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Yamanaka factors as previously described (7), and their complete characterization is described elsewhere (Byers B, BS, unpublished data, 2010).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 (HMDECs) 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 (~10^6 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 (2.5×10^5) 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 (5×10^5). 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% O_2) or normoxia (21% O_2) 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/cm^2/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 (4weeks) 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 (5×10^5 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 (5×10^5 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/mm^2 (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

Supplemental Table I:

Human Cell Type	Route of administration	Measurement outcome by comparison to control*	Reference
Bone marrow derived EPCs, 2 x 10^6 cells	Intravenous (IV) injection	Enhanced bone marrow-derived EPC incorporation into foci of corneal neovascularization at day 6 post hindlimb ischemia	Takahashi T et al, Nature Med 1999
Culture-expanded endothelial progenitor cells derived from human peripheral blood mononuclear cells, 5 x 10^5 cells	Intra-cardiac injection	Blood flow recovery and capillary density in the ischemic hindlimb were improved as shown by laser Doppler perfusion. Limb loss was reduced.	Kalka C et al, PNAS 2000
Bone marrow-derived mesenchymal stem cells, 1 x 10^7 cells	Percutaneous injection at 6 sites of the gastrocnemius muscle	Improved angiogenesis and blood flow in the gastrocnemius at 2 wks post-treatment	Ikenaga S et al, Journal of Surgical Research 2001
Early or late Endothelial progenitor cells (EPCs) from human peripheral blood mononuclear cells, 5 × 10^5 cells	Intra-ventricular injection	Both types of EPCs equally contribute to neovasculogenesis and improvement in limb perfusion at day 21 post-treatment.	Hur J et al, ATVB 2004
Umbilical cord-blood derived multipotent stem cells,1.3 × 10^6 cells	IM injection	Augmented arteriogenesis at day 28 post-treatment assessed by angiography.	Kim SW et al, Stem Cells 2006
$CD133^+$ progenitor cells from peripheral blood, 3 x 10^5 cells	IM injections in the ischemic thigh muscle	Increased intramuscular capillary density and number of arterioles per mm^2 of muscle section at 2 weeks post-treatment	Suuronen EJ et al, Circulation 2006
Human adipose stromal cells (hADSC) and bone marrow stromal cells (hBMSC), 1 x 10^6 cells	IM injection at 3 different sites of the ischemic leg (gastrocnemius, gracilis, and quadriceps muscles	Improved limb perfusion as assessed by laser Doppler imaging at day 14 post treatment. hADSC>hBMSC>saline	Kim Y et al, Cell Physiol Biochem 2007
Human IPSC-derived mesenchymal stem cells, Bone-marrow-derived mesenchymal stem cells, 3 x 10^6 cells	IM injection, 4 sites of the gracilis muscle in the medial thigh	Improved limb perfusion as assessed by laser Doppler imaging at day 21 post treatment. Also promoted vascular and muscle regeneration.	Lian Q et al, Circulation, 2010

		IPSC-MSC>BM-MSC>medium	
Human adipose tissue-derived stem cells, 1 x 10^6 cells	IM injection into 3separate regions from ankle up to thigh regions	Improved limb perfusion as measured by indocyanine green (ICG) perfusion imaging. Increased neovascularization and reduced muscle atrophy at 4 weeks post-treatment. hADSCs > medium	Kang Y et al, Microvascular Research 2010
Amniotic tissue-derived mesenchymal stem cells, 1 x 10^6 cells	IM injections at 3 different sites of the ischemic leg	Increased limb perfusion at 2 weeks post-treatment as assessed by laser Doppler imaging: cells>conditioned medium>vehicle	Kim and Choi, Heart Vessels 2011

*In most cases, control was vehicle. Exceptions are noted.