Co-administration of Endothelial and Smooth Muscle Cells derived from Human Induced Pluripotent Stem Cells as a Therapy for Critical Limb Ischemia

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Running Title: Ischemic disease therapy using human iPSC

Supplementary Material and Methods

Western blotting

Cells were washed twice with Hank's balanced salt solution (HBSS) and then lysed in lysis buffer (20 mM Tris-HCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM ethylenediamine-tetraacetic acid, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1% Triton X-100, pH 7.4). The cell lysates were centrifuged for 15 min at 4 °C, and the supernatants were used for western blotting. Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma-Aldrich) to ensure equal loading of the samples. After blocking with 5% non-fat milk for 30 min, the membranes were incubated with primary antibodies overnight, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence western blotting system (ECL, Amersham Biosciences, Piscataway, NJ, USA).

Cell migration assay

The iPSC-EC migration was assayed using a disposable 96-well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). The iPSC-EC were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and resuspended in EBM-2 at a concentration of 1×10^4 cells/ml. A chemotaxis chamber with an 8 μm pore size membrane filter was pre-coated overnight with 20 μg/ml rat-tail collagen at 4 °C. An aliquot (35 μL) of the iPSC-EC suspension was loaded into the upper chamber, and SMC-conditioned medium (SMC-CM) was then placed in the lower chamber. After incubation of the cells for 12 h at 37 °C under 5% CO_2, the filters were disassembled, and the upper surface of each filter was scraped free of cells by

wiping with a cotton swab. The number of cells that migrated to the lower surface of each filter was determined by counting the cells in five random locations under a microscope at ×100 magnification after staining with Hoechst 33342.

Tube formation assay

For assessing the tube formation of iPSC-EC, growth factor-reduced Matrigel (BD Biosciences) was added to 96-well culture plates and polymerized for 30 min at 37 °C. A total of 1.5×10^4 iPSC-EC was seeded on these Matrigel-coated plates and cultured in EBM-2 medium containing 0.5% FBS or supplemented with SMC-CM. After incubation of the cells at 37 °C under 5% CO_2 for 12 h, the capillary structures were photographed with a digital camera in four random microscopic fields and quantified by measuring the capillary length using the Image J software (version 1.50i).

Cell proliferation assay

The effects of SMC-CM on iPSC-EC proliferation were examined by counting the proliferating cell nuclear antigen (PCNA)+ cell population. Cells were seeded in 0.1% gelatin-coated 24-well culture plates and incubated with EBM-2 containing 0.5% FBS or SMC-CM for 24 h. The iPSC-EC was fixed in PBS containing 4% paraformaldehyde for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 10 min, and blocked with PBS containing 5% bovine serum albumin. The cells were then incubated with a rabbit anti-PCNA antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h and Alexa-488 goat anti-rabbit secondary antibody (Thermo Fisher Scientific) for 1 h. The cells were finally washed and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) with 40, 6-diamidino-2-phenylindole (DAPI), and images were obtained using a laser scanning confocal microscope

(Olympus FluoView FV1000).

Culture of human dermal fibroblasts

Human dermal fibroblasts (hDF) were isolated by mechanical and enzymatic digestion of human juvenile foreskin using a slight modification of a previously described protocol[1]. Written informed consent was obtained from all blood donors, and the experimental protocol was approved by the Institutional Review Board of Pusan National University Hospital. After removing the epidermis, the dermal tissue was cut into small pieces and washed three times with phosphate buffered saline (PBS) at room temperature. Thereafter, the pieces were incubated with 0.25% collagenase for 30 min at 37 °C with intermittent shaking. Supernatants were harvested and centrifuged at 300 × g for 5 min. The cell pellet was re-suspended in αminimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded in 10-cm culture plates and grown until they reached a confluent state in α-MEM containing 10% FBS. To obtain hDF-CM, the hDF was then washed twice with HBSS to remove serum components. The cells were incubated in 10 ml serum-free α-MEM for 48 h. The collected hDF-CM was filtered through a 0.45 μm syringe filter and immediately centrifuged at 500 ×g for 10 min and 10,000 ×g for 30 min to remove cells and debris.

Measurement of blood flow using a multi-spectral laser speckle contrast imaging system

For supplementary data (Supplementary Figure 4 and 5), blood flow of the ischemic and normal limbs was measured using a multi-spectral laser speckle contrast imaging (MS-LSCI) system[2] Blood flow was measured on days 0, 7, 14, 21, and 28 after induction of hindlimb ischemia.

The perfusion of the ischemic and non-ischemic limbs was calculated based on colored histogram pixels. Red and blue colors indicate high and low levels of blood flow, respectively. Blood perfusion is expressed as the ratio of ischemic versus non-ischemic limb blood flow. A ratio of 1 before operation indicates equal blood perfusion of both legs.

References

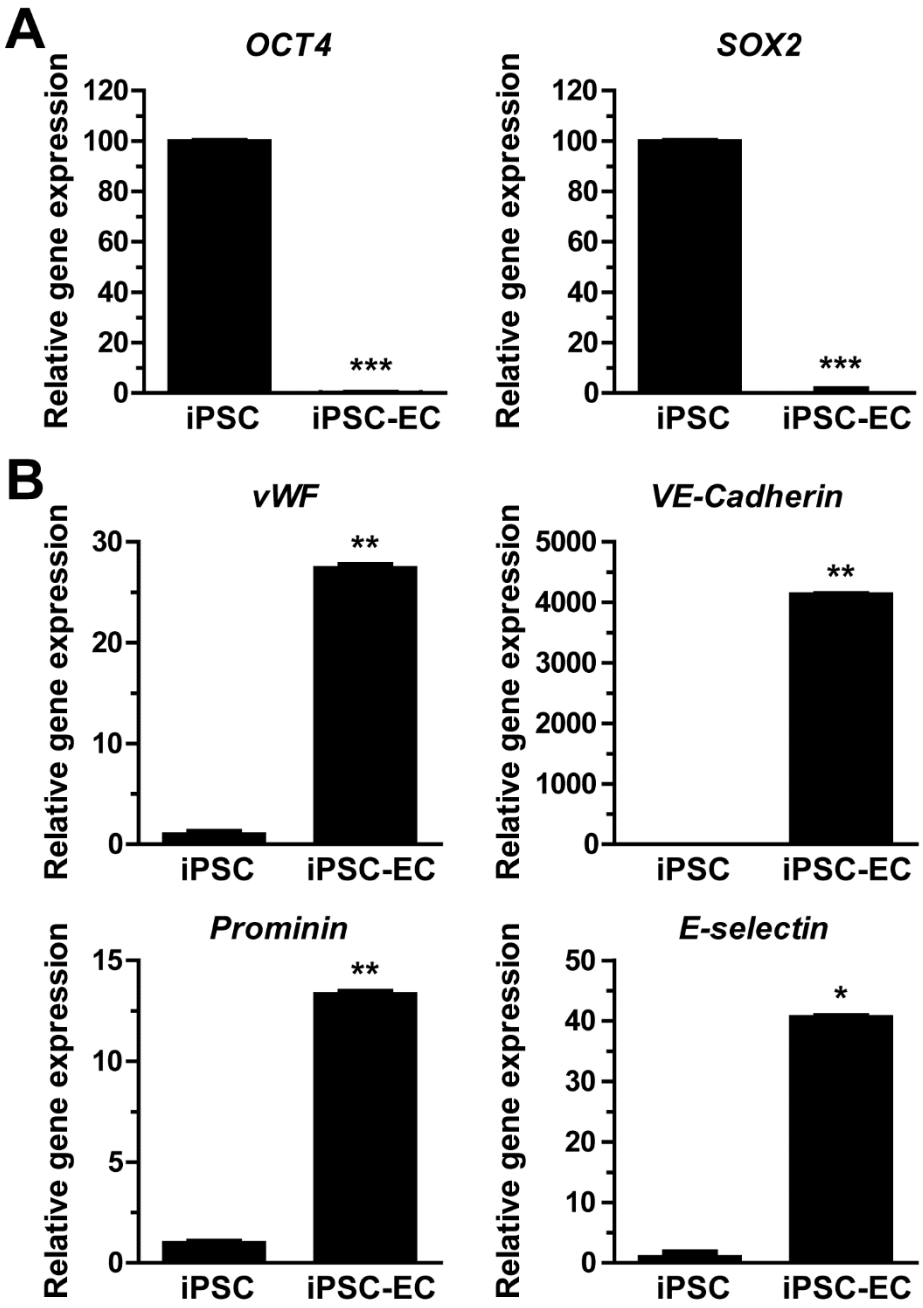
1. Rittie, L., and Fisher, G.J. Isolation and culture of skin fibroblasts. Methods Mol Med (2005). 117, 83-98. doi: 10.1385/1-59259-940-0:083.

2. Kim JW, Jang H, Kim GH, Jun SW, Kim CS. Multi-spectral laser speckle contrast images using a wavelength-swept laser. J Biomed Opt. 2019;24(7):1-9.

Supplementary Table 1. List of primer sequences for RT-PCR

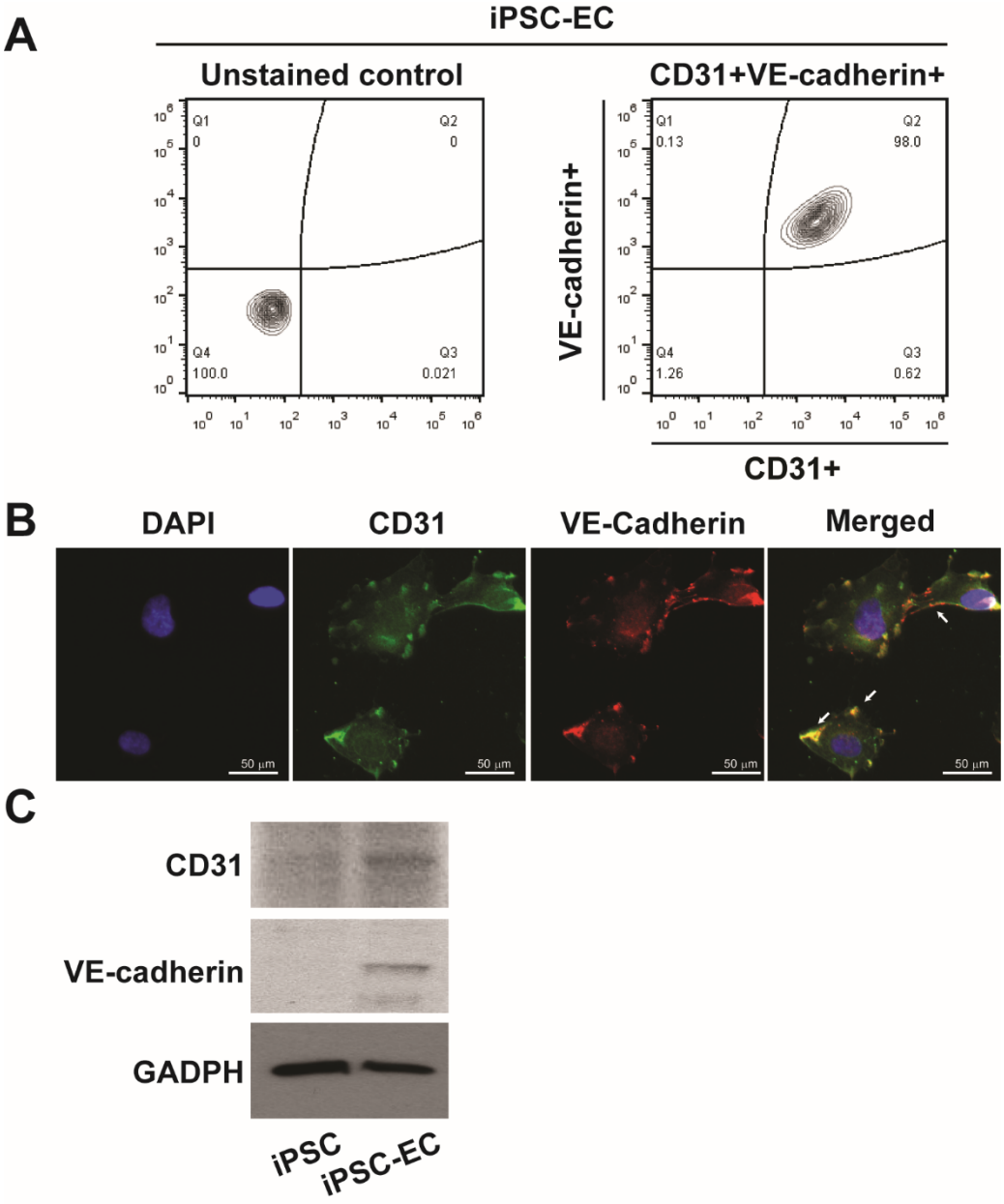
Gene	Primer sequences (5'-3')
OCT4	F: CGTGAAGCTGGAGGAGAAGGAGAAGCTG
	R: AAGGGCCGCAGCTTACACATGTTC
SOX2	F: GGGAAATGGGAGGGGTGCAAAAGAGG
	R: TTGCGTGAGTGTGGATGGGGATTGGTG
NANOG	F: CAAAGGCAAACAACCCACTT
	R: TCTGCTGGAGGCTGAGGTAT
Brachyury	F: AATTGGTCCAGCCTTGGAA
	R: TGCTCACAGACCACAGGC
MIXL1	F: GGGAGGAACACATCTTTTCT
	R: CATGGAAGTCAGAAAGGACA
TBX6	F: CCTAAACTGGATTGCTTCCT
	R: CTGAACTCCTTCCATAGCTC
CD34	F: TCAAATGTTCAGGCATCAGA
	R: TCTCCCCTGTCCTTCTTAAA
KDR	F: AGCCTACAAGTGCTTCTACC
	R: CGACTCCATGTTGGTCACTA
CD31	F: AGAGGCTGAGGTGAGATTAT
	R: GGATTCTCACTCTGTCACTC
SM22α	F: TTCTAGGGACTGCCTTTTTC
	R: GTGGTTTCATAGCATTGCTC
VEGFR1	F: GCCTCTGATGGTGATTGTTG
	R: GCTGCTGGTGACGCTATCTA
Desmin	F: AGTTGAAGGAAGAAGCAGAG
	R: CTCCTCTTCATGCACTTTCT
vWF	F: AGACTGTGATGATCGATGTG
	R: GTCCTCCTCTTAGCTGAATG
VE-Cadherin	F: AAGGAGAATATGTGGGCAAG
	R: CGTCATGAACTTTGATGGTG
Prominin	F: TCGTAAGAGCGAACTTGTAG
	R: CGATGTATTTCTCTCCCTGG
E-selectin	F: GGGAACTAGAGGGATACACT
	R: TATTGAAGTGGTGATGGGTG
GAPDH	F: GGTGAAGGTCGGAGTCAACGGA
	R: GAGGGATCTCGCTCCTGGAAGA

F: Forward primer; R: Reverse primer

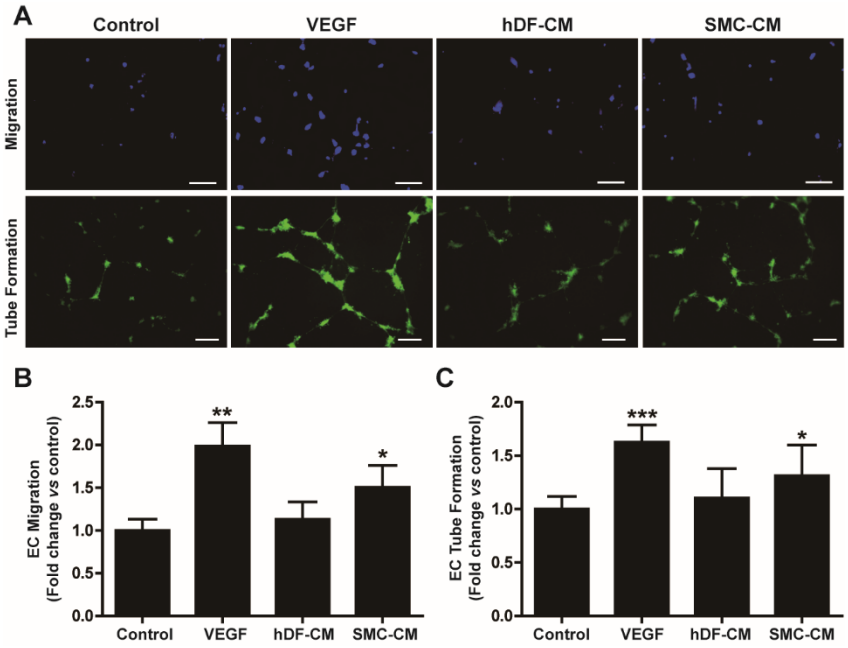


Supplementary Figure 1. Comparison of the markers of iPSC and EC differentiated from iPSC.

The mRNA levels of pluripotency markers (A, *OCT4 and SOX2*) and endothelial markers (B, *vWF, VE-Cadherin, Prominin, and E-selectin*) in iPSC and iPSC-EC were assessed by quantitative RT-PCR. Results are presented as mean ± SD. (n=3). *, $p < 0.05$; **, $p<0.01$; ***, $p<0.001$ *vs* iPSC.

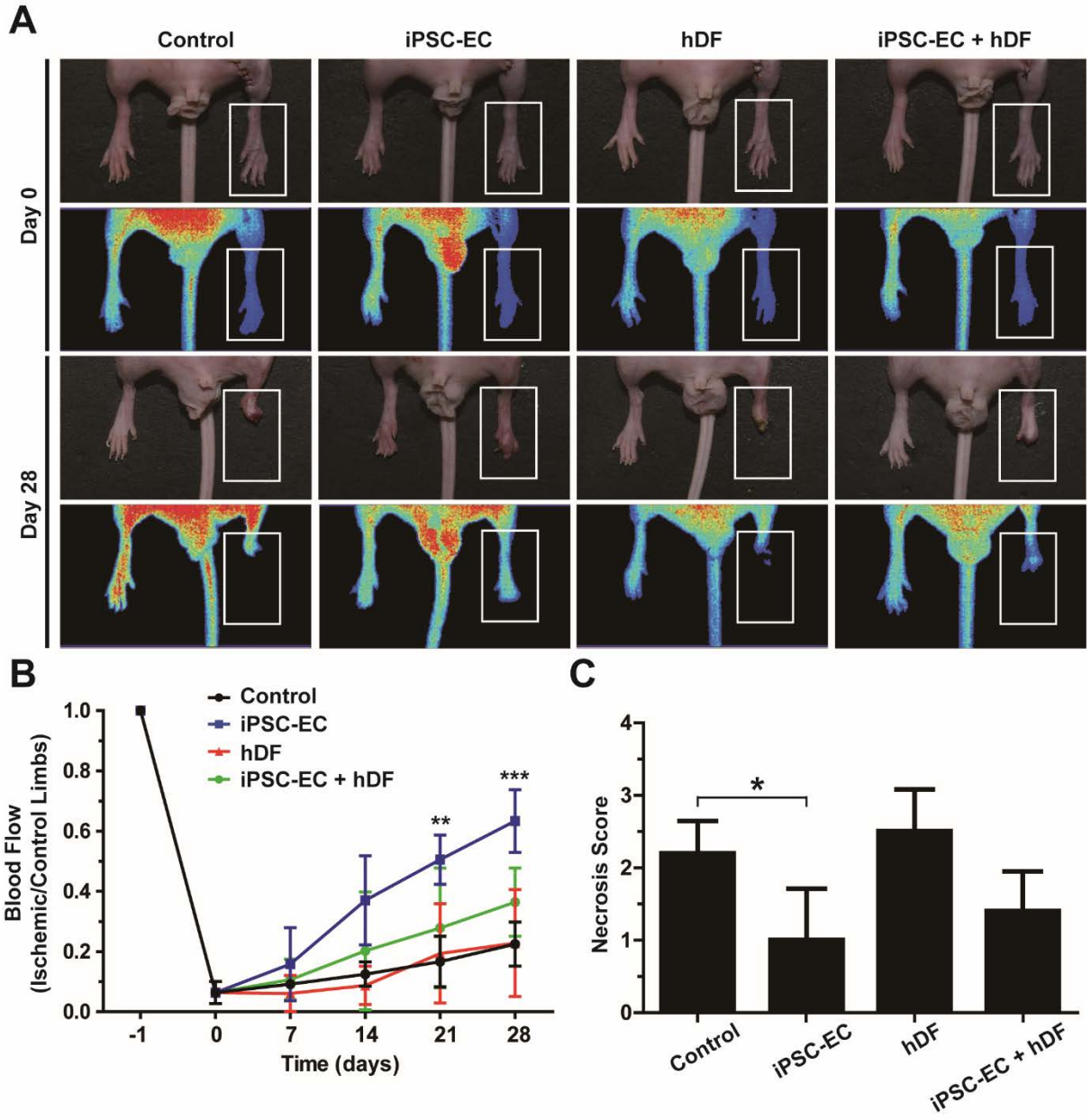


Supplementary Figure 2. Expression of VE-cadherin and CD31 in iPSC-EC.
(A) FACS analysis of iPSC-EC with antibodies against CD31 and VE-cadherin. The expression of CD31 and VE-cadherin in the iPSC-EC was analyzed by FACS analysis. (B) Immunostaining of iPSC-EC with anti-CD31 and anti-VE-cadherin antibodies. The images of CD31 (green color) and VE-cadherin (red color), nuclei (blue color), and merged image were shown. Arrows indicate co-localization of CD31 and VE-cadherin in plasma membrane. (C) Western blot analysis of iPSC-EC with anti-CD31 and anti-VE-cadherin antibodies. The protein levels of CD31, VE-cadherin, and GAPDH in lysates of iPSC and iPSC-EC were determined by Western blotting.



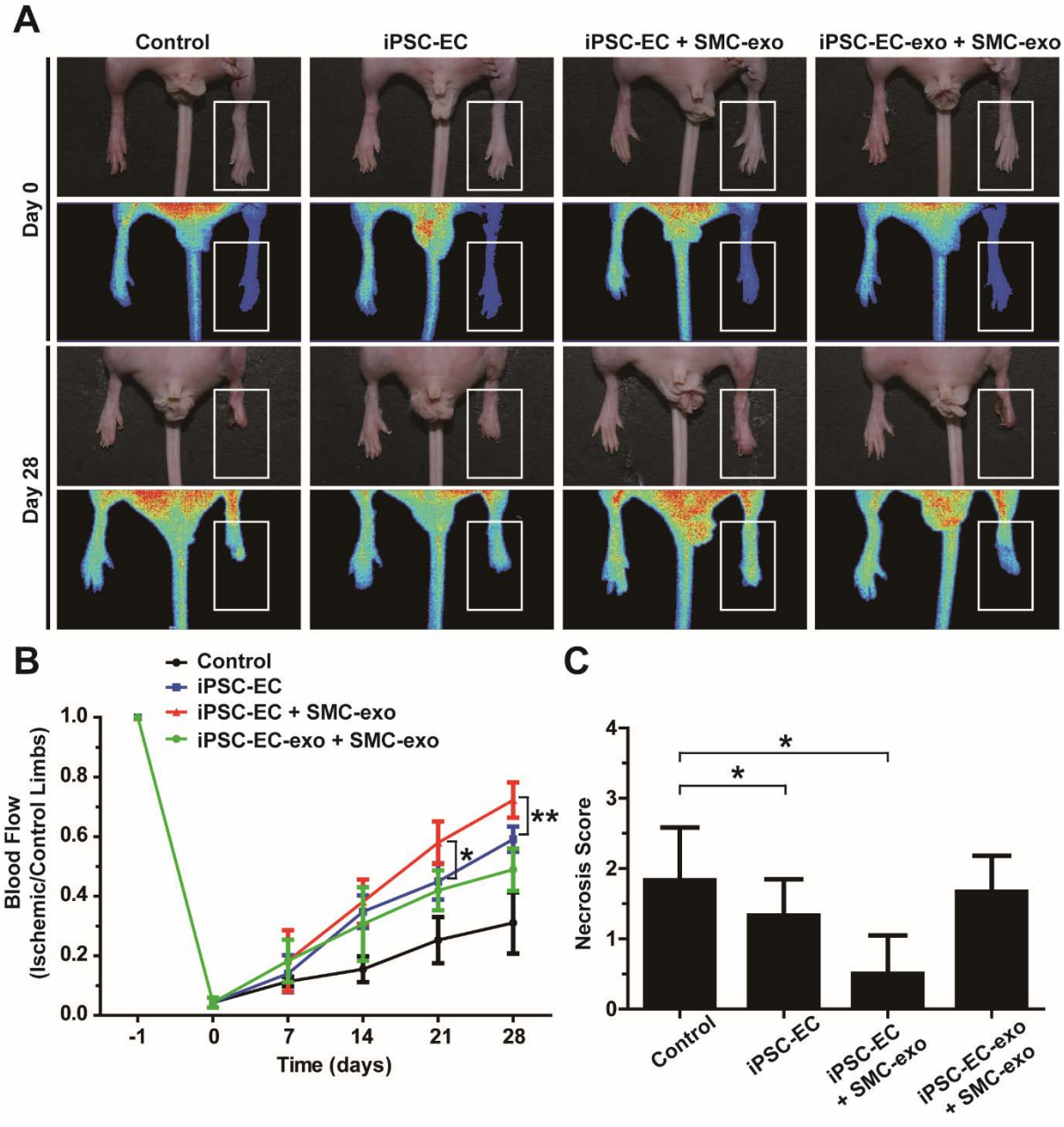
Supplementary Figure 3. Effects of human dermal fibroblast-conditioned medium on migration and tube formation of iPSC-EC.

(A) Representative images of chemotactic migration (upper panel) and tube formation (lower panel) of iPSC-EC in response to VEGF, hDF-CM, and SMC-CM are shown. Scale bar = 200 μm. (B) Migration of iPSC-EC was measured using a chemotaxis chamber in response to human recombinant VEGF protein (10 ng/ml), hDF-CM (20%), or SMC-CM (20%) after a 12-h incubation. (C) iPSC-EC was seeded onto a Matrigel-coated dish and treated with VEGF, hDF-CM, or SMC-CM for 12 h. Tube formation of iPSC-EC was quantified by measuring the length of the tubes formed Data indicate mean ± SD (n=4). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs* control.



Supplementary Figure 4. Effects of combined administration of iPSC-EC and hDF on blood perfusion and tissue necrosis in hindlimb ischemia

(A) Representative photographs and LDPI of mouse hindlimbs on day 0 and 28 after injection of HBSS (control), iPSC-EC, hDF, or iPSC-EC with hDF. (B) Quantitative analysis of the blood perfusion recovery measured using an LDPI analyzer. The LDPI ratio was calculated as the ratio of ischemic to non-ischemic hindlimb blood perfusion. (n=6–9 per experimental group). (C) Statistical analysis of the necrosis score on day 28. Results are presented as mean ± SD. (n=6-9). *, $p < 0.05$; ***, $p < 0.001$ *vs* control.



Supplementary Figure 5. Effects of combined administration of iPSC-EC and SMC-exosome on blood perfusion and tissue necrosis in hindlimb ischemia

(A) Representative photographs and LDPI of mouse hindlimbs on day 0 and 28 after injection of HBSS (control), iPSC-EC, iPSC-EC with SMC-exosome, or iPSC-EC-exosome with SMC-exosome. (B) Quantitative analysis of the blood perfusion recovery measured using an LDPI analyzer. The LDPI ratio was calculated as the ratio of ischemic to non-ischemic hindlimb blood perfusion. (n=6–9 per experimental group). (C) Statistical analysis of the necrosis score on day 28. Results are presented as mean ± SD. (n=6-9). *, $p < 0.05$; **, $p < 0.01$.