MATERIALS AND METHODS

Animal Groups and Experimental Design

All animals were randomly assigned to one of the following three groups: phosphate-buffered saline (PBS) control, mouse embryonic stem cells (mESCs) or mouse induced pluripotent stem cells (miPSCs) transplantation group. Additionally, to explore whether iPSC-derived enriched cardiomyocytes (iPSC-CMs) could improve metabolic function of the injured rodent heart, an additional 24 rats were divided into 3 groups (n=8 per group; all groups received tacrolimus daily): PBS control, hESC-CM or hiPSC-CM transplantation group. At day 3, 7, 14, 21 and 28 after cell delivery, all rats were accepted by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) micro-positron emission tomography (PET) and echocardiograph scans. Immunofluorescence staining was performed at every week after cell transplantation. Postmortem immunohistochemical staining and autoradiographic (ARG) detection were performed after the last PET scan. This animal protocol has been approved by the Institutional Animal Care and Use Committee and was in compliance with the standard of the Guide for the Care and Use of Laboratory Animals as formulated by the U.S. National Institutes of Health.

Generation of Stable Mouse iPSC and ESC Lines

Enhanced green fluorescent protein (EGFP)-labeled mouse iPSC cell lines reprogrammed by the lentivirus-mediated transduction of 4 transcription factors (*Oct4*-EGFP, *Sox2*-EGFP, *Klf4*-EGFP and *c-MYC*-EGFP) to mouse embryonic fibroblasts (MEF) were previously described (1). MEF cells were obtained from 13.5-d pregnant C57BL/6J mice. Approximately 2×10^5 MEF cells were mixed with a mixture of equal parts of the medium containing *Oct4*-EGFP, *Sox2*-EGFP, *Klf4*-EGFP and *c-MYC*-EGFP; seeded onto a 6-well culture dish precoated with feeder cells; and incubated overnight in MEF medium. The medium was replaced with ESC medium after 12h. ESC-like colonies with distinct edges appeared from days 13 to 15. On day 15, positive colonies with ESC-like morphologies were isolated with a glass needle and seeded onto new MEFs.

The establishment of EGFP-labeled mouse ESCs was previously described (2). The mouse ESCs used in this work were derived from the inner masses（ICMs）of day 3.5 blastocysts of 129S1/SvlmJ mice and cultured on feeder cells. ICMs were separated from zonae pellucidae and plated into 4-well plates precoated with feeder cells. Morphologically ESC-like colonies consisting of small juxtaposed spheroid cells with big nuclei were selected after 3-5 days of culture. EGFP-stable expressing mESCs were established by infection with lentivirus carrying EGFP. Twenty-four hours after transduction, the medium was replaced with fresh ESC culture medium (DMEM) (Gibco) and transduced mESCs were used in the following studies.

Stem Cell Culture

Stem cells were cultured as described previously (3). EGFP-labeled mouse iPSCs and ESCs were kept in a pluripotent, undifferentiated state with leukemia inhibitory factor (LIF) (Invitrogen) and maintained on a mitotically inactivated (mitomycin C) (Roche) embryonic fibroblast feeder layer. Stem cells were cultured in knockout Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum (Hyclone), 10% knockout serum replacement (Invitrogen), 2 mM L-glutamine (Invitrogen), 100x nonessential amino acids (Invitrogen), 1000x 2-mercaptoethanol (β-ME) (Invitrogen), 50 units/50 mg/mL penicillin/streptomycin (Invitrogen). Prior to administration, iPSC and ESC colonies were passaged up to four times without feeder cells on 60-mm culture dishes coated with 0.1% gelatin to eliminate contamination of the mouse embryonic fibroblasts.

H9 human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs) were cultured on feeder-free Matrigel-coated tissue culture dishes (BD Biosciences, San Jose, CA) with mTeSR-1 hESC Growth Medium (STEM CELL Technologies, Vancouver, Canada).

Cardiac Differentiation from hESCs or hiPSCs

hESCs or hiPSCs were differentiated into cardiomyocytes using a 2D monolayer differentiation protocol and maintained in a 5% CO_2/air environment (Thermo Fisher Scientific). Briefly, on day 0, hESC or hiPSC colonies were dissociated with Accutase (Invitrogen) into a single cell suspension and resuspended in mTeSR-1 media. Roughly 100,000 cells were then replated into one well of a 6-well dish precoated with Matrigel (BD) to ensure that single hESCs or hiPSCs were evenly dispersed in the dish. hESC or hiPSC monolayers were cultured for 2-3 days until ~95% confluency was achieved. For the next 5 days, hESCs or hiPSCs were treated with CHIR (Axon Medchem) to activate WNT signaling pathway and then IWR-1 (Merck) to inhibit WNT signaling pathway. Cells were then placed on RPMI+B27 with insulin (Invitrogen) until beating was observed. Beating monolayer cardiomyocytes were digested with Trypsin (Invitrogen) followed by mechanical dissociation using a 1000 μl pipettor and plated on Matrigel-coated coverslips. Primary antibodies consisted of mouse anti-human sarcomeric alpha-actinin (Abcam) and rabbit anti-human cardiac troponin T (cTNT) (Abcam). Goat anti-mouse IgG-TR (Santa Cruz Biotechnology) and goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology) were used as secondary antibodies. Imaging was performed using a DMIL-LED inverted tissue culture microscope running Nikon A1 Ti software with a 5x N-Plan Phase objective, a 10x Plan Fluotar phase objective, or an A1-R Resonant Confocal System running NIS Elements (C software on a Ti-E PFS microscope with a Plan Apo VC 60x Oil objective).

Animal Model of Myocardial Infarction (MI) and Stem Cell Administration

An animal model of MI was established by permanent coronary artery ligation as described

previously (4). To investigate the metabolic change after iPSC transplantation, 39 adult male Sprague-Dawley rats were divided into 3 groups (n=13 per group): PBS control, ESC or iPSC transplantation group. Furthermore, to explore whether iPSC-CMs could improve metabolic function of the injured rodent heart, an additional 24 rats were divided into 3 groups (n=8 per group; all groups received tacrolimus daily): PBS control, hESC-CM or hiPSC-CM enriched transplantation group. Briefly, all rats were intubated endotracheally and ventilated under general anesthesia by a continuous inhalation of isoflurane (2%). The pericardial space was exposed by left lateral thoracotomy through the fourth intercostal space. The left anterior descending coronary artery was ligated with a 6-0 silk suture. Thirty minutes after MI, 2×10^6 stem cells or stem cell-derived enriched cardiomyocytes were implanted by intramyocardial injection into the periinfarct area in five equal aliquots. Control animals received an equal volume of PBS without cells. Rats were then allowed to recover from anesthesia and returned to temperature-controlled individual cages. All animals were maintained under a 12-h light/dark cycle throughout the experiments and allowed enriched food and water. Prior to implantation, a 3-day tacrolimus (Astellas Ireland Co.) pretreatment was applied to the hESC-CM and hiPSC-CM enriched transplantation groups (1 mg/kg, i.m.). After implantation, both groups were treated daily (1 mg/kg, i.m.). Tacrolimus doses were injected into the quadriceps muscle, alternating daily between the left and right legs. The tacrolimus dosing scheme employed in this study was selected from similar approaches previously developed (5).

In Vivo ^{18}F-FDG Micro-PET Study and Image Analysis

Repeated ^{18}F-FDG micro-PET studies were performed at days 3, 7, 14, 21 and 28 after stem cell implantation. Rats were kept fasting overnight before ^{18}F-FDG injection of approximately 18.5 MBq (500 μCi) via the tail vein (6). Forty minutes later, rats were anesthetized by inhalation of 2% isoflurane and positioned prone in the micro-PET R4 scanner (Siemens Medical Solutions) for a 10-min static acquisition. The images were reconstructed with use of a modified back projection (MBP) algorithm. The region of interest (ROI) were manually drawn around the injection site and normal regions, and the maximal uptake of all selected planes was averaged (7-9). The lesion-to-normal (L/N) ratio was used for semiquantitative analysis. L/N ratio was calculated by the following formula: L/N ratio = maximal counts per pixel of lesion ROI/maximal counts per pixel of normal area. Then the change in L/N ratio was calculated with use of the following formula: change in L/N ratio on day N = (L/N ratio on day N - L/N ratio on day 3)/L/N ratio on day 3 ×100% (N=3, 7, 14, 21 and 28).

Echocardiography

Myocardial contractile function was evaluated by using echocardiography on the same day as the ^{18}F-FDG micro-PET scan, as previously described (10). Echocardiography was performed under general anesthesia at days 3, 7, 14, 21 and 28 after cell administration. Transthoracic 2D and M-mode echocardiography images were obtained at the level of the papillary muscle using a Vevo

2100 system (VisualSonics Inc., Toronto, Canada). The left ventricular (LV) end-diastolic and end-systolic diameters (LVDd and LVDs, respectively) were measured in M-mode in the short-axis view. LV ejection fraction (EF) was expressed as a percentage and calculated as 100 × (LVEDV - LVESV)/(LVEDV), where the LVEDV is the left ventricular end-diastolic volume and LVESV is the left ventricular end-systolic volume. The data were calculated for at least 3 continuous cardiac cycles. Change in LVEF was calculated by using the following formula: change in LVEF value on day N = (LVEF on day N - LVEF of day 3)/LVEF on day 3 ×100% (N=3, 7, 14, 21 and 28).

Autoradiograph (ARG) Imaging

The ARG imaging was performed after the last PET scan. Six rats were sacrificed immediately after the last micro-PET study. The hearts were quickly excised and put into the cryostat (Bright Instrument Company Ltd.) for about 60 min at –20°C. Cardiac slices (120 μm thick) were cut using a skiving machine (Bright Instrument Company Ltd.) and kept in contact with an imaging plate (BAS-IP MS 2040, Fujifilm) for 10 min. The exposed plates were scanned with a bioimaging analyzer (FLA-7000, Fujifilm), and the images were viewed with a Dell computer (Image Reader FLA-7000 version 1.12, Fujifilm). The pixel size was 50 μm. The ARG images of the hearts were analyzed with Image Gauge Software (Version 3.0, Fujifilm).

Postmortem Immunohistochemical Staining

After the last micro-PET scan, 30 rats (5 per group) were euthanized and transcardially rinsed with saline followed by 4% paraformaldehyde in phosphate buffer. The whole heart was fixed in 4% paraformaldehyde. The sections were permeabilized with 0.04% Triton X-100 and blocked with 10% normal goat serum and 0.5% bovine serum albumin in PBS for 1h and then treated overnight at 4°C with primary antibodies: rabbit polyclonal antibody against von Willebrand factor (vWF) (1:200 dilution; DAKO); mouse monoclonal antibody against a-smooth muscle actin (α-SMA) (1:300 dilution; DAKO); and mouse monoclonal antibody against cardiac troponin I (cTNI) and cTNT (1:100 dilution; Abcam). The sections were visualized by secondary antibodies such as goat anti-rabbit IgG or goat anti-mouse IgG until a brown reaction was produced.

Immunohistochemical studies were performed to determine whether the transplanted stem cells can induce myogenesis, angiogenesis and fibrillation. cTNI and cTNT were used as a myocardial cell marker, vWF as the endothelial cell marker, and α-SMA as the smooth muscle cell or myofibroblast marker. The number of cells positively stained with vWF was counted, and the mean values and SDs were calculated. To evaluate the average integral optical density (IOD) of cTNI, cTNT or α-SMA, five microscopic fields were selected, and the IODs of cTNI, cTNT and α-SMA were obtained using Image-ProPlus 5.0 software (Media Cybernetics).

Immunofluorescence Staining

Serial immunofluorescent analysis was processed for tracking the fate of EGFP-labeled transplanted cells at days 3, 7, 14, 21 and 28. Cardiomyocytes, microvessels and arterioles were determined by cTNT, vWF and α-SMA respectively. We assessed evidence for direct differentiation of stem cells into cardiomyocytes, endothelial cells and smooth muscle cells by dual antibody staining. Sections were blocked and incubated overnight at 4°C with the primary antibodies rabbit polyclonal antibody against vWF (1:200 dilution; DAKO); α-SMA (1:300 dilution; DAKO); and cTNT (1:100 dilution; Abcam). After washes in PBS, sections were incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 568, 1:500; Invitrogen) for 1 h at room temperature. Sections were washed and counterstained with the nuclear dye 4, 6-diamino-2-phenylindole. Fluorescence-labeled sections were viewed with use of a confocal microscope (Olympus).

Statistical Analyses

All data were presented as mean ± SD. Quantitative analysis of both ^{18}F-FDG PET and echocardiography images were performed. One-way ANOVA was performed to analyze the group differences, and Pearson correlation was used to examine the correlation between changes of L/N ratio and EF value. The level of statistical significance was set at a *P* value of less than 0.05. All statistical analyses were performed with SPSS software (version 16.0, SPSS Inc.).

RESULTS

Immunohistochemical and Immunofluorescent Analysis

Serial immunofluorescence studies on miPSCs and mESCs found more cTNT$^+$ and cTNT$^+$ cells within 21 days (at days 3, 14 to 21) than on day 28; and more vWF$^+$ and α-SMA$^+$ cells within 14 days than after day 21 (Supplemental Figs. 4-8).

In addition, no teratoma was noted in injected hearts by hematoxylin and eosin (H.E.) staining at day 28.

Cardiac Differentiation from hESCs or hiPSCs

We observed spontaneous beating as early as 10-12 days after initiation of cardiac differentiation (Supplemental Videos 1 and 2). We then dissociated the monolayer CMs into single cells and observed that both the enriched hESC-CMs and the enriched hiPSC-CMs maintained spontaneous beating, and positive staining for cardiac-specific markers cTnT and sarcomeric α-Actinin (Supplemental Fig. 1). We usually yielded cTnT-positive CMs at a ratio of 50%-60%.

REFERENCES

1. Takahashi K, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc.* 2007;2:3081-3089.

2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A.* 1981;78:7634-7638.

3. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126:663-676.

4. Hu X, Yu SP, Fraser JL, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg.* 2008;135:799-808.

5. Peduto G, Rinsch C, Schneider BL, Rolland E, Aebischer P. Long-term host unresponsiveness to encapsulated xenogeneic myoblasts after transient immunosuppression. *Transplantation.* 2000;70:78-85.

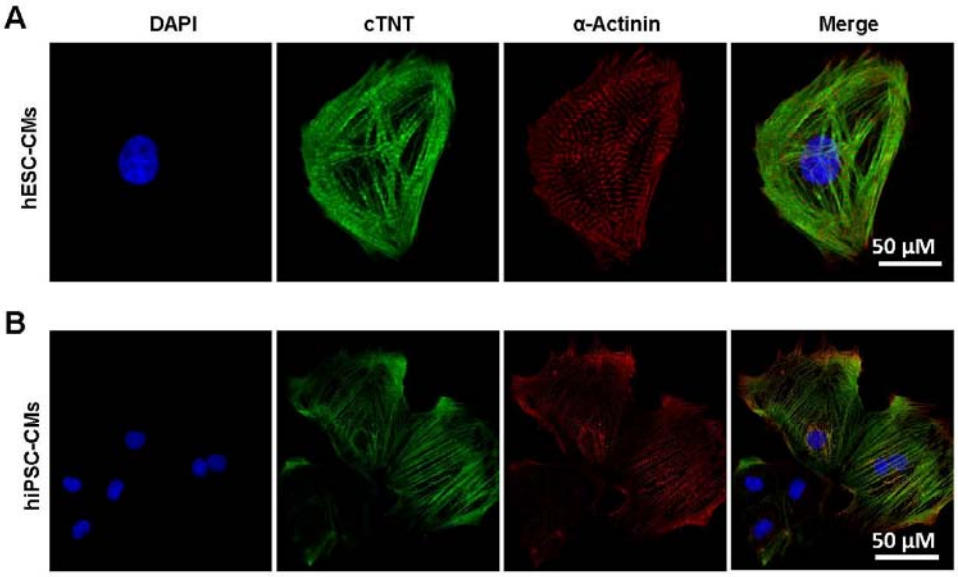
6. Zhang S, Dutton JR, Su L, Zhang J, Ye L. The influence of a spatiotemporal 3D environment on endothelial cell differentiation of human induced pluripotent stem cells. *Biomaterials.* 2014;35:3786-3793.

7. Nekolla SG, Reder S, Saraste A, et al. Evaluation of the novel myocardial perfusion positron-emission tomography tracer 18F-BMS-747158-02: comparison to 13N-ammonia and validation with microspheres in a pig model. *Circulation.* 2009;119:2333-2342.

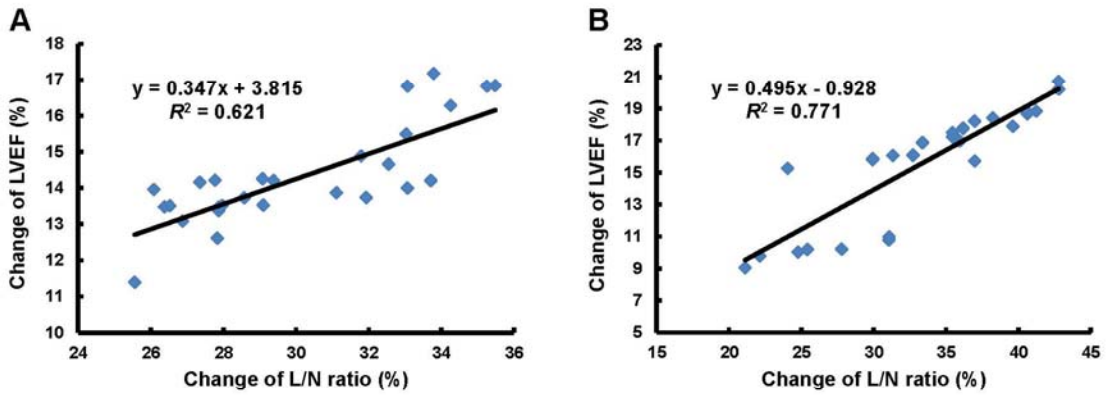
8. Morooka M, Kubota K, Kadowaki H, et al. 11C-methionine PET of acute myocardial infarction. *J Nucl Med.* 2009;50:1283-1287.

9. Perin EC, Tian M, Marini FC, et al. Imaging long-term fate of intramyocardially implanted mesenchymal stem cells in a porcine myocardial infarction model. *PLoS One.* 2011;6:e22949.

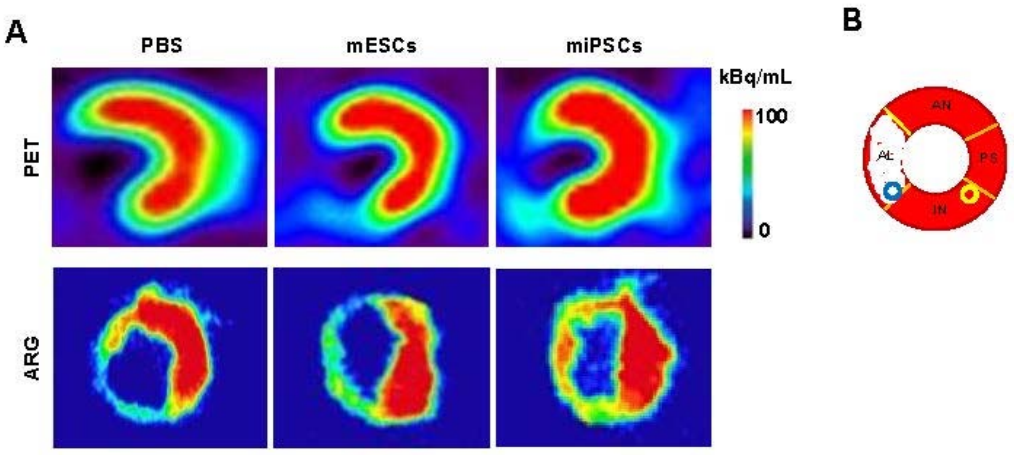
10. Flynn A, Chen X, O'Connell E, O'Brien T. A comparison of the efficacy of transplantation of bone marrow-derived mesenchymal stem cells and unrestricted somatic stem cells on outcome after acute myocardial infarction. *Stem Cell Res Ther.* 2012;3:36.



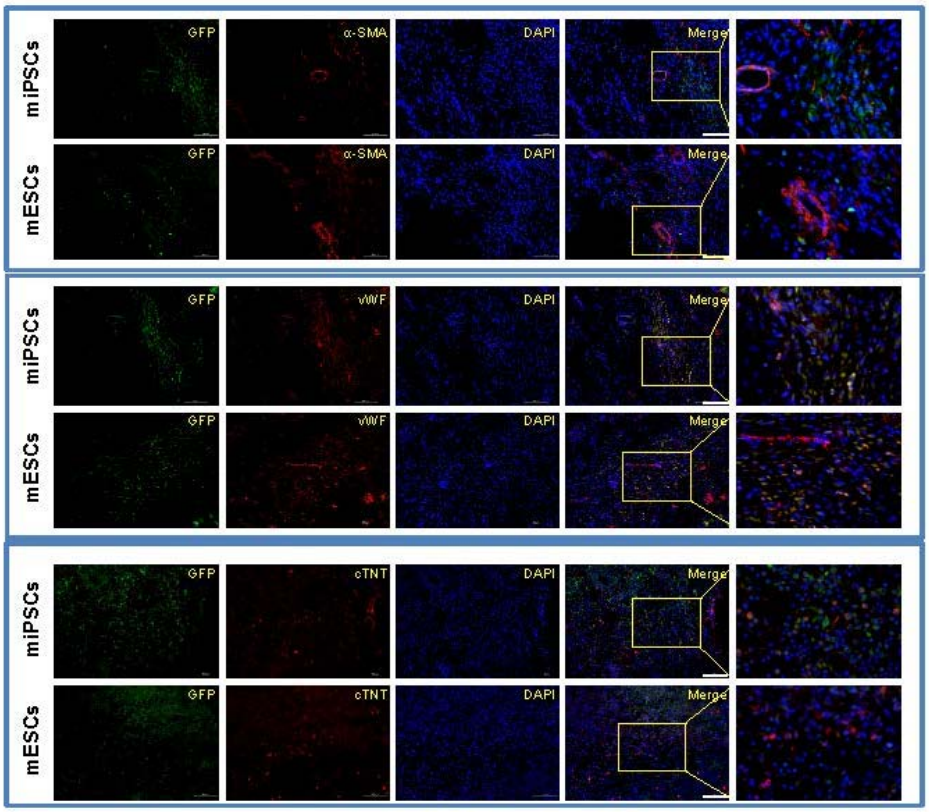
Supplemental Figure 1. Immunofluorescence staining of cardiac markers cTnT (green) and α-actinin (red) in single hESC-CMs or hiPSC-CMs. DAPI indicates the nuclear staining (blue). (A) cTnT (green) and α-actinin (red) staining in single hESC-CMs at 60x magnification. (B) cTnT (green) and α-actinin (red) staining in single hiPSC-CMs at 60x magnification.



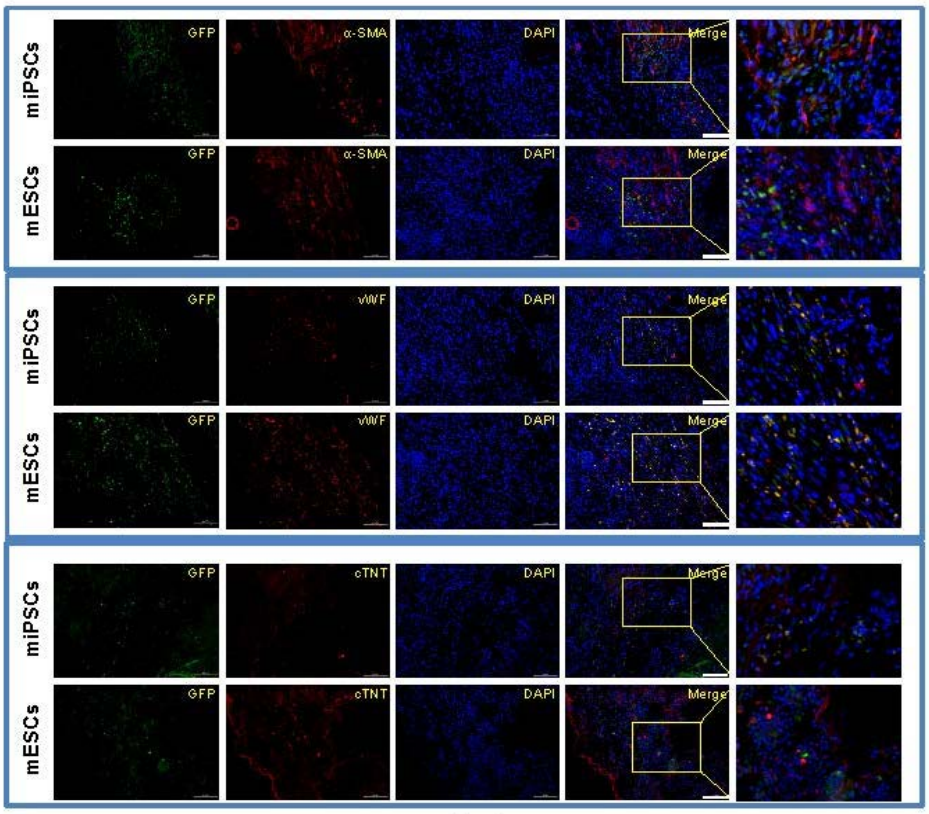
Supplemental Figure 2. (A) Correlation between the changes of L/N ratio and LVEF value in ESC group. Pearson correlation coefficient, R^2=0.621; *P*< 0.001. (B) Correlation between the changes of L/N ratio and EF value of the iPSC group. Pearson correlation coefficient, R^2=0.771; *P*< 0.001.



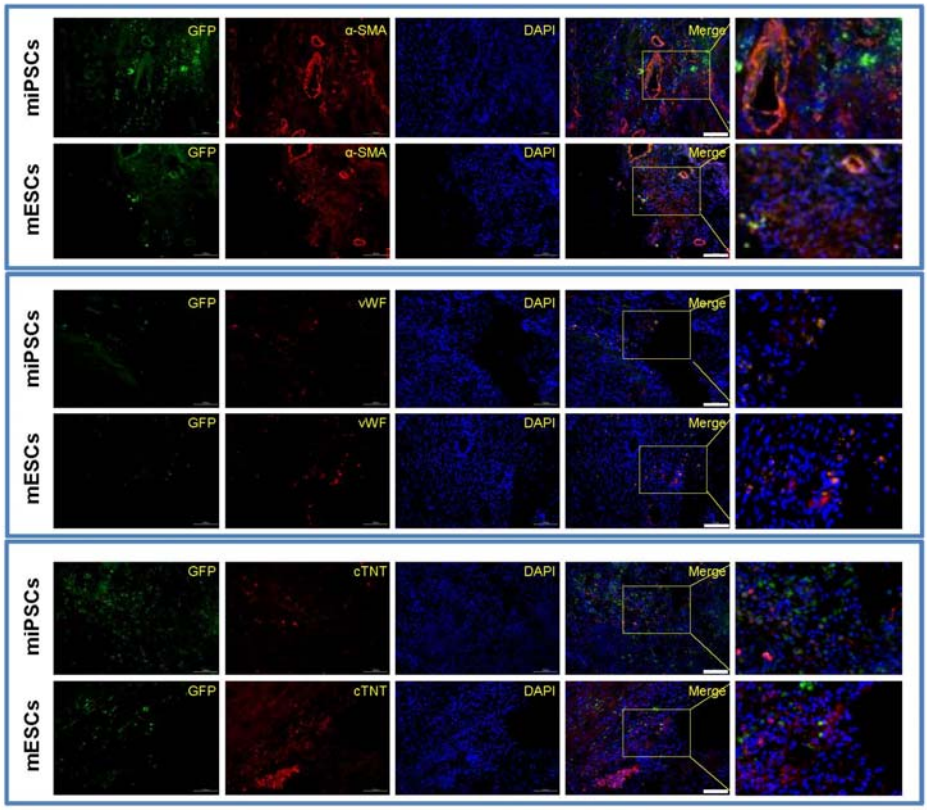
Supplemental Figure 3. (A) Axial slices of autoradiographic (ARG) and PET images. Scale was set according to signal intensity. (B) Schematic of MI.



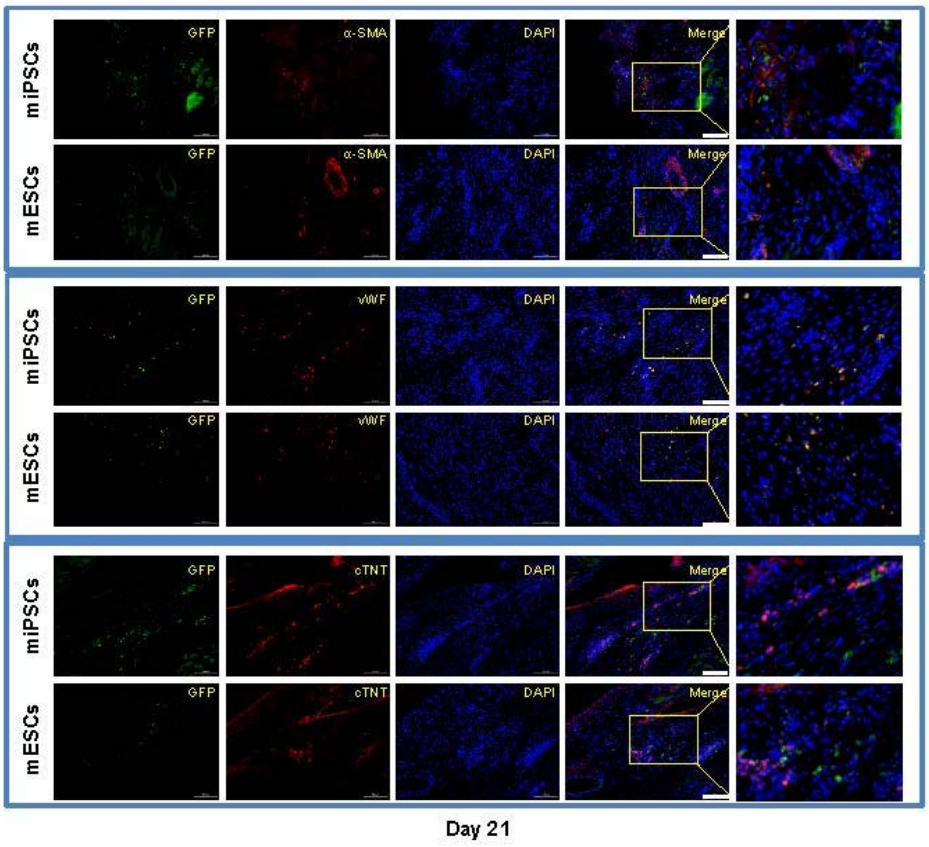
Supplemental Figure 4. Immunofluorescence staining of α-SMA$^+$, vWF$^+$ and cTnT+ stem cells. Representative images of ischemic region in miPSCs and mESCs or PBS-injected rats with magnifications as indicated at day 3. Scale bar denotes 100 μm. Magnified areas within the yellow rectangles are presented in the rightmost column.



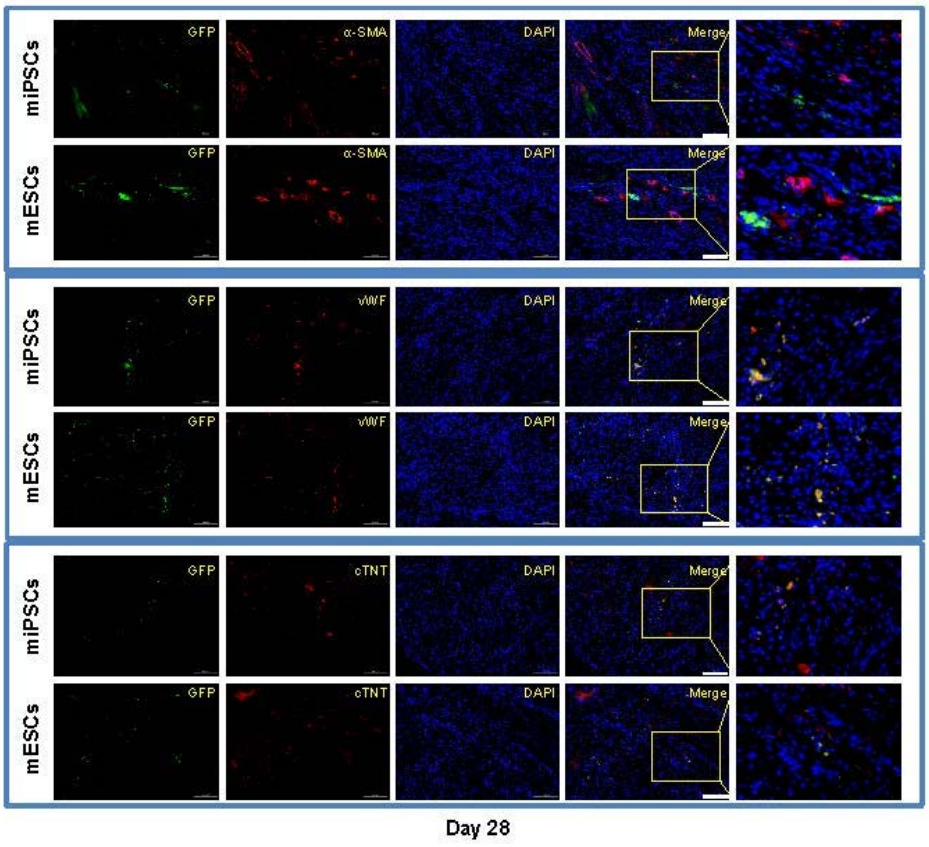
Supplemental Figure 5. Immunofluorescence staining of α-SMA$^+$, vWF$^+$ and cTnT$^+$ stem cells. Representative images of ischemic region in miPSCs and mESCs or PBS-injected rats with magnifications as indicated at day 7. Scale bar denotes 100 μm. Magnified areas within the yellow rectangles are presented in the rightmost column.



Supplemental Figure 6. Immunofluorescence staining of α-SMA$^+$, vWF$^+$ and cTnT+ stem cells. Representative images of ischemic region in miPSCs and mESCs or PBS-injected rats with magnifications as indicated at day 14. Scale bar denotes 100 μm. Magnified areas within the yellow rectangles are presented in the rightmost column.



Supplemental Figure 7. Immunofluorescence staining of α-SMA$^+$, vWF$^+$ and cTnT+ stem cells. Representative images of ischemic region in miPSCs and mESCs or PBS-injected rats with magnifications as indicated at day 21. Scale bar denotes 100 μm. Magnified areas within the yellow rectangles are presented in the rightmost column.



Supplemental Figure 8. Immunofluorescence staining of α-SMA$^+$, vWF$^+$ and cTnT$^+$ stem cells. Representative images of ischemic region in miPSCs and mESCs or PBS-injected rats with magnifications as indicated at day 28. Scale bar denotes 100 μm. Magnified areas within the yellow rectangles are presented in the rightmost column.