SUPPLEMENTAL INFORMATION

Effects of Cellular Origin on Differentiation of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells

Shijun Hu[1, 2, 3, *], Ming-Tao Zhao[1, 2, 3, *], Fereshteh Jahanbani[4], Ning-Yi Shao[1, 2, 3], Won Hee Lee[1, 2, 3], Haodong Chen[1, 2, 3], Michael P. Snyder[4], Joseph C. Wu[1, 2, 3]

[1]Stanford Cardiovascular Institute; [2]Department of Medicine, Division of Cardiology; [3]Institute for Stem Cell Biology and Regenerative Medicine; [4]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA.

[*]Contributed equally to this work.

Running title: Effects of cellular origin on endothelial cell differentiation

Extended Methods

Isolation and maintenance of human fibroblast cells. Freshly isolated human fetal skin was rinsed with DPBS and transferred into a 1.5 ml tube. The tissue was minced in 1 mg/ml of collagenase I (Roche) in DMEM medium (Life Technologies) for 4 hours at 37°C. The dissociated fibroblast cells were plated and maintained in the medium containing DMEM (Life Technologies), 10% FBS (Life Technologies), GlutaMAX (Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), 50 U/ml penicillin (Life Technologies), and 50 g/ml streptomycin (Life Technologies). Cell culture was maintained in a humidified incubator in 5% CO_2 at 37°C.

Isolation and maintenance of human cardiac progenitor cells. Human CPCs were isolated based on a previously described protocol (1). Fetal hearts were subject to Langendorff perfusion with Tyrode solution containing collagenase and protease. CPCs were isolated by magnetic-activated cell sorting using anti-Sca-1 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. Sca-1^+ cells were eluted from the column by washing with DPBS supplemented with 2% fetal bovine serum and then cultured on 0.1% gelatin-coated dishes in M199 (Life Technologies)/EGM-2 (Lonza) (ratio 3:1) supplemented with 10% FBS (Life Technologies), 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml epithelial growth factor (EGF), 5 ng/mL insulin-like growth factor (IGF-1), and 5 ng/ml hepatocyte growth factor (HGF).

Lentivirus production and transduction. The 293T cells (Life Technologies) were plated at 80% confluency on 100-mm dishes and transfected with 12 μg of lentivirus vectors (OCT4, SOX2, C-MYC and KLF4) plus 8 μg of packaging psPAX2 and 4 μg of pMD2.G plasmids using

Lipofectamin 2000 (Life Technologies). The supernatant containing viruses was collected at 36 and 48 hours after transfection. The viruses were concentrated using PEG-it Virus Concentration Solution (System Biosciences) and resuspended in PBS.

Human iPSC derivation. Human FBs, ECs, and CPCs were seeded at the appropriate density and reached 60-70% confluent on the day of reprogramming. The cells were then transfected with lentivirus carrying constructs with four Yamanaka factors (OCT4, SOX2, C-MYC, and KLF4). The medium was changed on the next day and cells were kept in their respective native medium for another 3-4 days until confluent. The reprogrammed cells were then split and cultured in mTeSR-1 medium on Matrigel-coated plates. The iPSC colonies appeared around 7-10 days post reprogramming. Individual colonies were manually picked up and transferred to each well of 12-well plates. The reprogramming efficiency was calculated by the number of iPSC colonies divided by the counts of initial seeding somatic cells. When iPSC colonies reached over 80% confluency, they were dissociated and replated onto 6-well plates. Typically stable iPSC clones were obtained after passage 5.

Alkaline phosphatase staining. Alkaline phosphatase (AP) staining was conducted as previous studies using Quantitative Alkaline Phosphatase ES Characterization Kit (Millipore) according to the manufacturer's instruction (2, 3).

Immunofluorescence staining. Cells were washed with PBS and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized by 0.1% TritonX-100 in PBS and washed with PBS. The non-specific binding sites were blocked with PBS plus 10% serum of the species

where the secondary antibodies were raised. The cells were subsequently incubated with primary and secondary antibodies, then extensively washed with PBS (3x) after antibody incubation. The primary antibodies included TRA-1-60 (Millipore, catalog number MAB4360), OCT4 (Santa Cruz Biotechnology, catalog number sc-5279), NANOG (Santa Cruz Biotechnology, catalog number sc-33759), and SSEA4 (Millipore, catalog number MAB4304), CD31 (Thermo Scientific, catalog number RB-10333), CD144 (BD Biosciences, catalog number 561567), Cardiac Troponin T (Thermo Fisher Scientific, catalog number MA5-12960), MEF2C (Abcam, catalog number ab43796), NESTIN (Millipore, catalog number MAB5326), and SOX2 (Biolegend, catalog number 656102). The secondary antibodies were either Alexa Fluor 488- or Alexa Fluor 594- conjugated (Thermo Fisher Scientific).

Spontaneous in vitro differentiation. For embryoid body (EB) formation, iPSC colonies were dissociated with Accutase (Sigma-Aldrich), and seeded onto ultra-low attachment 6-well plates in Knockout DMEM medium (Life Technologies) supplemented with 20% Knockout Serum Replacement (Life Technologies), GlutaMAX, 0.1 mM non-essential amino acids, 0.1 mM β–mercaptoethanol, 50 U/ml penicillin, and 50 g/ml streptomycin. After 5 days, EBs were transferred to adherent, gelatin-coated plates and cultured in the same medium for another 8 days.

Teratoma formation. One million undifferentiated iPSCs were harvested and suspended in 100 μL of Matrigel (BD Biosciences) and delivered by a 28.5-gauge syringe to the sub-renal capsule of 8-week old SCID beige mice. Tumors were collected eight weeks after cell injection and subjected to hematoxylin and eosin (H&E) staining.

Bisulfite sequencing. Genomic DNA was extracted by a DNeasy Blood & Tissue kit (Qiagen) and then was subjected to sodium bisulfite treatment using an EZ DNA Methylation-Direct™ kit (Zymo Research). Bisulfite-treated DNA was amplified using bisulfite sequencing specific primers designed by the MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The amplified product was cloned into a pGEM-T Easy vector (Promega) for further Sanger sequencing. For each cell type, at least 10 clones were sequenced. The sequencing results were analyzed by using the online BiQ analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de).

Karyotype analysis. Karyotype analyses were carried out at the Cytogenetics Laboratory at Stanford Hospital and Clinics. iPSCs with 80% confluence were treated with 0.1 μg/mL colcemid for induction of mitotic arrest and were subsequently harvested. The dissociated cells were then subject to hypotonic shock, and fixed with a methanol: acetic acid mixture (3:1). For each iPSC line, 20 metaphase-stage cells were analyzed by the standard G-banding method.

Fluorescence-activated cell sorting (FACS). Flow cytometry was performed using a BD Aria II (BD Biosciences) at the Stanford Shared FACS Facility and data were analyzed by FlowJo software (Tree Star Inc). BD Pharmingen Alexa Fluor 488 mouse anti-human CD31 and Alexa Fluor 647 mouse anti-human CD144 monoclonal antibodies were used for endothelial cell staining. The mouse IgG isotype-matched antibodies were used for background fluorescence (blank controls).

LDL uptake and tube formation. For LDL uptake assay, iPSC-ECs were incubated with 10 μg/ml of DiI-Ac-LDL (Invitrogen) at 37°C for 6 hours. After washing with PBS twice, LDL uptake was detected by fluorescence microscopy. For tube formation, primary ECs or iPSC-ECs were seeded in a 24-well plate coated with Matrigel and incubated at 37°C for 12 hours.

Quantitative RT-qPCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and then reverse transcribed to cDNA using High-Capacity RNA-to-cDNA kit (Life Technologies). Real-time qPCR was performed using Taqman Universal PCR Master mix on StepOne Plus Real-time PCR System (Life Technologies). Taqman gene expression assays are listed in **Table S1**. Primers for total and endogenous pluripotency genes were purchased from Clontech Laboratories (catalog number 631966). The endogenous primers target the 5'UTR of pluripotency genes whereas total primers target the coding exons. The exogenous transgene from the vector doesn't have 5'UTR thus doesn't show signal after PCR using endogenous primers.

Bioluminescence imaging of transplanted animals. Bioluminescence imaging (BLI) was performed using the Xenogen IVIS Spectrum system (Xenogen, CA). Recipient mice were anesthetized with isoflurane, and were intraperitoneally injected with D-Luciferin (200 mg/kg body weight). Mice were imaged on days 1, 7, and 14 after surgery. Peak signals from a fixed region of interest (ROI) were obtained and quantified in photons/s/cm^2/sr as previously described (4).

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Supplemental Table

Table S1. Taqman Gene Expression Assays.

Genes	**Taqman Assays**
PECAM1	Hs00169777_m1
NOS3	Hs01574659_m1
CTGF	Hs01026927_g1
HGF	Hs00300159_m1
VEGFB	Hs00173634_m1
KDR	Hs00911700_m1
ACTA2	Hs00426835_g1
VIM	Hs00185584_m1
ICAM1	Hs00164932_m1
IL8	Hs00174103_m1
TNFA	Hs01113624_g1
ANGPT2	Hs01048042_m1
ID1	Hs03676575_s1
POU5F1	Hs00999634_gH

Figure S1

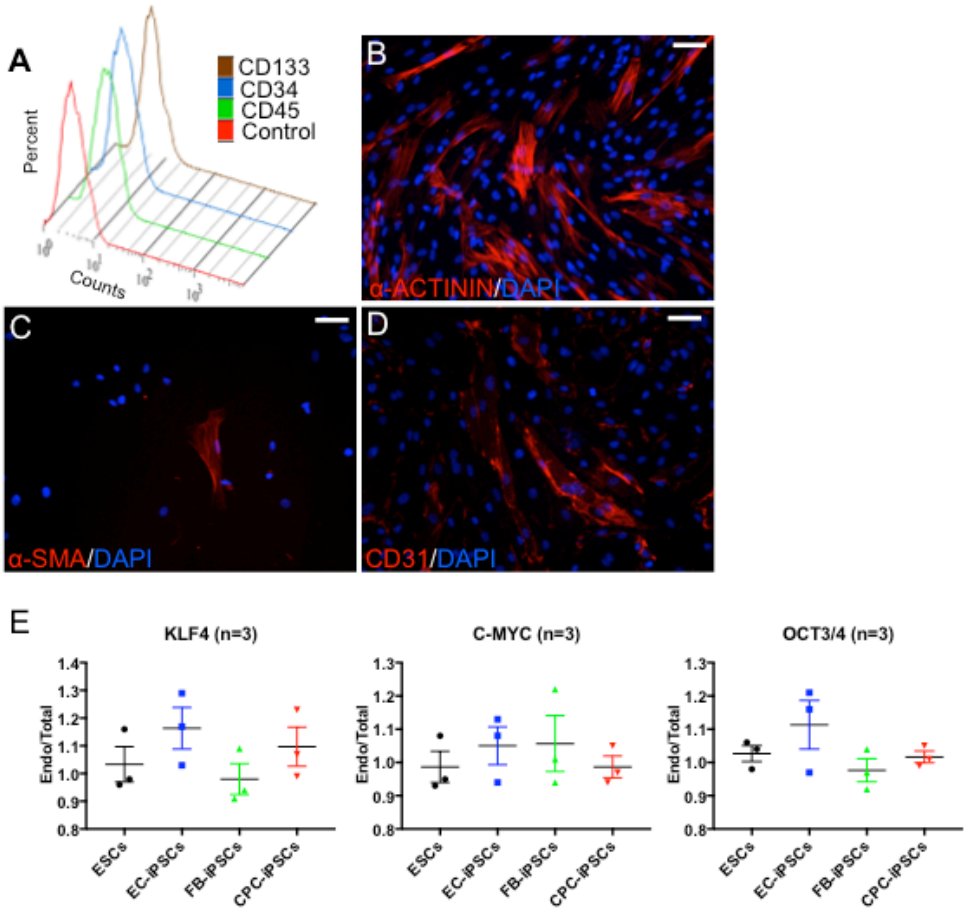


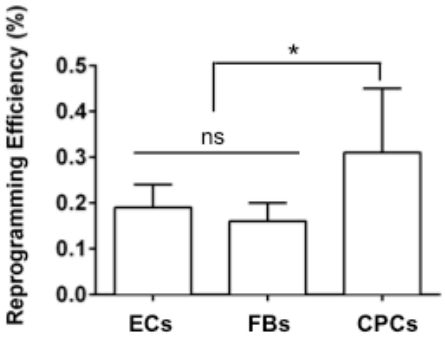
Figure S1. The multipotent lineage differentiation of human fetal cardiac progenitor cells. **(A)** Fetal CPCs were positive for CD105 (endoglin) and Sca-1, but negative for CD133 (hematopoietic stem cell marker), CD34 (endothelial progenitor cell marker), and CD45 (leukocyte marker). Additionally, fetal CPCs could differentiate into three different lineages, including cardiomyocytes (**B**), smooth muscle cells (**C**), and endothelial cells (**D**), thus confirming the multipotent differentiation ability of human CPCs. (E) Relative gene expression levels of pluripotency genes (KLF4, C-MYC and OCT3/4) in human iPSCs and ESCs. Scale bars: 50 μm.

Figure S2

A

Cells	Passage # to initiate reprogramming
F1-ECs	3
F1-FBs	4
F1-CPCs	5
F2-ECs	3
F2-FBs	3
F2-CPCs	4

B



C

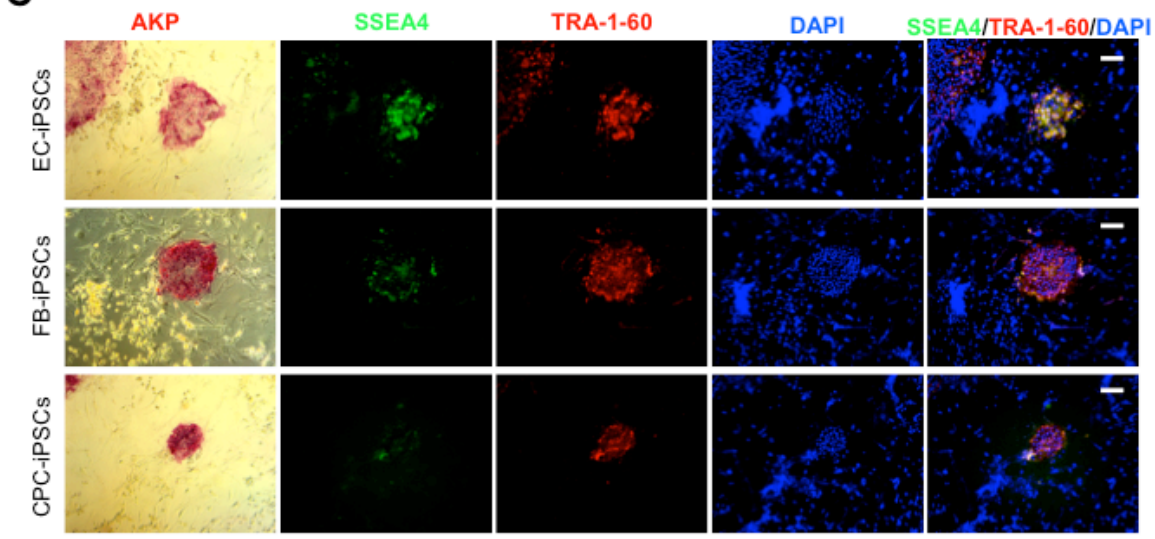


Figure S2. Reprogramming of human fetal ECs, FBs, and CPCs into iPSCs. (**A**) The somatic cells were between passage 3-5 when initiating reprogramming. (**B**) CPCs showed higher reprogramming efficiency than the isogenic FBs and ECs ($P<0.05$). (**C**) The emerging iPSC colonies were cultured on MEF feeders and were positive for embryonic stem cell markers: alkaline phosphatase (AKP), SSEA4, and Tra-1-60. Scale bars: 100 μm. * $P<0.05$, one-way ANOVA. ns: not statistically significant, unpaired t-test.

Figure S3

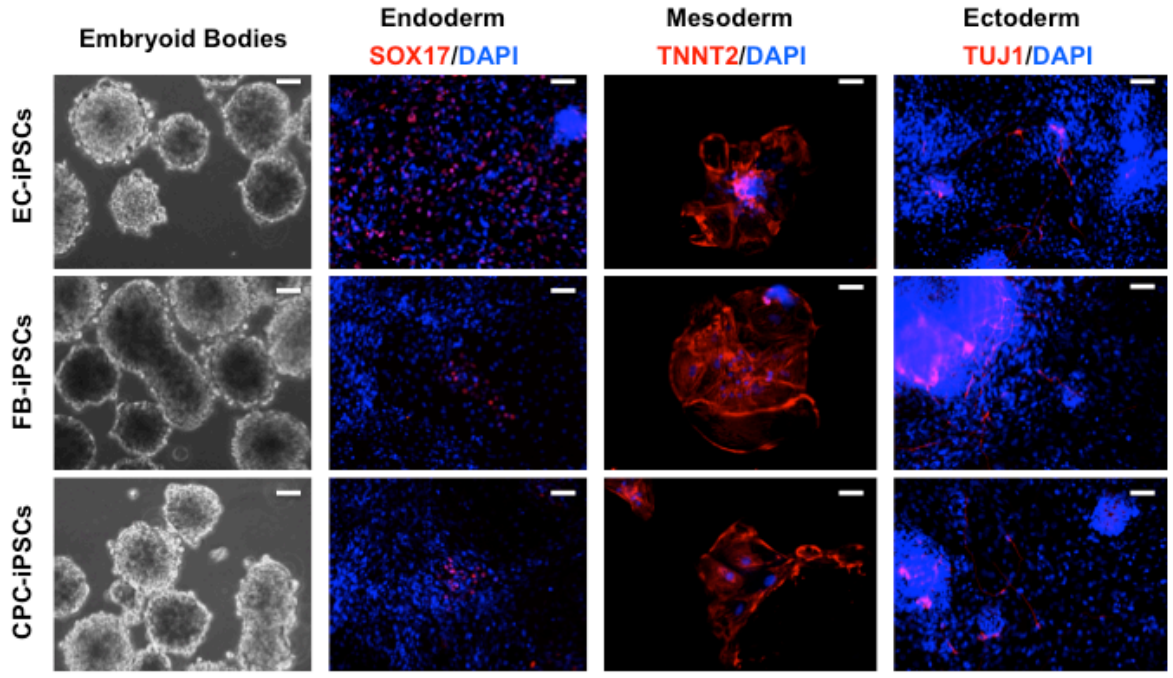


Figure S3. In vitro differentiation of EC-iPSCs, FB-iPSCs, and CPC-iPSCs. All tested iPSCs could spontaneously form floating embryoid bodies (EBs). Detailed dissection of EBs indicates they differentiated into the derivatives of three germ layers: endoderm (SOX17), mesoderm (TNNT2), and ectoderm (TUJ1). Scale bars: 50 μm.

Figure S4

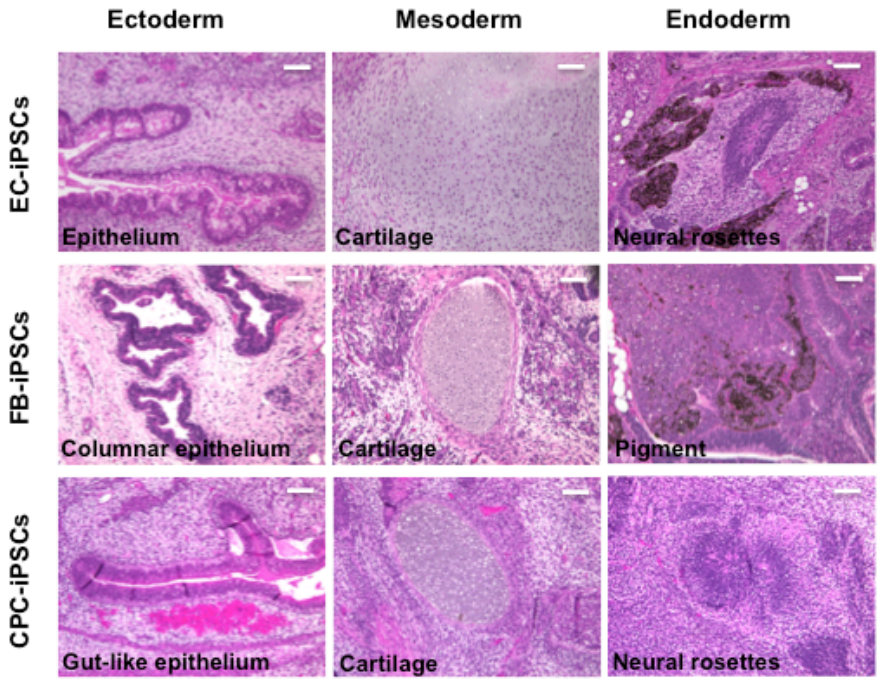


Figure S4. In vivo differentiation of human iPSCs by teratoma formation. All tested iPSC lines formed teratomas after injection into kidney capsule of SCID mice. The mature teratoma included the derivatives of three germ layers (endoderm, mesoderm, and ectoderm), further confirming the pluripotent differentiation capacity of these iPSCs in vivo. Scale bars: 50 μm.

Figure S5

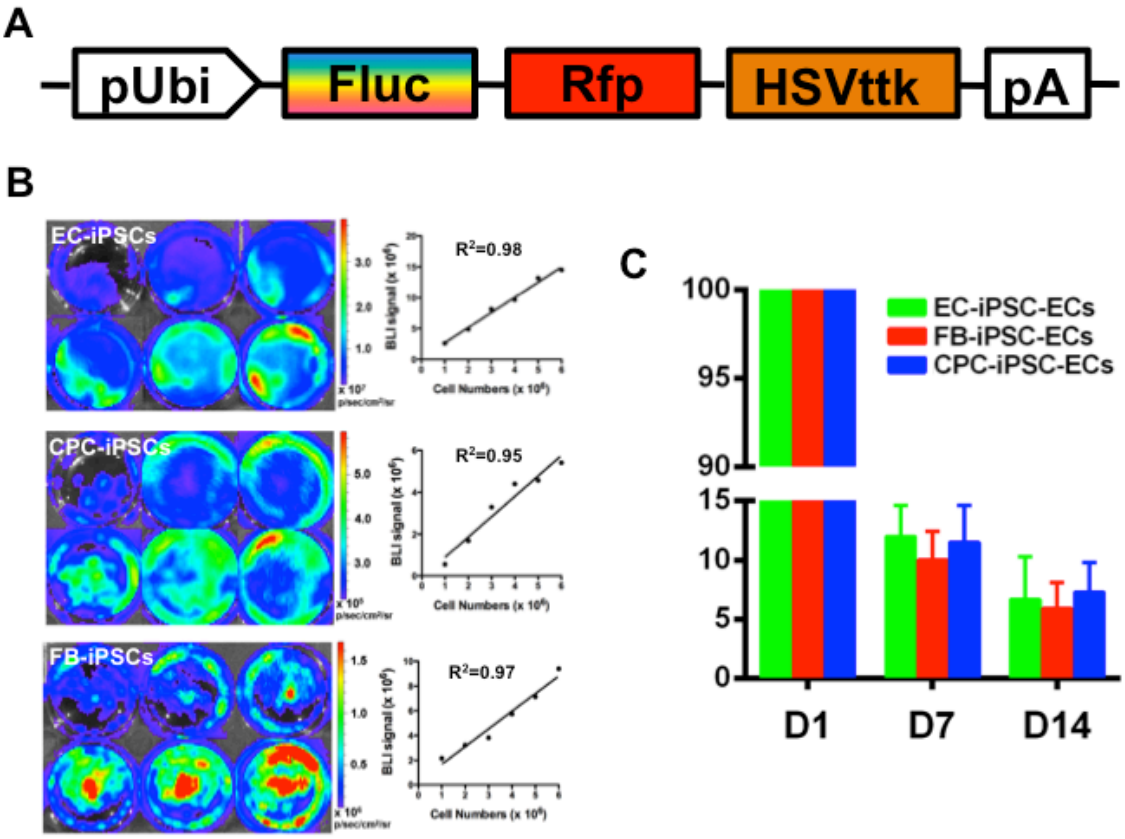


Figure S5. Bioluminescent imaging of transgenic iPSC-ECs. (**A**) A triple fusion construct was used to stably transfect human iPSCs derived from fibroblasts, endothelial cells, and cardiac progenitor cells (FB-iPSCs, EC-iPSCs, and CPC-iPSCs). (**B**) These transgenic iPSC-ECs showed linear correlation between Fluc signal intensity and cell number in vitro, which is vital for accurately tracking cell fate in vivo. The labeled iPSC-ECs were then transplanted into mouse ischemic hindlimb. (**C**) The normalized BLI signals (relative to day 1) were similar among different sources of iPSC-ECs at different time points after transplantation, indicating comparable in vivo cell survival rates.