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Table 1: Evaluation of fatal arrhythmia by 24-hour ECG monitoring post-transplantation.

Group	Heart Rate, bpm			PAC	PVC			VT	Vf
	Max beat	Min beat	Mean beat		Singlet	Couplet	Triplet		
Sham (n=6)	181±42	83(52-118)	102(62-137)	208(126-297)	35(6-65)	0	0	0	0
EPC-only (n=6)	189±31	100(53-132)	113(71-163)	254(102-377)	55(21-83)	0	0	0	0
CSC-only (n=6)	171±29	55(50-79)	97(45-101)	120(44-201)	19(9-58)	0	0	0	0
CSC-EPC (n=6)	172±17	83(42-92)	101(48-169)	250(145-377)	48(15-77)	0	0	0	0

Abbreviations: ECG, electrocardiogram; PAC, premature atrial contraction; PVC, premature ventricular contraction; VT, ventricular tachycardia; Vf, ventricular fibrillation; EPC, endothelial progenitor cell; CSC, cardiac stem cell.

FIGURE LEGENDS

Figure 1: Characterization of the CSC sheet *in vitro*.

(A), Representative double immunostaining for c-kit (red) and phalloidin (green) of cultured CSCs at the second passage. **(B)**, C-kit positivity was markedly decreased by passage culture. **(C)**, FACS analysis of cultured CSCs at the fifth passage. **(D)**, Cultured CSCs at the fifth passage expressed myocyte structural protein, a characteristic of cardiac progenitor cells. Phalloidin (green), Troponin I (red). **(E)**, RT-PCR analysis of CSCs at the fifth passage. **(F)**, Detached CSC sheet. **(G)**, Representative immunostaining for Ki67 or Connexin 43. Abbreviations: CSC, cardiac stem cell; FACS, fluorescence-activated cell sorting; RT-PCR, reverse transcription polymerase chain reaction.

Figure 2: Global LV function assessed by multi-detector CT and conductance catheter. **(A-C)**, multi-detector CT parameters (**A**, EF, **B**, ESV, **C**, EDV) before and 8 weeks after cell transplantation (n=6 each). **(D)**, Representative P-V loops during IVC occlusion for each group at 8 weeks post-treatment (n=5 each). ESPVR of the CSC-EPC group was the greatest followed by that of the CSC-only group, then the EPC-only group, and then the sham group ($P<0.001$, ANOVA). **(E)**, dP/dt max, dP/dt min, τ, and EDP (n=5 each). $^{*}P<0.05$ versus sham, $^{\dagger}P<0.05$ versus EPC-only, $^{\ddagger}P<0.05$ versus CSC-only. Abbreviations: CT, computed tomography; EF, ejection fraction; ESV, end-systolic volume; EDV, end-diastolic volume; P-V, pressure-volume; IVC, inferior vena cava; ESPVR, end-systolic pressure-volume relationship; CSC, cardiac stem cell; EPC, endothelial progenitor cell; EDP, end-diastolic pressure.

Figure 3: Region and layer-specific systolic LV function and myocardial perfusion assessed by speckle-tracking and real-time contrast echocardiography.

(A,B), Epicardial **(A)** and endocardial **(B)** WMIs in the ischemic area before, 4 and 8 weeks after cell transplantation (n=6 each). At 8 weeks post-treatment, the epicardial WMI of the CSC-EPC and CSC-only groups was significantly greater than that of the EPC-only or sham group (P=0.001, Kruskal-Wallis test), while the endocardial WMI of the CSC-EPC group was the greatest followed by that of the EPC-only group, and then the CSC-only and sham group (P<0.001, ANOVA). **(C,D)**, Representative epicardial **(C)** and endocardial **(D)** radial strain images at end-systole in the CSC-EPC and sham groups. **(E)**, Representative contrast echocardiography 2D-imaging visualized by Volmac software in each group. **(F)**, Myocardial perfusion scores 8 weeks post-treatment (n=6). Myocardial perfusion score in the ischemic zone was significantly greater in the CSC-EPC than in the sham group (P<0.001, ANOVA) $^{*}P<0.05$ vs. sham, $^{\dagger}P<0.05$ vs. EPC-only, $^{\ddagger}P<0.05$ vs. CSC-only. Abbreviations: LV, left ventricular; 2D, 2-dimensional; CSC, cardiac stem cell; EPC, endothelial progenitor cell; WMI, wall motion index.

Figure 4: Engraftment of transplanted CSCs and EPCs, and neovascularization of the ischemic wall.

(A), Engraftment of the transplanted DiI-red-labeled CSC sheets at 3 weeks post-treatment. **(B)**, Quantification of the CSC-sheet engrafted area in the CSC-only and CSC-EPC groups 1, 3, and 8 weeks post-treatment. **(C)**, Quantification of the vWF-positive capillary density at the ischemic epicardium and endocardium in each group at 3 weeks post-treatment. The number of vWF-positive capillaries in the ischemic epicardium of the CSC-EPC and CSC-only groups was significantly greater than in the EPC-only or sham group (P<0.001, ANOVA), while the

number of capillaries in the ischemic endocardium was significantly greatest in the CSC-EPC, followed by the EPC-only group, and then by the CSC-only and sham groups (P<0.001, ANOVA). **(D)**, Representative immunostaining for vWF at the ischemic epicardium and endocardium at 3 weeks post treatment in each group. Migration of CSCs into the ischemic endocardium was observed only in the CSC-EPC group, and not in the CSC-only group. Yellow and white arrows indicate CSCs and EPCs, respectively. Abbreviations: CSC, cardiac stem cell; EPC, endothelial progenitor cell; vWF, von Willebrand factor.

Figure 5: Possible mechanism of CSC migration.

(A), RT-PCR analysis in each group at 3 weeks post-treatment. The mRNA levels of swine-specific SDF-1 (P<0.001, Welch's ANOVA) and CXCR4 (P<0.001, Welch's ANOVA) were markedly greater in the CSC-EPC group than in the other groups (n=4 each). **(B)**, Representative immunostaining for SDF-1 in the CSC-EPC group at 3 weeks post-treatment. DiI-red-labeled CSCs (middle panel), SDF-1 (green) (right panel), and merged image (left panel). Abbreviations: CSC, cardiac stem cell; SDF-1, stromal cell-derived factor 1; RT-PCR, reverse transcription polymerase chain reaction; CXCR4, C-X-C chemokine receptor type 4.

Figure 6: Histological assessment of interstitial fibrosis, capillary density, and myocyte hypertrophy 8 weeks after cell transplantation.

(A), Representative Masson's trichrome staining in a section through the entire heart. **(B)**, Representative periodic acid-Schiff staining of the remote zone. **(C)**, Representative immunostaining for vWF in the peri-ischemic zone. **(D)**, Quantification of fibrosis in each group (n=6 each). **(E)**, In the CSC-EPC group, the thickness of the LV wall was well preserved compared with the sham group (P<0.01, ANOVA). **(F)**, Quantification of the cell

diameter of myocytes (n=6 each). **(G)**, Quantification of capillary density at the peri-ischemic zone (n=6 each). The number of vWF-positive capillaries of the CSC-EPC group was the greatest, followed by the CSC-only and EPC-only groups, and then the sham group ($P<0.001$, ANOVA). $^{*}P<0.05$ vs. sham, $^{\dagger}P<0.05$ vs. EPC-only, $^{\ddagger}P<0.05$ vs. CSC-only. Abbreviations: vWF, von Willebrand factor; CSC, cardiac stem cell; EPC, endothelial progenitor cell; LV, left ventricular.

Figure 7: Phenotypic fate of transplanted CSCs and EPCs at 8 weeks post-treatment. **(A,B)**, Representative immunostaining with human FISH and cTn-I **(A)** or vWF **(B)** in the CSC-EPC group. Small numbers of cardiomyocytes **(A)** and endothelial cells **(B)** with a human genome were present in the native myocardium. **(C,D)**, Representative immunostaining with swine FISH and cTn-I **(C)** or vWF **(D)** in the CSC-EPC group. All the cardiomyocytes **(C)** and endothelial cells **(D)** that were positive for human genomic markers were also positive for porcine markers; thus, they had chimeric nuclei. Yellow and white arrows indicate human and swine genomic markers, respectively. Abbreviations: CSC, cardiac stem cell; EPC, endothelial progenitor cell; FISH, Fluorescence in-situ hybridization; cTn-I, cardiac troponin I; vWF, von Willebrand factor.

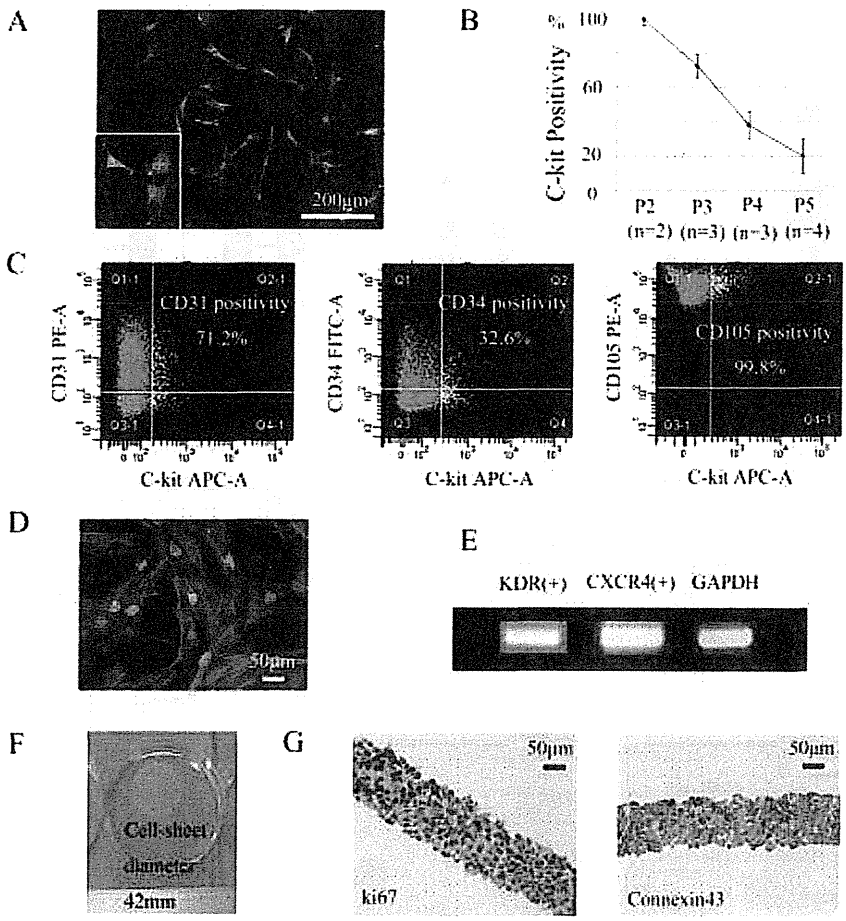

Figure 1

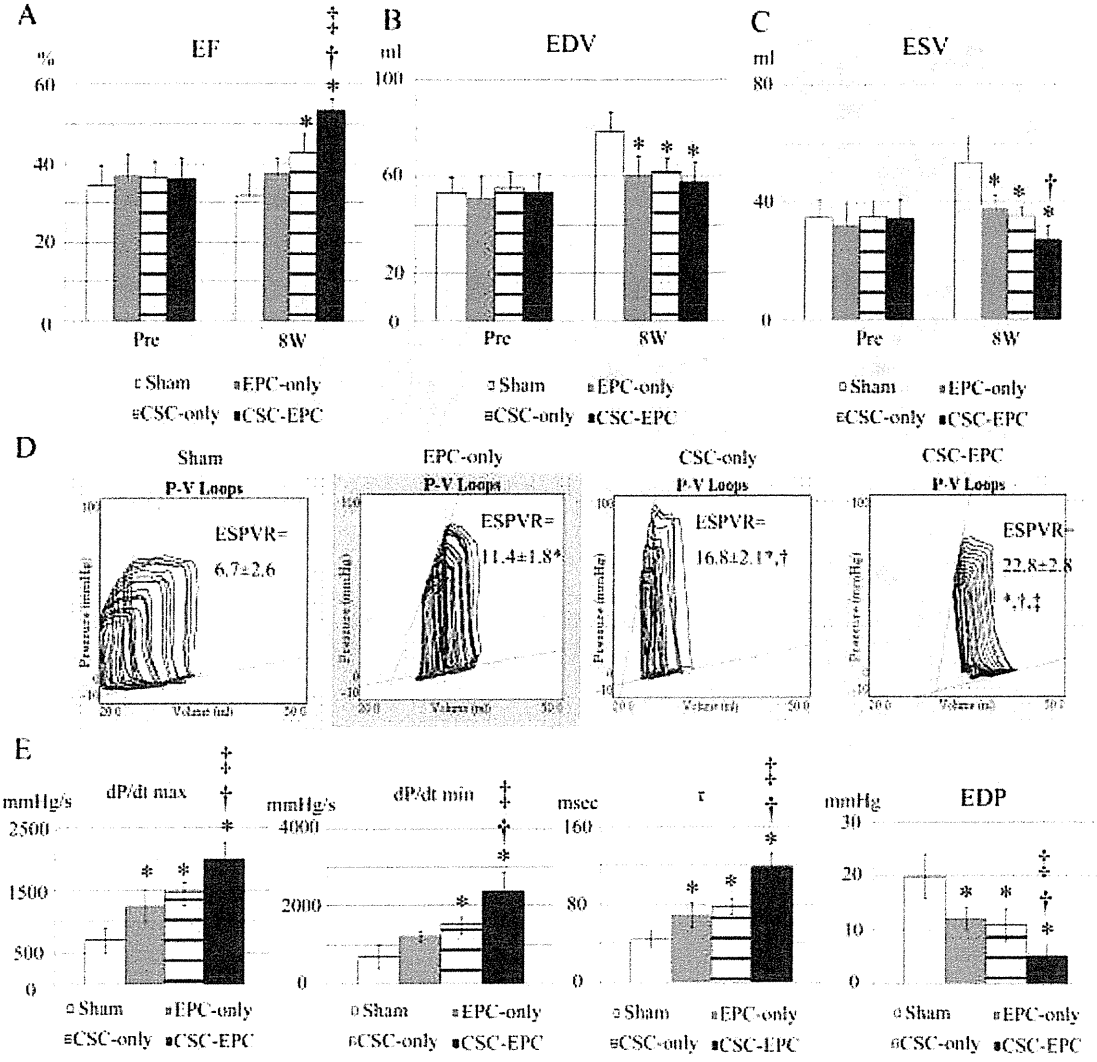

Figure 2

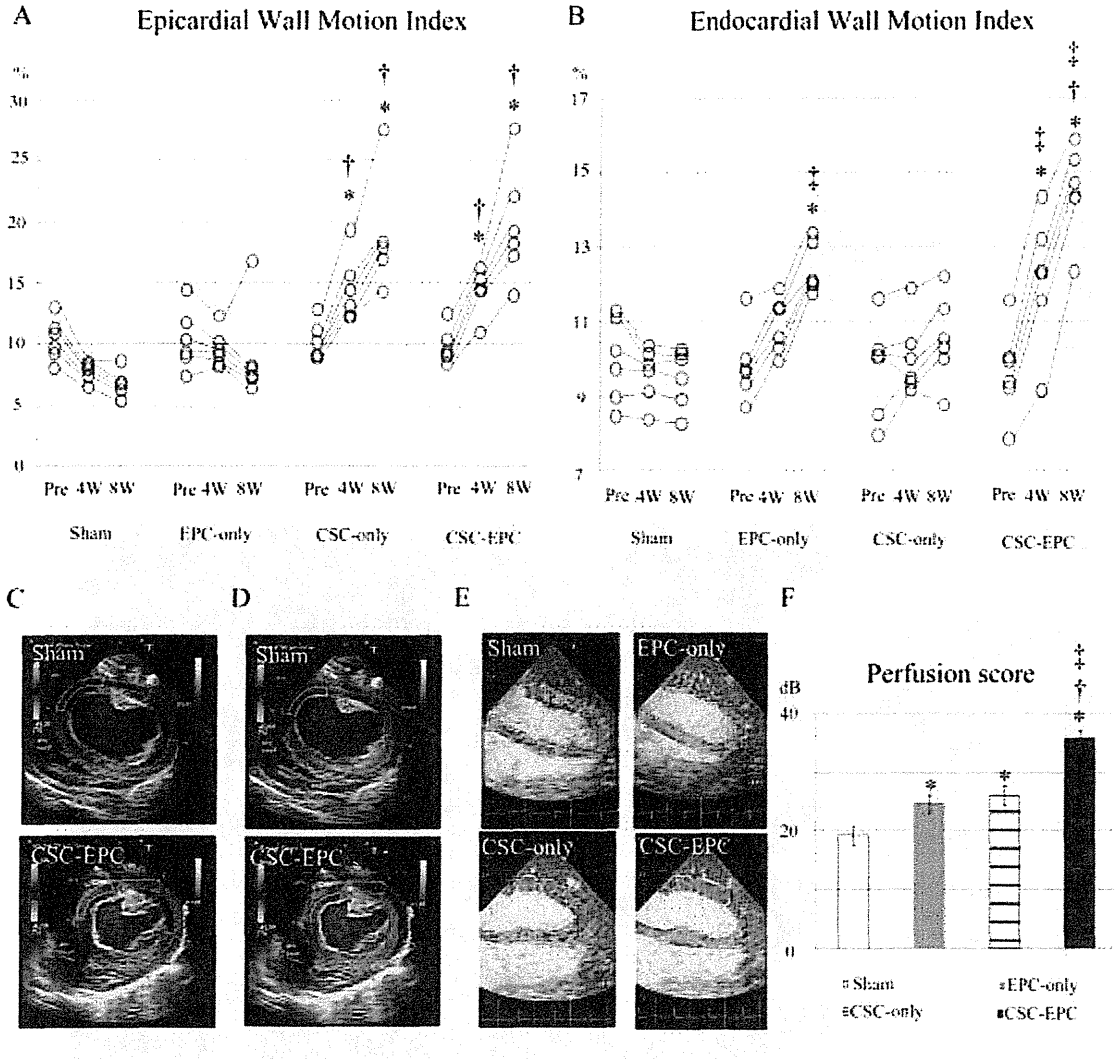

Figure 3

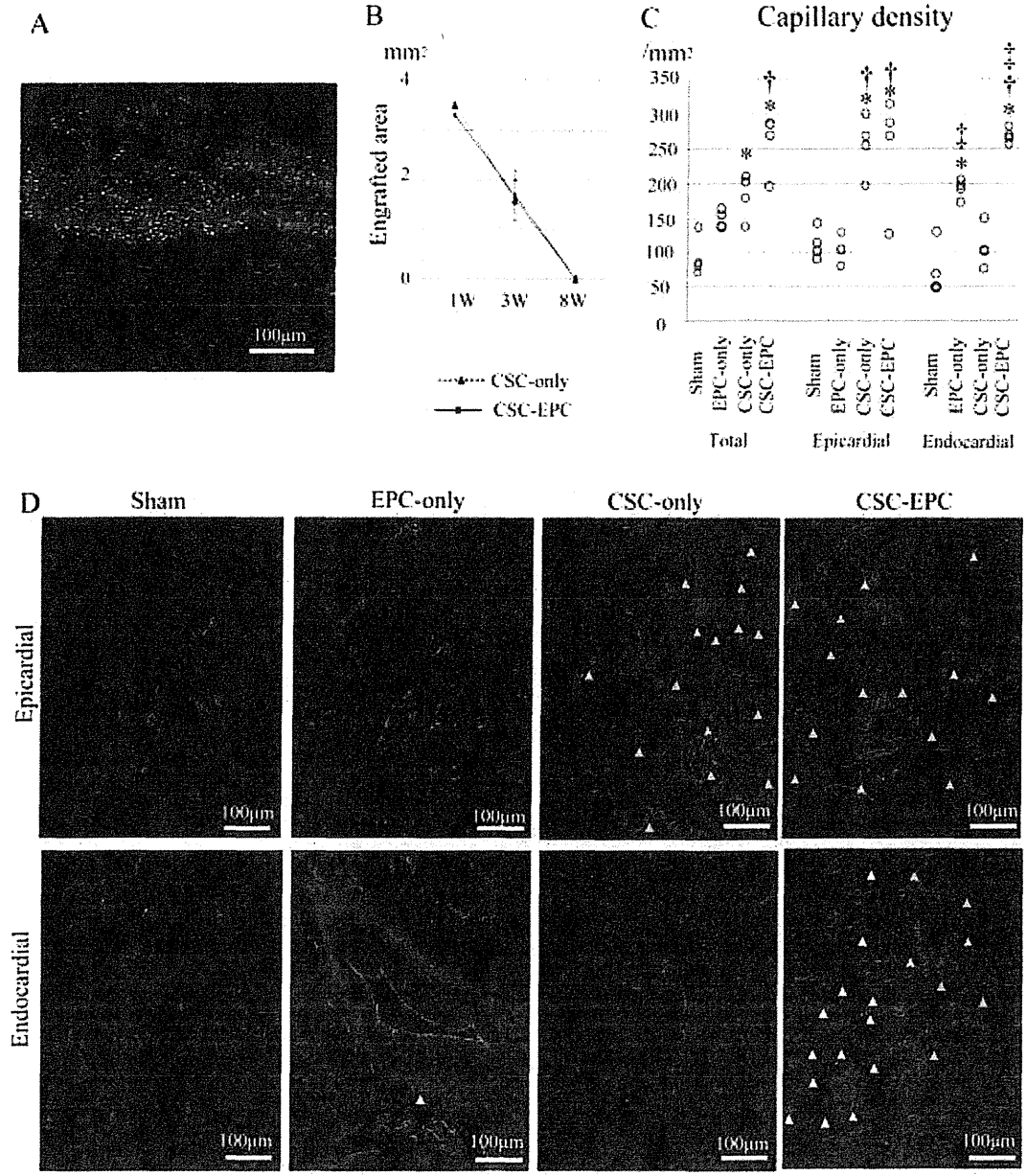

Figure 4

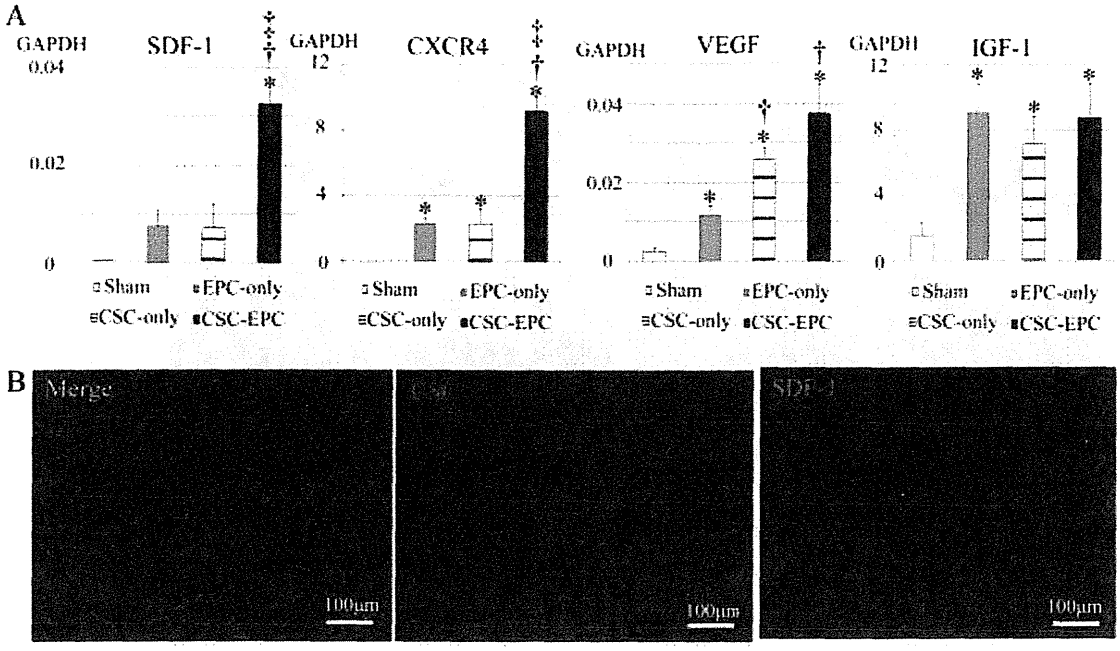

Figure 5

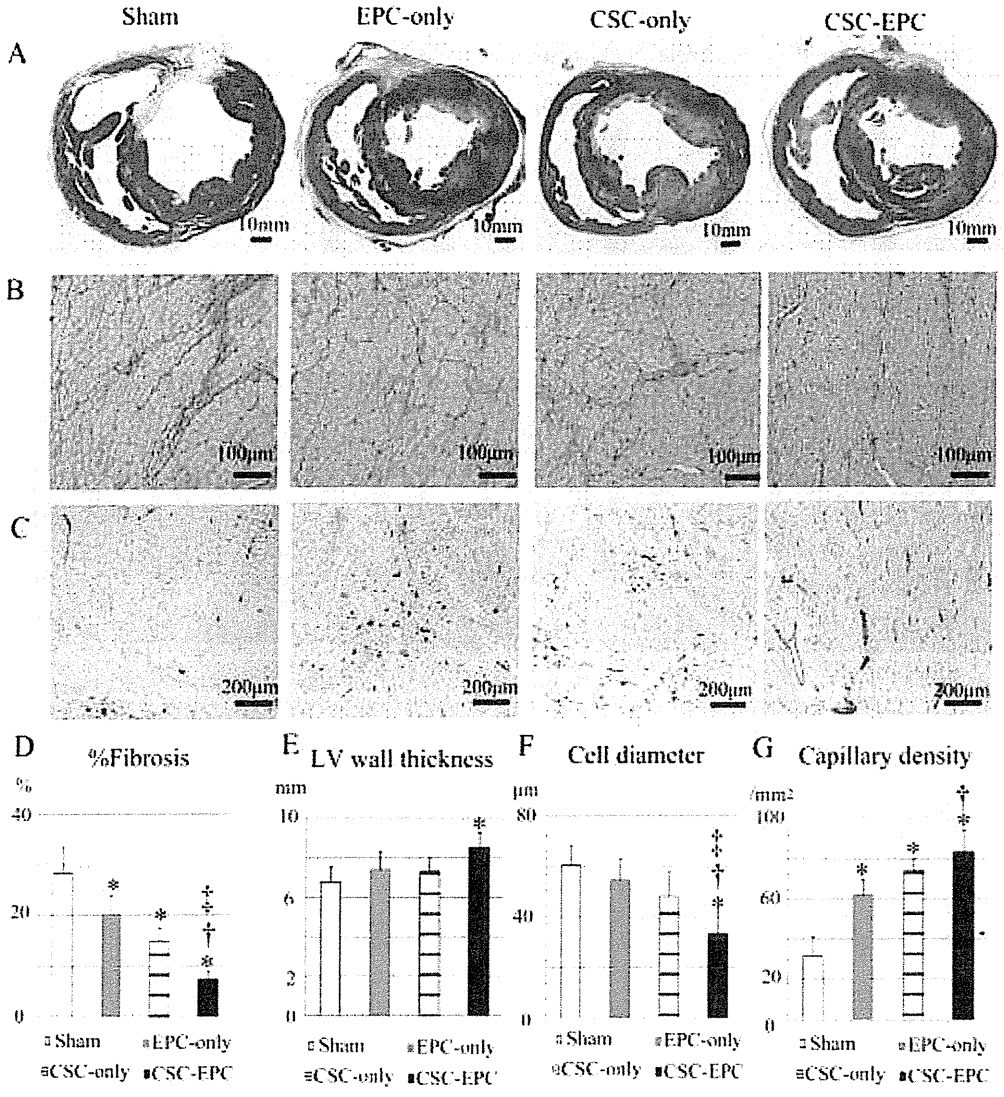

Figure 6

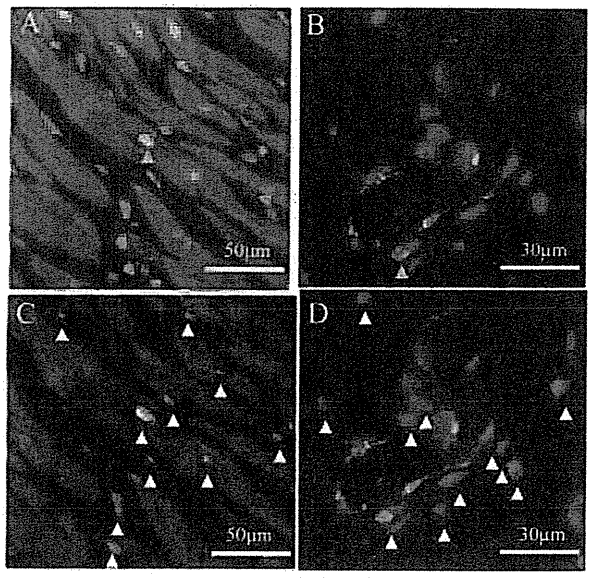

Figure 7

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ORIGINAL ARTICLE
Regenerative Medicine

Human Cardiac Stem Cells With Reduced Notch Signaling Show Enhanced Therapeutic Potential in a Rat Acute Infarction Model

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Background: Because human cardiac stem cells (CSC) have regeneration potential in damaged cardiac tissue, there is increasing interest in using them in cell-based therapies for cardiac failure. However, culture conditions, by which CSCs are expanded while maintaining their therapeutic potential, have not been optimized. We hypothesized that the plating cell-density would affect proliferation activity, differentiation and therapeutic potential of CSCs through the Notch signaling pathway.

Methods and Results: Human CSCs were plated at 4 different densities. The population doubling time, C-KIT positivity, and dexamethasone-induced multidifferentiation potential were examined in vitro. The therapeutic potential of CSCs was assessed by transplanting them into a rat acute myocardial infarction (AMI) model. The low plating density (340 cells/cm^2) maintained the multidifferentiation potential with greater proliferation activity and C-KIT positivity in vitro. On the other hand, the high plating density (5,500 cells/cm^2) induced autonomous differentiation into endothelial cells by activating Notch signaling in vitro. CSCs cultured at low or high density with Notch signal inhibitor showed significantly greater therapeutic potential in vivo compared with those cultured at high density.

Conclusions: CSCs cultured with reduced Notch signaling showed better cardiomyogenic differentiation and therapeutic potentials in a rat AMI model. Thus, reducing Notch signaling is important when culturing CSCs for clinical applications. (*Circ J* 2014; **78:** 222–231)

Key Words: Cardiac stem cells; Cell culture; Notch signaling

Cardiac failure is a major cause of reduced quality of life and mortality.[1,2] Although cardiac tissue is known to have limited regeneration capacity, it has been shown that damaged cardiac tissue is regenerated by cardiac stem cells (CSCs), which are identified as C-KIT-positive cells in the heart,[3] through their proliferation and differentiation into functional cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells (ECs), and through release of a variety of factors that activate native healing processes.[4] Although transplantation of autologous stem cells into the heart has been proven to enhance this regenerative capacity of the damaged heart,[5] transplantation of CSCs that have been expanded in vitro may have promise in maximizing the regeneration process.[6] The magnitude of the therapeutic effect of CSC transplantation is determined by the cell preparation and delivery method, though the protocol for preparing CSCs has not been fully established.[7] Determination of the culturing protocol will be critical for the clinical application of patient-derived CSCs that may be isolated from limited biopsy samples.

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The cell preparation protocol of CSCs involves multiple steps, including enzymatic digestion of tissues, cell isolation, and cultivation, that will affect the fundamental behavior and therapeutic potential of CSCs.[8,9] Although the cell isolation protocol has been intensively studied,[3,10–13] the cell cultivation protocol has not. Among the many parameters of the culture

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conditions, it has been suggested that cell density at plating modulates fundamental behavior of stem cells such as proliferation and differentiation, indicating that the plating density may affect the therapeutic potential of CSCs.[14,15] In addition, it was reported that Notch signaling in direct cell-cell communication may be associated with fundamental behavior of cells under cultivation, including the lineage specification of cardiac progenitor cells.[16–18] We hypothesized that the plating cell-density would affect proliferation activity, differentiation and therapeutic potential of human CSCs by regulating Notch signaling, with the aim of exploring the optimal CSC cultivation protocol for treating cardiac failure.

Methods

Isolation and Cultivation of CSCs

All procedures were in accordance with the ethical standards of the institutional committee on human experimentation (control number: 729-4). CSCs were isolated from the right atrium of 3 patients with dilated cardiomyopathy (12–55 years old; data from the 12-year-old patient are mainly used in this report). In short, the cells were separated from the tissue by enzymatic digestion [37°C with 1 mg/ml of collagenase (17454, Serva Electrophoresis, Heidelberg, Germany) in Ham's F12 medium] after dissecting fat and fibrous tissues and mincing. The digestion was performed for a total of 5 reactions (60 rpm×20 min/reaction, 8 ml/reaction in 50 ml tube) then overnight digestion (for 12 h) was performed for the remaining debris with 0.1 mg/ml of collagenase solution (37°C, 60 rpm, 10 ml in 50 ml tube). After each reaction, the supernatant was collected and then the cells were collected by centrifugation (4°C, 500 g, 5 min) and plated on a normal 10-cm culture dish (353003, BD Biosciences, Franklin Lakes, NJ, USA) with complete medium [Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; SH30406.02, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 5 mU/ml human erythropoietin (E5627-10UN, Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml basic fibroblast growth factor (100-18B, PeproTech, Rocky Hill, USA), 0.2 mmol/L L-glutathione (G6013, Sigma-Aldrich) with antibiotics].[6] The medium was changed on day 2. On day 5, the cells were collected with trypsin (T3924, Sigma-Aldrich) and replated on 10-cm dishes at 170 cells/cm^2. At the second passage (P1), the cells were collected with non-enzymatic solution (C5914, Sigma-Aldrich), labeled with anti-C-KIT antibody (130-091-735, Miltenyi Biotec, Bergisch Gladbach, Germany) followed by FcR blocking reagent (130-059-901, Miltenyi Biotec) in 3% FBS/PBS, and subjected to FACS (FACS Aria, BD Biosciences) to isolate the CSCs. As the negative control sample, mouse IgG1-phycoerythrin (130-092-212, Miltenyi Biotec) was used. The dead cells were excluded from the sample by using 7AAD (559925, BD Biosciences). After sorting, the CSCs were passaged (340 cells/cm^2) every 5 days. At P5, CSCs were sorted again as described. The purified CSCs were plated on normal culture dishes at different densities with and without 100 nmol/L of gamma secretase inhibitor XXI (GSI; Merck, Darmstadt, Germany)[16] and passaged every 5 days.

qPCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase digestion (RNase-Free DNase set, Qiagen). The extracted RNA was subjected to reverse transcription (Omniscript reverse transcriptase, Qiagen) with random primers (Invitrogen-Life Technologies, Carlsbad, CA, USA). qPCR (quantitative real-time PCR) was performed using predesigned TaqMan primers/probes [assay ID; GAPDH: Hs99999905_m1, P21: Hs00355782_m1, P53: Hs01034249_m1, ETS1: Hs00901425_m1, TIE2: Hs00945155_m1, HES1: Hs00172878_m1, C-KIT: Hs00174029_m1, IL8: Hs99999034_m1, VEGFA: Hs00900055_m1, cTnT: Hs00165960_m1, PDGFRB: Hs01019589_m1, HGF: Hs00900070_m1, Applied Biosystems-Life Technologies, Carlsbad, CA, USA] and a 7500 Fast real-time PCR system (Applied Biosystems). GAPDH was used as the internal control.

Tube Formation Assay

CSCs in EBM2 (CC-3156, Lonza, Basel, Switzerland) supplemented with 0.2% FBS were plated onto a Matrigel (BD Biosciences) -coated 96-well plates (7.5×10^3 cells per well) and incubated for 16 h.[19] Subsequently, the total tube length per well was measured[20] by analytic software (BZII, Keyence, Osaka, Japan).

Immunocytofluorescence Analysis of Cell Differentiation

αMEM supplemented with 10% FBS and 10^{-8} mol/L dexamethasone was used to induce differentiation of CSCs.[3] In short, CSCs were replated (3,400 cells/cm^2) onto a cover slip coated with 0.1% gelatin and incubated under 5% CO_2 at 37°C for 7 days. The cells were fixed with 4% paraformaldehyde and labeled with primary antibodies against αSA (alpha sarcomeric actin, A2172, Sigma-Aldrich), anti-αSMA (alpha smooth muscle actin, A2574, Sigma-Aldrich), or anti-TIE2 (T6577, Sigma-Aldrich). The samples were visualized with appropriate secondary antibodies and counterstained with DAPI.

Rat Acute Myocardial Infarction (AMI) Model and Cell Transplantation

The animal study protocols were approved by the Animal Care and Use Committee of the Osaka University (21-030-2). The left coronary artery (LCA) was permanently ligated in nude rats (F344/NJcl-rnu/rnu, 8-week-old females, CLEA Japan, Tokyo, Japan) under inhalation anesthesia with 2.0% isoflurane through endotracheal intubation.[21] Immediately after the ligation, 8×10^4 cells[3] or 3×10^6 cells (for immunostaining against HNA/MLC and qPCR for VEGFA/HGF (Hepatocyte growth factor)) per rat were transplanted by intramuscular injection into the infarct's border zone.

Transthoracic Echocardiography (TTE)

TTE was performed under inhalation anesthesia with 1.5% isoflurane as described previously.[21]

Histological Examination

Rat hearts were collected after retrograde infusion of phosphate-buffered saline (PBS) supplemented with 50 mmol/L potassium chloride and 100 units/ml heparin. The hearts were embedded in OCT (Sakura Finetek Japan, Tokyo, Japan), cut into 5-μm sections, and fixed with 4% paraformaldehyde.[22] The sections were then stained with Masson's trichrome or immunohistologically against von Willebrand factor (vWF; A0082, Dako, Glostrup, Denmark), HNA (human nuclear antigen, MAB1281, Millipore, MA, USA), or MLC (myosin light chain, ab79935, Abcam, MA, USA) by similar methods to those mentioned earlier.

To calculate the percentage of left ventricle (LV) that was fibrotic, the total LV and fibrotic areas (blue-colored) were traced and measured using analytical software (BZII, Keyence). The percentage of MLC-positive cells was calculated as the number of HNA and MLC double-positive cells divided by the number of HNA-positive cells in a high-power magnification area (×200).