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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.

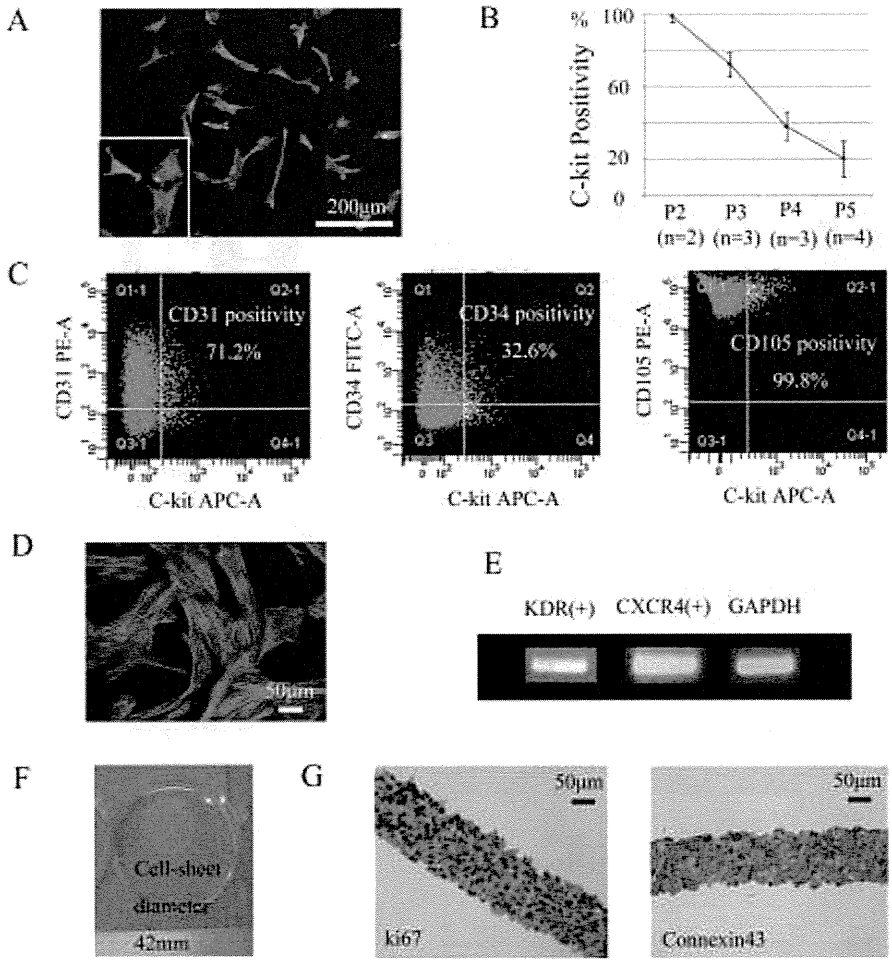
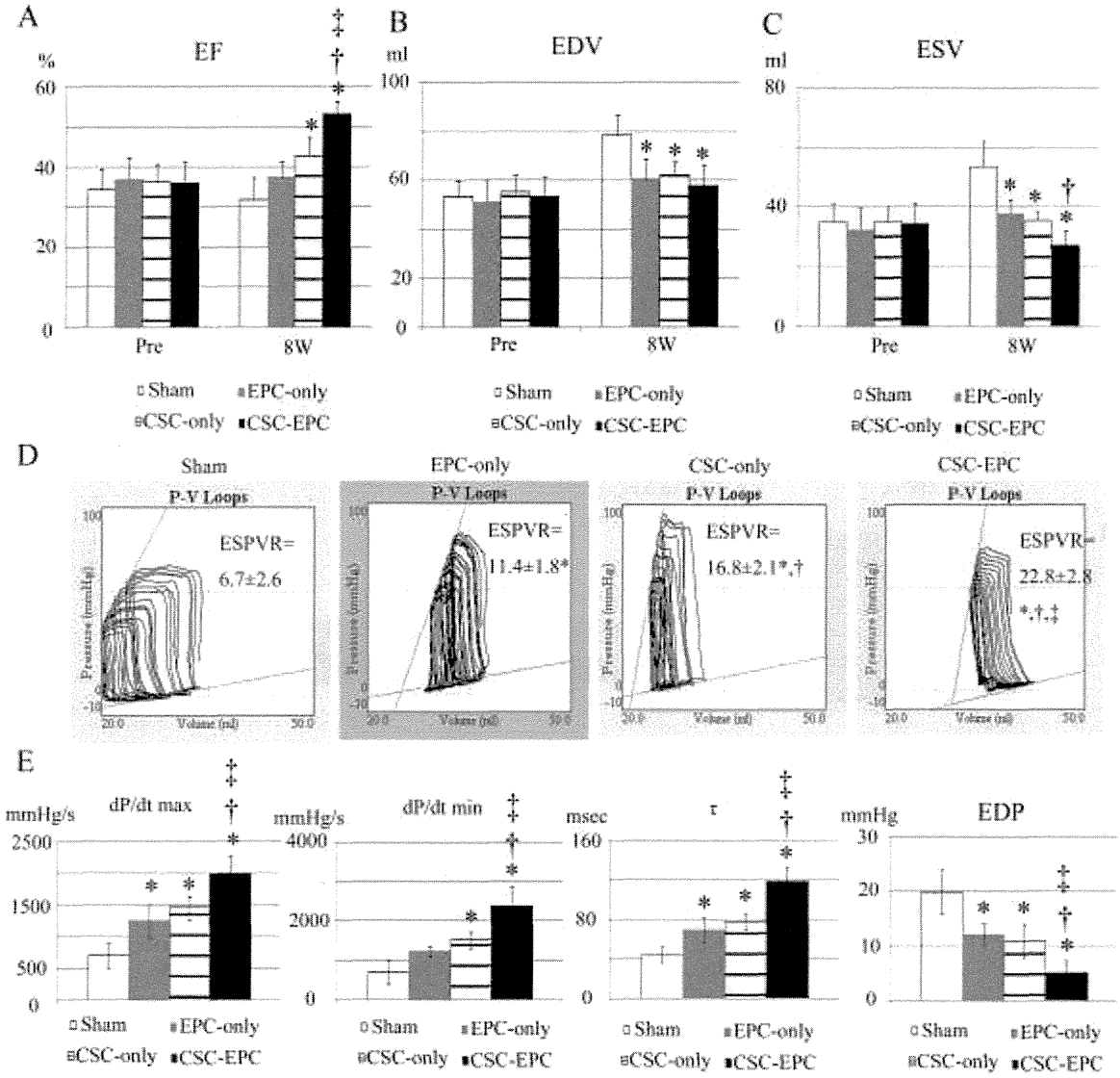


Figure 1



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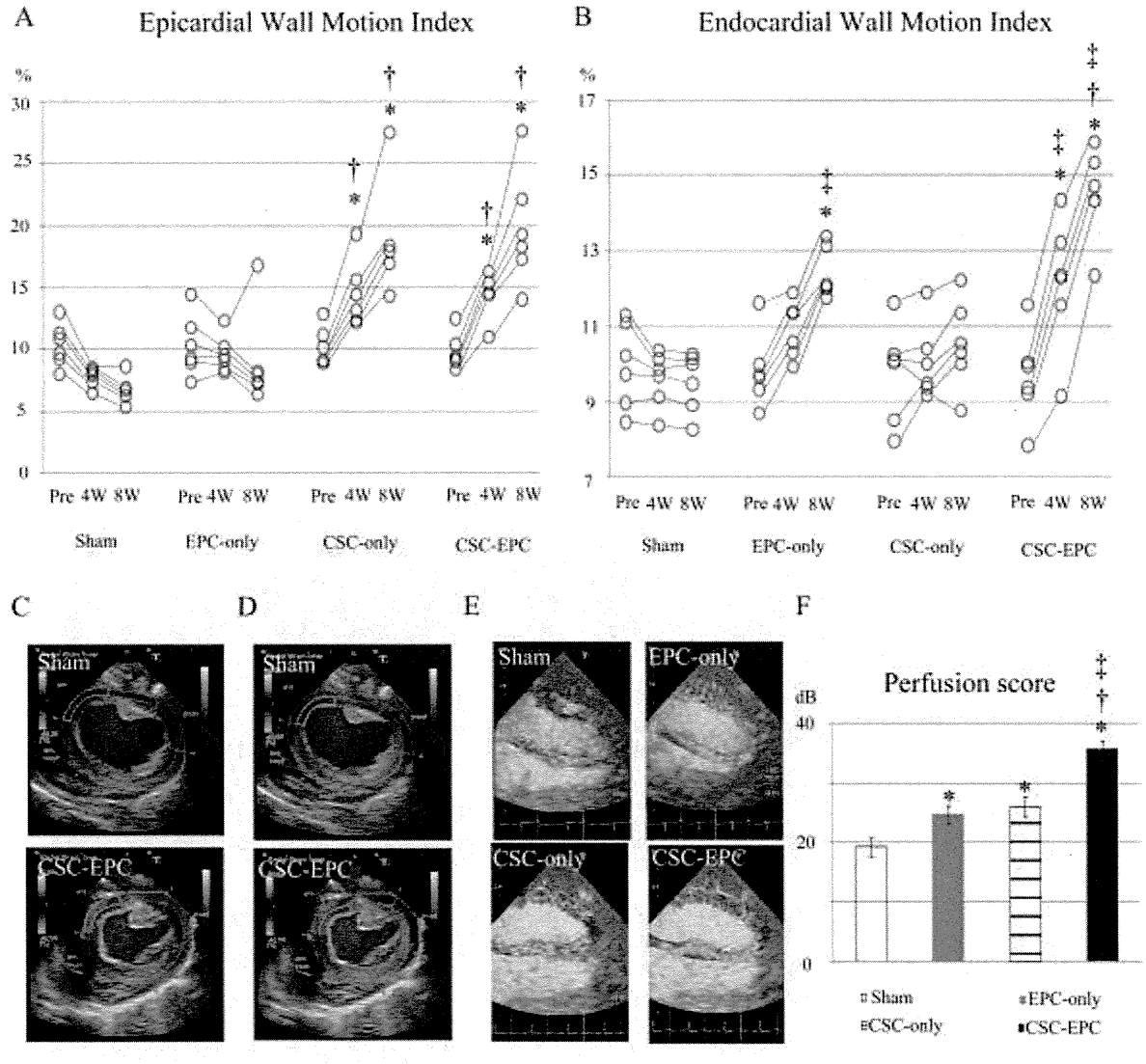

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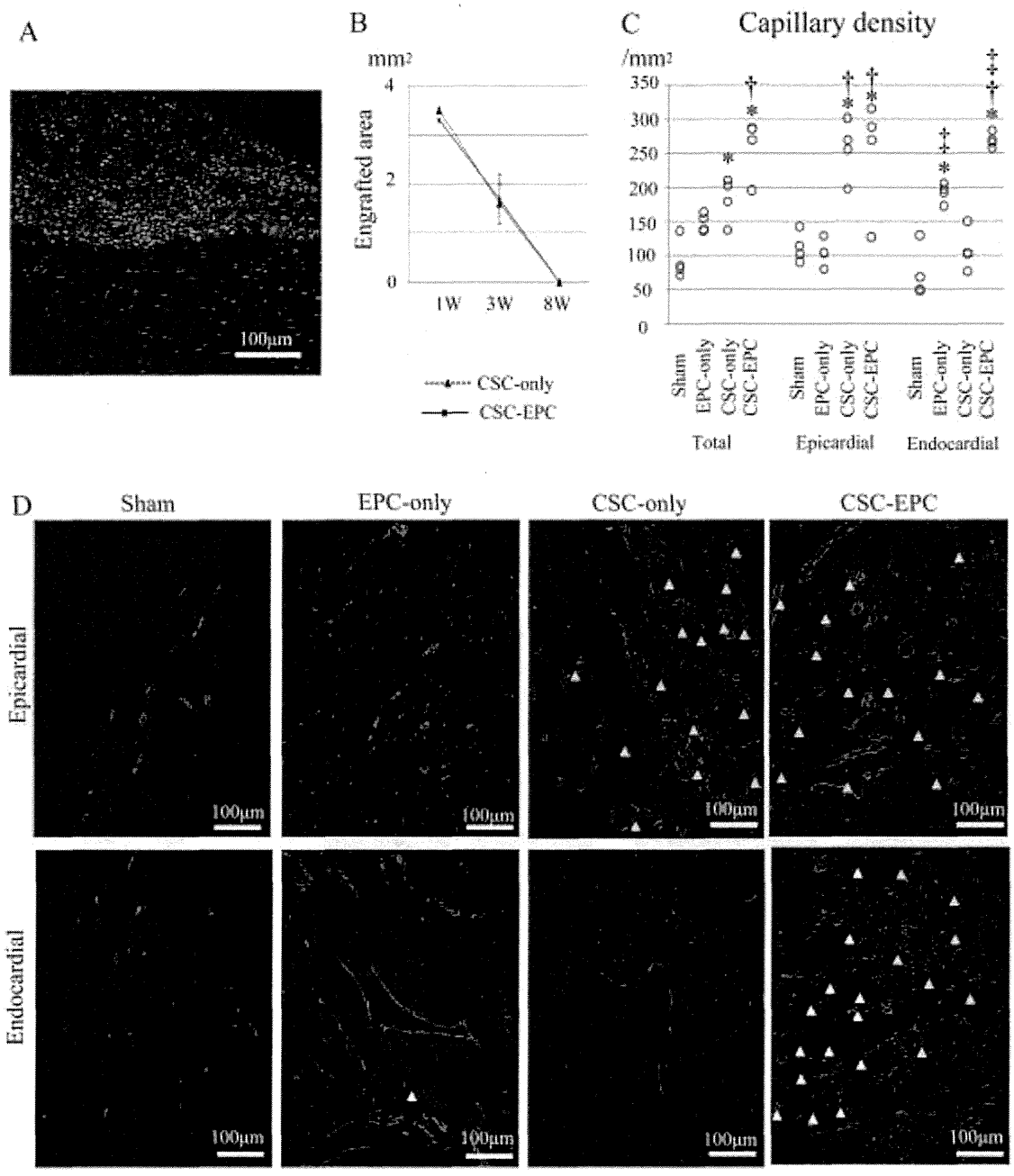

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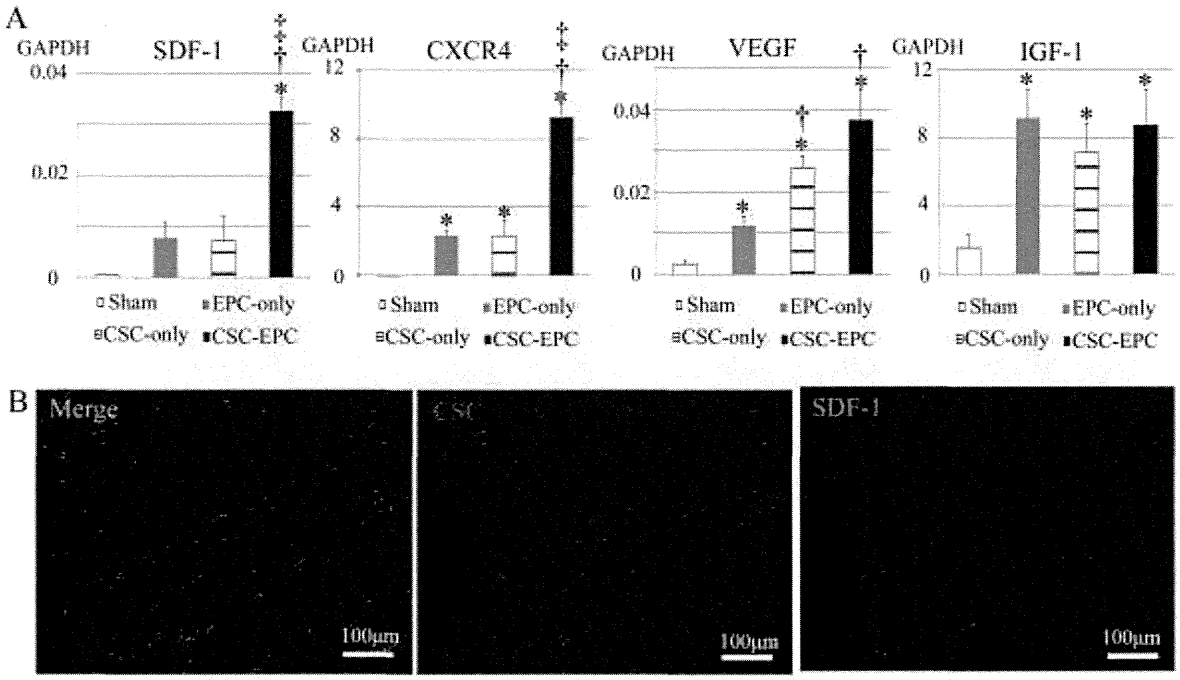

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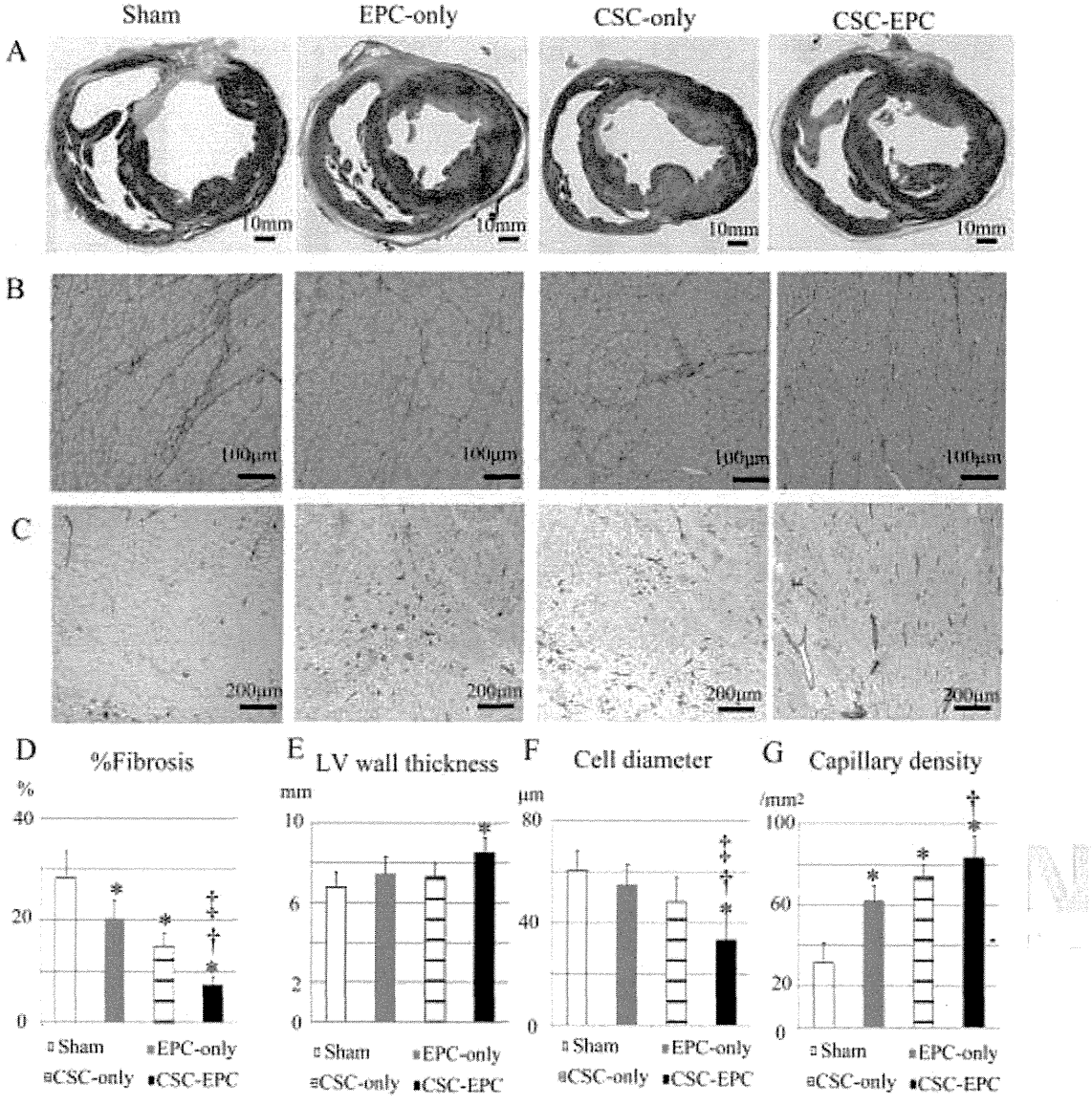

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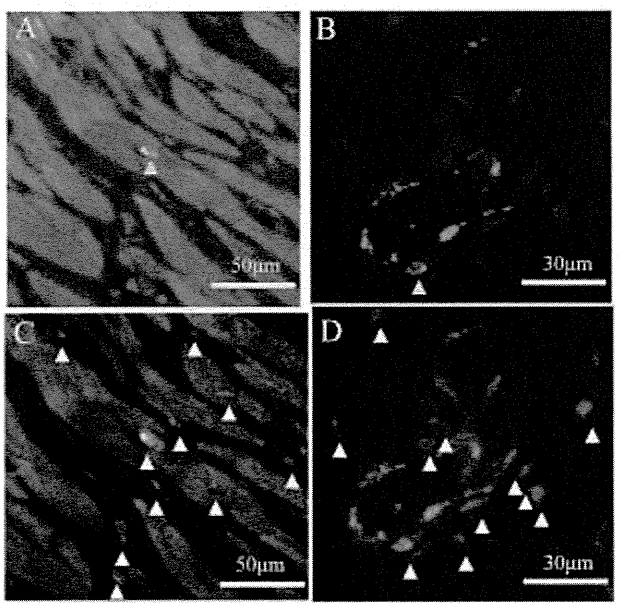

Figure 7

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ORIGINAL ARTICLE

Impact of cardiac stem cell sheet transplantation on myocardial infarction

Sfoug Alshammary · Satsuki Fukushima · Shigeru Miyagawa · Takenori Matsuda · Hiroyuki Nishi · Atsuhiro Saito · Sokichi Kamata · Takayuki Asahara · Yoshiki Sawa

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Abstract
Purpose Myocardial infarction (MI) remains a major cause of mortality because of the limited regenerative capacity of the myocardium. Transplantation of somatic tissue-derived cells into the heart has been shown to enhance the endogenous healing process, but the magnitude of its therapeutic effects is dependent upon the cell-source or cell-delivery method. We investigated the therapeutic effects of C-Kit positive cardiac cell (CSC) cell-sheet transplantation therapy in a rat model of MI.
Methods and results CSCs of human origin were sorted and cultured to generate scaffold-free CSC cell-sheets. One-layered or 3-layered cell-sheets were transplanted into nude rats 1 h after left coronary artery ligation. We observed a significant increase in the left ventricular ejection fraction and a significant decrease in left ventricular systolic dimension at 2 and 4 weeks in the 3-layer group, but not in the 1-layer or sham groups. Consistently, there was less accumulation of interstitial fibrosis in the 3-layer group than in the 1-layer or sham groups. Moreover, capillary density was significantly greater in the 3-layer group than in the 1-layer or sham groups.
Conclusions The 3-layered cell-sheet improved cardiac function associated with angiogenic and anti-fibrotic effects. Thus, CSC is a promising cell-source to use with the cell-sheet method for the treatment of cardiac failure, as long as a sufficient number of cells are delivered.

Keywords Cardiac · Stem cell · Myocardial infarction

S. Alshammary · S. Fukushima · S. Miyagawa · T. Matsuda · H. Nishi · A. Saito · S. Kamata · Y. Sawa (✉)
Department of Cardiovascular Surgery,
Osaka University Graduate School of Medicine,
2-2 Yamadaoka, Suita 565-0871, Japan
e-mail: sawa-p@surg1.med.osaka-u.ac.jp

T. Asahara
Institute of Biomedical Research and Innovation, Kobe, Japan

Introduction

The limited regenerative capacity of the myocardium accounts for the fact that cardiac failure related to myocardial infarction (MI) remains a major cause of morbidity and mortality worldwide, despite major advances in medical and/or interventional treatments [1]. The treatment of cardiac failure relies on strategies that are designed to target and/or limit residual or persistent myocardial ischemia, additional myocardial damage, pathological cardiac remodeling, and hemodynamic impairment, including cardiac dyssynchrony [2]. On the other hand, the transplantation of somatic tissue-derived stem/progenitor cells into the heart has been shown to enhance the endogenous healing process of the damaged myocardium, while the magnitude of the therapeutic effects are dependent on the cell-source, cell-number, cell-delivery method, and target cardiac pathology [3–5]. It has been shown that the transplantation of C-kit-positive heart-derived cells into the MI heart yields functional recovery, mediated by proliferation and differentiation into the heart-composing cells in situ, and by releasing cardioprotective factors that activate native healing processes [6]. However, the optimal preparation and delivery method of CSCs into the heart has not been established.

The cell-sheet method, in which aggregated cells in a sheet shape cultured under a thermoresponsive dish are attached to the epicardial surface [7], has been shown to deliver a large scale of cultured cells with minimal damage to the cells or native cardiac tissues [8]. This enhances its therapeutic effects and minimizes inflammation-related

complications, representing a promising cell-delivery method in CSC transplantation therapy [9]. However, there are concerns about potential ischemia of the implanted cell-sheet, which would limit cellular function, survival, and therapeutic potential. According to a previous study, a 3-layered cell-sheet generated by skeletal myoblasts showed greater therapeutic effects than a 1-layered cell-sheet, while a 5-layered cell-sheet did not enhance the effects, possibly because of ischemia in the implanted cell-sheet [10]. Based on the hypothesis that the therapeutic potential of CSC cell-sheet treatment might be dependent on the number of layers of the cell-sheet, we investigated the therapeutic effects of CSC cell-sheet transplantation therapy on MI hearts using a rat model.

Methods

All studies using human tissues and experimental animals were carried out under approval of the institutional ethical committee. Human tissues were collected only after obtaining written informed consent. This investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, revised 1996). All experimental procedures and evaluations were carried out in a blinded manner.

Isolation and culture of C-Kit-positive human cardiac cells and cell-sheet generation

Discarded cardiac tissue samples were taken from the left ventricular apex of a 31-year-old man with dilated cardiomyopathy, requiring daily cardiovascular procedures in Osaka University Hospital. Cardiac cells were dissociated from the tissues, cultured, and then sorted for C-kit using FACSAria (BD Biosciences) to yield C-Kit positive cardiac cells, which were then cultured for expansion with multiple passages. The cells were then incubated in thermoresponsive dishes (35 mm UpCell, CellSeed, Tokyo, Japan) at 37 °C for 2 days prior to transplantation, when the cells were incubated at 25 °C to induce their spontaneous detachment, to yield a mono-layered scaffold-free CSC cell-sheet that included 1.5×10^6 cells (Fig. 1a). The 3-layered cell-sheet was generated by filling up the mono-layered cell-sheet, as described previously [10].

Generation of AMI model and CSC cell-sheet transplantation

Thirty-nine athymic female nude rats, 8 weeks of age, were subjected to permanent ligation of the left coronary artery

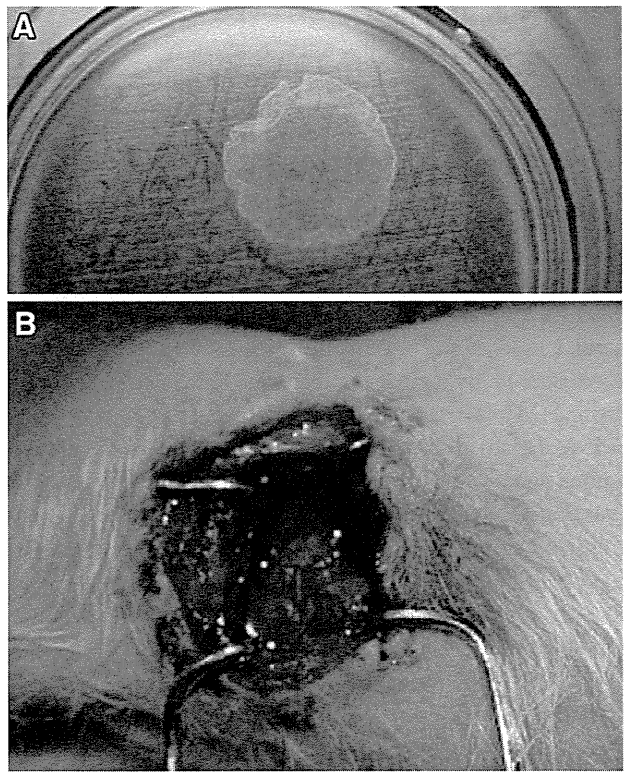

Fig. 1 A mono-layered cell-sheet was generated by c-kit positive cardiac cells of human origin on thermoresponsive dishes in vitro (**a**). A mono-layered or 3-layered cell-sheet was transplanted over the left ventricular free wall of the rat heart, which had been subjected to ischemia by permanent ligation of the corresponding coronary artery, 1 h prior to the treatment (**b**)

(LCA) under general anesthesia with endotracheal intubation and isoflurane inhalation, as previously described [10]. LCA ligation-related death occurred prior to treatment in 16 %. The rats that survived for 50 min after the ligation were randomly assigned to the following three treatment groups: transplantation of a 3-layered cell-sheet ($n = 12$), transplantation of a l-layered cell-sheet ($n = 10$), or a sham operation ($n = 11$). In the two transplantation groups, the cell-sheet was attached directly to the epicardial surface of the ischemic/infarct area (Fig. 1b) [10]. The cell-sheet was large enough to cover all of the ischemic or infarcted area. By 20 min after the transplantation, when the cell-sheets were properly fixed to the cardiac surface, the chest was closed and the rats were allowed to recover in individual temperature-controlled cages until they were killed 28 days after the treatment.

Transthoracic echocardiography

Transthoracic echocardiography was performed under isoflurane inhalation, using a system equipped with a 12 MHZ transducer (GE Healthcare). Diastolic and systolic dimensions of the left ventricular diastolic and

systolic dimensions (LVDD and LVDS, respectively) were measured at the papillary muscle level by the M-mode, while the LV ejection fraction (LVEF) was calculated by the following formula: $(LVDD^3–LVDS^3)/LVDd^3 \times 100$ [10, 11].

Histology

The ventricles were immerse-fixed in 4 % paraformaldehyde, embedded in paraffin, and cut into 5 micrometres using a microtome for histological studies. The sections were stained by hematoxylin–eosin (HE) or Masson trichrome (MT) and assessed by optical microscopy (Olympus, Tokyo, Japan). Metamorph software was used to separate stained and non-stained myocardium by MT staining and to quantitatively calculate each area. The sections were labeled immunohistologically by polyclonal anti-von Willebrand factor antibody (vWF, DAKO, Glostrup, Denmark), and visualized by the LSABTM kit (DAKO), which is an automated immunostaining system based on the LSAB Lepto strept avidin–biotin-peroxidase method. The sections were labeled immunohistologically by the anti-human-specific HLA antibody or anti-cardiac troponin (cTn) I antibody, visualized by corresponding secondary antibodies that were counterstained by DAPI, and assessed by confocal microscopy (Olympus).

Statistics

Values are expressed as mean ± SEM. The three groups were compared with 1-way or 2-way ANOVA as appropriate, followed by the Fisher protected least-significant difference test, or the Kruskal–Wallis test, followed by the post hoc pairwise Wilcoxon-Mann–Whitney U test, as appropriate. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA).

Results

Functional recovery following CSC cell-sheet transplantation

Scaffold-free CSC cell-sheet was prepared from primary C-kit positive cardiac cells of human origin, cultivated in thermoresponsive dishes. We transplanted the 1-layered or 3-layered cell-sheets onto the epicardial surface of the nude rat 1 h after the permanent LCA ligation. A sham operation was performed for the control group. Cardiac performance was serially assessed by transthoracic echocardiography just after the treatment (baseline), and then 1, 2, and 4 weeks after the treatment.

Before any intervention, the LVEF, LVDD, and LVDS did not differ significantly among the groups (Fig. 2). However, for 4 weeks after treatment, the LVEF showed a significantly progressive reduction, while the LVDD and LVDS showed a significantly progressive increase in the sham group and the 1-layer group. Conversely, in the 3-layer group, the LVEF showed a significant increase, and the LVDS showed a significant decrease 2 and 4 weeks following the transplantation, while the LVDD did not change significantly in this group over the 4 weeks. Notably, the LVEF in the 3-layer group was significantly greater than that in the 1-layer group or sham group, while the LVDS in the 3-layer group was significantly lower than that in the 1-layer group or sham group. The LVDD did not differ significantly among the groups at any time.

Histological reverse LV remodeling following CSC cell-sheet transplantation

We assessed gross structure, interstitial fibrosis and capillary distribution in the myocardium 4 weeks after the CSC cell-sheet transplantation to qualitatively and semi-quantitatively explore the degree of LV remodeling in each group by HE staining, Masson Trichrome staining, and immunohistolabelling for von Willebrand factor, respectively. The infarcted area, in which the cell-sheet was transplanted, was clearly thicker in the 3-layer group than in the 1-layer or sham groups, as assessed by the HE staining (Fig. 3a–c). In addition, the myocardial structure in the peri-infarcted area was better preserved in the 3-layer group than in the 1-layer group or the sham group. There seemed to be less accumulation of interstitial fibrosis in the peri-infarcted and infarct-remote myocardium of the 3-layer group than in the 1-layer group or sham groups (Fig. 3d–f). In fact, computer-based morphometry confirmed significantly less fibrosis in the 3-layer groups than in the 1-layer group or sham group (Fig. 4a). Capillary density in the peri-infarcted myocardium was significantly greater in the 3-layer group than in the 1-layer group or sham group (Fig. 4b).

Phenotypic fate of the transplanted CSCs in the heart

The transplanted CSCs in the heart were phenotypically assessed by immunohistolabelling for human-specific HLA, which clearly dissected the transplanted cells in the native cardiac tissue. While the transplanted cells were rarely present in the 1-layer group 4 weeks after transplantation, the 3-layer group showed abundant human-specific HLA-positive transplanted cells in the tissues epicardially attached to the native cardiac tissue, which were assumed to consist of the remaining transplanted cell sheet and accumulated cells of native origin (Fig. 5a).

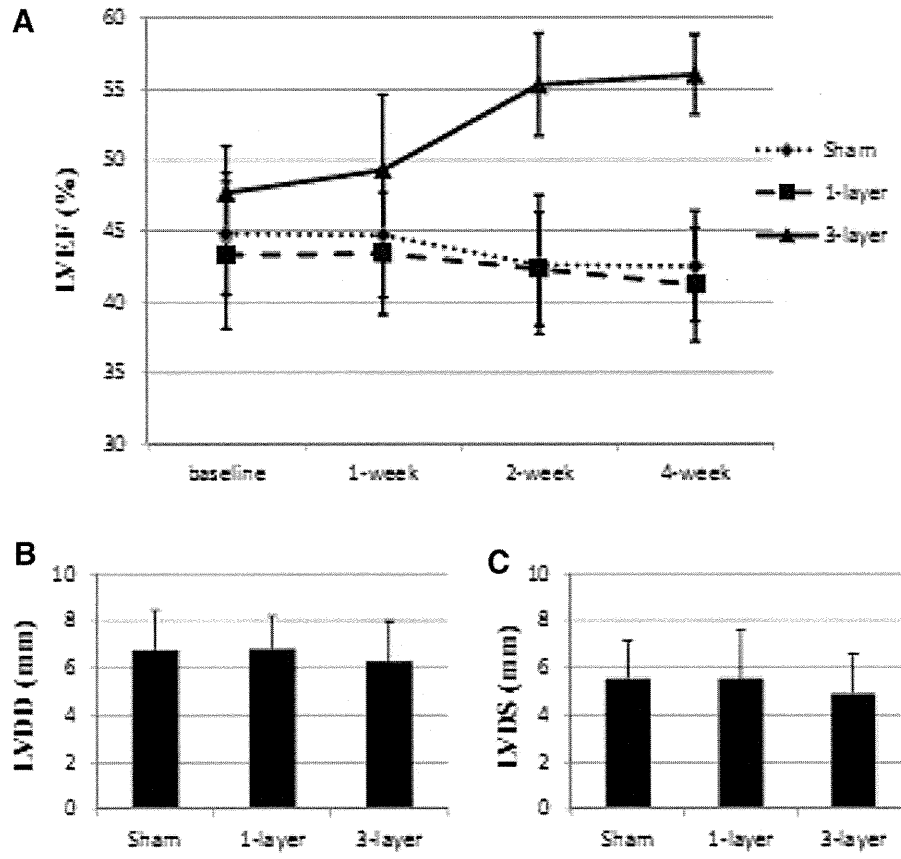

Fig. 2 Cardiac performance measures, such as left ventricular ejection fraction (LVEF) (**a**), LV diastolic dimension (LVDD, **b**), and LV systolic dimension (LVDS, **c**), were assessed echocardiographically immediately after treatment and then 1, 2, and 4 weeks after treatment (sham operation vs. 1-layer cell-sheet transplantation vs. 3-layer cell-sheet transplantation)

Notably, some human-specific HLA-positive transplanted cells were present in the native myocardium, suggesting the migration of transplanted cells into the native cardiac tissue (Fig. 5b–d).

Discussion

This study demonstrated clearly that the transplantation of CSC cell-sheets to treat the MI heart yielded significant recovery of cardiac performance in a cell-sheet layer dependent manner. Consistently, the hearts transplanted with the multi-layered cell-sheet showed significantly more preserved gross myocardial structure, reduced interstitial fibrosis, and increased capillary density than the hearts transplanted with a mono-layered cell-sheet. Moreover, the differentiation of heart-composing cells, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells, was greater in the hearts transplanted with the multi-layered cell-sheet than in those transplanted with the mono-layered cell-sheet.

The transplanted cell-source is known to be a major determinant of the therapeutic effects of cell transplantation therapy for cardiac failure [10–12]. The transplantation of skeletal myoblast transplantation predominates anti-fibrotic effects, whereas that of bone marrow-derived cell transplantation predominates neoangiogenesis in the ischemic/infarcted myocardium. These effects are mediated by indirect effects, in which cell transplantation upregulates a variety of cardioprotective factors to enhance the native healing process, although differentiation of the transplanted cells into the functional heart-composing cells, such as cardiomyocytes or vascular cells rarely occur following the transplantation of skeletal myoblasts or bone marrow-derived cells [13, 14]. In contrast, the transplantation of CSCs has been shown to yield therapeutic effects both directly and indirectly [15, 16]. This study showed that the transplantation of CSCs induced both anti-fibrotic and neoangiogenic effects in a transplanted cell number-dependent manner, indicating that CSCs might have released soluble factors to activate the anti-fibrotic and angiogenic process of the native myocardium following the transplantation. Moreover, the differentiation into the cardiomyocytes and vascular cells, shown in this study, suggests potential direct contribution of these cells to functional recovery, although the magnitude of these direct effects on the global cardiac function remains unclear.

The number of transplanted cells is also an important contributor to the therapeutic effects. Although the cell-sheet method has been shown to deliver more cells into the heart than other delivery methods, such as intramyocardial or intracoronary injection [10], ischemia in the transplanted

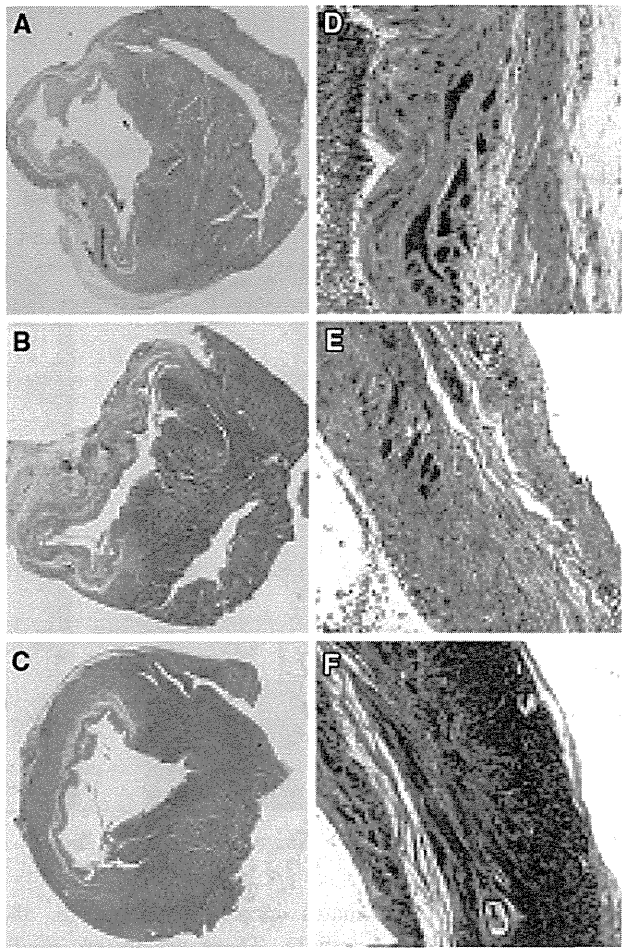

Fig. 3 The gross structure of the heart 4 weeks after treatment was assessed by H&E staining. The sham group (**a**) and the 1-layer group (**b**) showed a large infarcted area in the left ventricular (LV) free wall, but the 3-layer group (**c**) showed a better preserved LV free wall. Interstitial fibrosis 4 weeks after the treatment was assessed by Masson Trichrome staining, which showed more accumulated fibrosis in the sham group (**d**) and the 1-layer group (**e**) than in the 3-layer group (**f**)

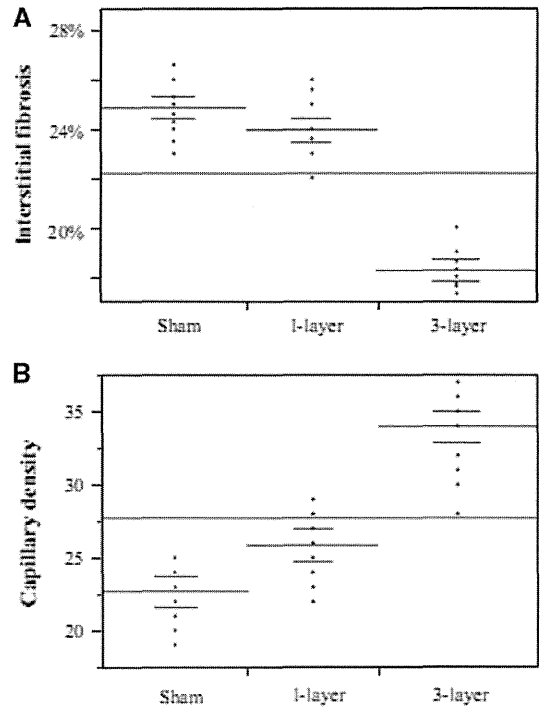

Fig. 4 Masson Trichrome staining revealed a significantly lower percentage of fibrosis 4 weeks after the treatment in the 3-layer group than in the sham or 1-layer groups ($P < 0.05$ vs. the sham and the 1-layer group). Capillary density 4 weeks after treatment, assessed by immunohistochemistry for von Willebrand factor, was significantly greater in the 3-layer group than in the sham or 1-layer groups ($P < 0.05$ vs. the sham and the 1-layer group)

cell-sheet might be a critical limiting factor to the effects. In fact, it was reported that ischemia-related cell-necrosis occurs in the transplanted cells in accordance with the number of cell-sheets filled up [10, 17]. Furthermore, our researchers reported previously that the therapeutic effects of skeletal myoblast cell-sheets increased with the number of layers, but plateaued at five layers, possibly because of ischemia-related functional impairment of the transplanted cell-sheet, although skeletal myoblasts are known to be highly resistant to ischemic stimuli [10, 18, 19]. This study showed that the therapeutic effects of the CSC cell-sheet increased up until three layers, despite poor vascular support after acute infarction of the cell-sheet transplanted area, warranting 3-layered CSC cell-sheet transplantation for treating ischemia-related cardiac failure. Integration of the transplanted CSC cell-sheet into the native myocardium is also a concern of this treatment, as the cell-sheet was simply attached to the epicardial surface. However, this study unveiled that the transplanted cells migrated into the native myocardium and differentiated to heart-composing cells, although the biological mechanisms of this migration process remain unclear.

This study is limited by fact that we used a rodent model transplanted with cells of human origin. The difference in factors related to biological actions between the rat and the human might have modulated the therapeutic effects of this treatment, although a number of previous reports would justify using this model to mimic the clinical scenario [17, 20]. Moreover, using the cells from one patient in the in vivo study might not be appropriate to investigate the effects of CSC of human origin in general, although the cellular behavior did not seem to differ among more than five patients in vitro (data not shown), in accordance with previous reports [21].

In conclusions, the 3-layered cell-sheet improved cardiac function associated with angiogenic and anti-fibrotic effects in a rat model. Thus, the delivery of a sufficient number of CSCs by a cell-sheet method represents a promising treatment for cardiac failure, although further optimization is essential.

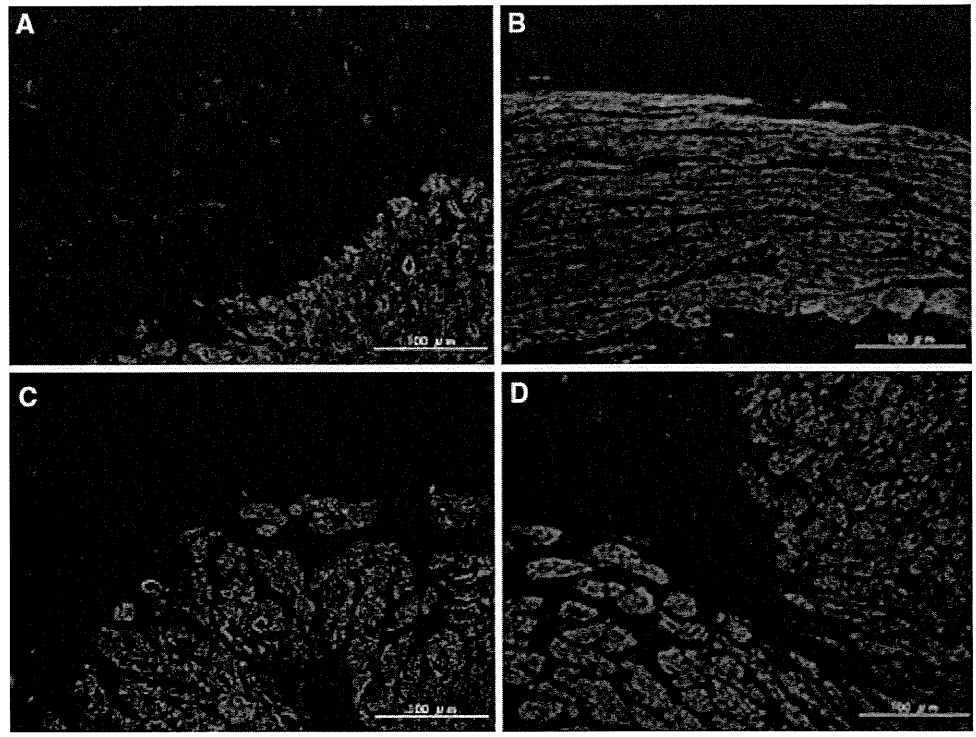

Fig. 5 The presence and distribution of transplanted CSCs of human origin were immunohistologically assessed using human-specific anti-HLA antibody. By 4 weeks after transplantation, the 3-layer group showed abundant human-specific HLA-positive transplanted cells in tissues that were epicardially attached to the native cardiac tissue (**a**). Some human-specific HLA-positive transplanted cells were present in the interstitium of the native myocardium (**b–d**)

Conflict of interest There are no relationships or conflicts of interest related to this manuscript.

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