by the hiPSC-ECs in normoxic and hypoxic conditions according to the manufacturer’s instructions. In brief, hiPSC-ECs were grown in hypoxia (1% O₂) or normoxia (21% O₂) for 24 hours (n=2). The conditioned media was pooled from each well, passed through 0.2µm sterile filters, and incubated with the antibody cocktail (1:1000) for 1 hour at room temperature. Nitrocellulose membranes, containing the capture antibodies, were blocked using the assay specific blocking solution. Thereafter, the sample/detection antibody cocktail mixture was added to the membranes and incubated overnight at 4°C on a rocking platform. Each membrane was washed 3 times with 1X wash buffer for 10 min of a rocking platform before incubation with Streptavidin-HRP (1:2000) for 30 min at room temperature. The membranes were then washed prior to incubation with ECLplus (Amersham, Buckinghamshire, UK). The membranes were exposed to X-ray film for 3 minutes. The array data was quantified by densitometry using Image J software.

Transduction of hiPSC-ECs and fibroblasts with double fusion reporter construct

For non invasive tracking in vivo, the hiPSC-ECs and fibroblasts were transduced with a lentiviral vector (LV-pUb-Fluc-GFP) carrying an ubiquitin promoter driving firefly luciferase (Fluc) and enhanced green fluorescence protein (GFP) as described previously (9). They were then purified using FACS by GFP-positive and CD31-positive expression. In order to determine the correlation between cell density and fluc activity, hiPSC-ECs of varying densities were incubated with reporter probe, D-luciferin (150µg/ml) and bioluminescence imaging (BLI) was then performed using In Vivo Imaging System Spectrum (IVIS Spectrum; Caliper Life Sciences, Hopkinton, Mass). The BLI intensity was expressed in units of photons/cm²/second/steradian (p.cm⁻².s⁻¹.sr⁻¹).
Mouse hindlimb ischemia model and cell transplantation

Unilateral hindlimb ischemia was induced by ligating the femoral artery of (4-6 months) male NOD SCID mice as we have reported (10). The animals were randomly assigned to groups receiving either saline or cell intramuscular (IM) injection, specifically in the gastrocnemius muscle (n=8 each group) and were observed for 14 days. In a separate investigation we assessed longer term (4 weeks) effect of hiPSC-EC or fibroblast transplantation. The animals were randomly assigned into groups receiving saline (n=4), hiPSC-ECs (n=7) or human fibroblasts (n=7). The hiPSC-ECs or fibroblasts (5 x 10^5 cells per mouse) were suspended in 150µl of saline and delivered by IM injection into the animals immediately after induction of hindlimb ischemia. After 7 days, an additional treatment (5 x 10^5 cells) was delivered to the cell-treatment group by intramuscular (IM) injection. All animal studies were approved by our Administrative Panel on Laboratory Animal Care.

BLI for cell survival and localization analysis

At indicated time points, animals were injected with D-luciferin (375 mg/kg) into the peritoneum, and BLI imaging was performed using the IVIS-Spectrum according to our previous studies (11). Bioluminescence intensity of the ischemic limb was quantified in units of p.cm^{-2}.s^{-1}.sr^{-1}.

Laser Doppler Blood Perfusion

Perfusion of the ischemic and non-ischemic hindlimb was assessed using the PeriScan PIM3 laser Doppler system (Perimed AB, Sweden) (10). The mice were prewarmed to core temperature of 37°C and the blood perfusion was measured pre- and post-operatively on day 0 and again on days 4, 7 and 14. The level of perfusion for both ischemic and non-ischemic hindlimbs were quantified using the mean pixel value within the region of interest and the relative changes in blood perfusion were expressed as the ratio of the ischemic over the non-ischemic perfusion value.
Capillary density measurement

After euthanization of the animals, the gastrocnemius tissue was excised from both the ischemic and control hindlimbs and then snap frozen in O.C.T. compound (Sakura Finetek, Japan) for cryosectioning. For murine capillary density analysis, 4 sections from each mouse hindlimb was stained using a mouse-specific CD31 (BD Pharmingen), followed by Alexafluor-594 secondary antibody. Capillary density was assessed by counting the number of capillaries in 5 high-powered fields in each of 4 tissue sections and then expressing the data as capillaries/mm² (11). Survival of transplanted cells was visualized by staining with a vWF antibody that reacts with both murine and human capillaries. The hiPSC-ECs were detected by their coexpression of GFP and vWF. The transplanted cells were also detected using a human specific VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) antibody and the Animal Research Kit (Dako Cytomation, Carpinteria, CA).

Supplemental Figure Legends

**Suppl Fig I**: Histological characterization of the hiPSC line. A) [First row] Alkaline phosphatase staining (left panel) and light microscopy of a hiPSC colony (right panel)  [Second and third rows] Nuclear staining (DAPI) and immunofluorescence staining for markers of pluripotentiality including SSEA3, Tra1-60, Tra1-81, Nanog, SSEA4, SSEA-1 B) hiPSCs form teratoma when injected into subcutaneously into the SCID mouse. The tissues were stained with hematoxylin and eosin to document cells originating from the three germ layers; gut epithelium (endoderm), cartilage (mesoderm) and neural tube (ectoderm). Scale bar: 100μm

**Suppl FigII**: Schematic of the double fusion reporter construct used to transduce the hiPSC-ECs for non-invasive tracking. For non invasive tracking in vivo, the hiPSC-ECs and fibroblasts
were transduced with a lentiviral vector carrying an ubiquitin promoter driving firefly luciferase (Fluc) and enhanced green fluorescence protein (GFP)

**Suppl Fig III**: Comparison of the correlation between the cell number and the bioluminescence imaging (BLI) signal (R=0.99) for hiPSC-ECs and fibroblasts.

**Suppl Fig IV**: Comparison between the localization and survival of hiPSC-ECs and fibroblasts in the ischemic limbs after 28 days. A) hiPSC-ECs or fibroblasts were delivered by IM injection of the ischemic limb and were tracked non-invasively by BLI. B) Quantification of BLI signals in the ischemic limb of mice after cell injection. Dashed line is the threshold for positive BLI signal.

**Suppl Fig V**: Comparison between the BLI values for hiPSC-ECs and fibroblasts injected into the ischemic limbs over 28 days. The BLI signal is expressed in each group as percent change relative to day 0.

**Suppl Fig VI**: Comparison between the improvements in blood perfusion in the ischemic hindlimb after hiPSC-EC or fibroblast transplantation. A) Images of laser doppler perfusion imaging at day 28 after treatment. A greater increase in perfusion is observed in the ischemic limb (arrow) of the mouse that received hiPSC-EC transplantation by comparison to the saline-treated animal. B) Perfusion ratio of ischemic limbs at day 28 after treatment. The perfusion ratio was greater in mice that received hiPSC-EC transplantation (n=8) compared to those that received saline (n=4) or fibroblast (n=7).

**Suppl Fig VII**: Immunohistochemical staining for human VE-cadherin in the mouse ischemic hindlimb shows hiPSC-ECs incorporating into the microvasculature. Scale bar: 50μm

**Suppl Fig VIII**: Proteomic analysis of angiogenesis-related proteins, with comparison of human ECs and hiPSC-ECs under normoxia or hypoxia.

**Suppl Fig IX**: Quantification of BLI signals in the ischemic limb after single hiPSC-EC injection
<table>
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<td>Bone marrow derived EPCs, 2 x 10^6 cells</td>
<td>Intravenous (IV) injection</td>
<td>Enhanced bone marrow-derived EPC incorporation into foci of corneal neovascularization at day 6 post hindlimb ischemia</td>
<td>Takahashi T et al, Nature Med 1999</td>
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<td>Culture-expanded endothelial progenitor cells derived from human peripheral blood mononuclear cells, 5 x 10^5 cells</td>
<td>Intra-cardiac injection</td>
<td>Blood flow recovery and capillary density in the ischemic hindlimb were improved as shown by laser Doppler perfusion. Limb loss was reduced.</td>
<td>Kalka C et al, PNAS 2000</td>
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<td>Bone marrow-derived mesenchymal stem cells, 1 x 10^7 cells</td>
<td>Percutaneous injection at 6 sites of the gastrocnemius muscle</td>
<td>Improved angiogenesis and blood flow in the gastrocnemius at 2 wks post-treatment.</td>
<td>Ikenaga S et al, Journal of Surgical Research 2001</td>
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<td>Early or late Endothelial progenitor cells (EPCs) from human peripheral blood mononuclear cells, 5 x 10^5 cells</td>
<td>Intra-ventricular injection</td>
<td>Both types of EPCs equally contribute to neovasculogenesis and improvement in limb perfusion at day 21 post-treatment.</td>
<td>Hur J et al, ATVB 2004</td>
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<td>Umbilical cord-blood derived multipotent stem cells, 1.3 x 10^6 cells</td>
<td>IM injection</td>
<td>Augmented arteriogenesis at day 28 post-treatment assessed by angiography.</td>
<td>Kim SW et al, Stem Cells 2006</td>
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<td>CD133^+ progenitor cells from peripheral blood, 3 x 10^5 cells</td>
<td>IM injections in the ischemic thigh muscle</td>
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<td>Suuronen EJ et al, Circulation 2006</td>
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<td>Human adipose stromal cells (hADSC) and bone marrow stromal cells (hBMSC), 1 x 10^6 cells</td>
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<td>Kim Y et al, Cell Physiol Biochem 2007</td>
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<td>Lian Q et al, Circulation, 2010</td>
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<td>Human adipose tissue-derived stem cells, $1 \times 10^6$ cells</td>
<td>IM injection into 3 separate regions from ankle up to thigh regions</td>
<td>Improved limb perfusion as measured by indocyanine green (ICG) perfusion imaging. Increased neovascularization and reduced muscle atrophy at 4 weeks post-treatment. hADSCs &gt; medium</td>
<td>Kang Y et al, Microvascular Research 2010</td>
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<td>Amniotic tissue-derived mesenchymal stem cells, $1 \times 10^6$ cells</td>
<td>IM injections at 3 different sites of the ischemic leg</td>
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<td>Kim and Choi, Heart Vessels 2011</td>
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</tbody>
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*In most cases, control was vehicle. Exceptions are noted.*
Supplementary 1

A

B

Endoderm  Mesoderm  Ectoderm
Supplementary 2

Lentiviral Construct

5' LVLTR → pUbiquitin → fluc → gfp → SIN LTR 3'
Supplementary 3

**hiPSC-EC**

![Image of hiPSC-EC](image)

- **A**: Heat map of cell density (X10^3)
- **B**: Graph showing bioluminescence (p/s/cm^2/sr-1) vs. cell density (X10^3)

**Fibroblast**

![Image of Fibroblast](image)

- **A**: Heat map of cell density (X10^3)
- **B**: Graph showing bioluminescence (p/s/cm^2/sr-1) vs. cell density (X10^3)

R² = 0.99

**Note:** The graphs compare bioluminescence intensity with cell density for hiPSC-EC and Fibroblast cells, showing a strong linear relationship in both datasets.
Supplementary 4

(A) Images showing the distribution of hiPSC-EC and Fibroblast cells over time. The images are color-coded to indicate the intensity of luminescence, with a scale ranging from 0.2 to 1.0 X 10^5 ps^-1 cm^-2 sr^-1.

(B) Graphs depicting the change in bioluminescence over time for hiPSC-EC and Fibroblast cells. The y-axis represents bioluminescence in units of ps^-1 cm^-2 sr^-1, and the x-axis represents time in days. Arrows indicate the days when cells were added.
Supplementary 4

4-week Bioluminescence Data

% Change in Bioluminescence

C

hiPSC-EC
Fibroblast

Cells

Cells

Supplementary 4
Supplementary 5

A  Day 28 iPSC-EC  Day 28 fibroblast  Day 28 saline

B

Mean Perfusion Ratio (Ischemic/Control)

PBS  hiPSC-EC  Fibroblast

* P=0.003  # P=0.009
Supplementary 6

Human Capillaries (VE-cadherin)
**Supplementary 7**

Protein fold change normalized to normoxia HMDECs

- **Normoxia HMDECs**
- **Hypoxia HMDECs**
- **Normoxia hiPSC-ECs**
- **Hypoxia hiPSC-ECs**

Protein fold change normalized to normoxia IPSC-ECs
Supplementary 8

Bioluminescence (p/cm$^2$/s/sr)

hiPSC-EC No Reinjection (n=4)
Supplementary 1: Histological characterization of the hiPSC line. A) [First row] Alkaline phosphatase staining (left panel) and light microscopy of a hiPSC colony (right panel) [Second and third rows] Nuclear staining (DAPI) and immunofluorescence staining for markers of pluripotentiality including SSEA3, Tra1-60, Tra1-81, Nanog, SSEA4, SSEA-1 B) hiPSCs form teratoma when injected into subcutaneously into the SCID mouse. The tissues were stained with hematoxylin and eosin to document cells originating from the three germ layers; gut epithelium (endoderm), cartilage (mesoderm) and neural tube (ectoderm). Scale bar: 100μm

Supplementary 2: Schematic of the double fusion reporter construct used to transduce the hiPSC-ECs for non-invasive tracking.

Supplementary 3: Comparison of the correlation between the cell number and the BLI signal (R=0.99) for hiPSC-ECs and fibroblasts.

Supplementary 4: Comparison between the localization and survival of hiPSC-ECs and fibroblasts in the ischemic limbs after 28 days. A) hiPSC-ECs or fibroblasts were delivered by IM injection of the ischemic limb and were tracked non-invasively by BLI. B) Quantification of BLI signals in the ischemic limb of mice after cell injection. Dashed line refers to the threshold for positive BLI signal. C) In vivo BLI signal expressed as percent change relative to day 0.

Supplementary 5: Comparison between the improvements in blood perfusion in the ischemic hindlimb after hiPSC-EC or fibroblast transplantation. A) Images of laser doppler perfusion imaging at day 28 after treatment. A greater increase in perfusion is observed in the ischemic limb (arrow) of the mouse that received hiPSC-EC transplantation by comparison to the saline-treated animal. B) Perfusion ratio of ischemic limbs at day 28 after treatment. The perfusion ratio was greater in mice that received hiPSC-EC transplantation (n=8) compared to those that received saline (n=4) or fibroblast (n=7).

Supplementary 6: Immunohistochemical staining for human VE-cadherin in the mouse ischemic hindlimb shows hiPSC-ECs incorporating into the microvasculature. Scale bar: 50μm

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