(SH30406.02, Hyclone, Thermo Fisher Scientific), 0.2 mM L-Glutathione (G6013, Sigma Aldrich), 10 ng/ml recombinant human bFGF (100-18B, PeproTech), and 5 mU/ml erythropoietin (E5627-10UN, Sigma Aldrich) (30).

Preparation of CSC sheet and endothelial progenitor cells

Cultured CSCs were characterized by fluorescence-activated cell sorting (FACS) analysis, labeled by DiI-red (Molecular Probes, Eugene, OR) (33), and then incubated on 10-cm thermoresponsive dishes (Cell Seed Inc., Tokyo, Japan) at 37°C for 12 hours. The DiI-red labeled CSCs spontaneously detached from the dish surface following incubation at 20°C for 30 minutes, yielding a CSC sheet. Each CSC sheet was approximately 42 mm in diameter and 100-μm thick. Granulocyte colony stimulating factor-mobilized EPCs of human origin (AllCells, MPB-017F, Emeryville, CA) were labeled with DiI-blue *in vitro* (Molecular Probes) (33). The following monoclonal antibodies were used: C-kit APC (A3C6E2(clone), 130-091-733, Miltenyi Biotec), CD105 PE (FAB10971P, R&D Systems, Minneapolis, MN), CD34 FITC (555821, BD Biosicences), CD31 PE (FAB3567P, R&D Systems), 7AAD PerCP-Cy5-5 (51-68981E(559925), BD Biosicences), IgG1-FITC isotype controls (555748, BD Biosicences), IgG1-APC isotype controls (130-092-214, Miltenyi Biotec), and IgG1-PE isotype controls (IC002P, R&D Systems).

Generation of the swine chronic ischemic injury model and cell transplantation

A 2.5-mm ameroid constrictor (Tokyo Instruments, Inc, Tokyo, Japan) was placed around the proximal left anterior descending artery *via* a left thoracotomy in female swine (Clawn miniature, 1-year-old, 25kg) (Japan Farm, Inc, Kagoshima, Japan). A total of 65 swines were

then cared for in a temperature-controlled individual cage with a daily intake of 5 mg/kg cyclosporin A (Novartis, East Hanover, NJ) (15). Multi-detector CT identified 52 pigs that developed an LVEF between 30 and 40% at 21 days post-ameroid placement. Since eight of these pigs died prior to cell transplantation, so a total of 44 pigs were randomly divided into 4 treatment groups (n=11 in each): sham operation (sham group), CSC-sheet transplantation only (CSC-only group), intramyocardial injection of EPCs (EPC-only group), and CSC-sheet transplantation plus EPC injection (CSC-EPC group). After a median sternotomy under general anesthesia, 3-layered CSC sheet (total 1×10^8 cells) was placed on the epicardium of the ischemic area (LAD region), and stitched in place around the edge. EPCs (total 2.5×10^6 cells) were intramyocardially injected into 12 different sites of the ischemic and peri-ischemic area. After the transplantation and/or intramyocardial injection was completed, the pericardium was closed. The pigs were taken care of for 1 day (n=1 each), 3 (n=4 each) or 8 weeks (n=6 each), when they were sacrificed in a humane manner.

Continuous electrocardiogram monitoring

The electrocardiogram (ECG) was monitored during 5 days post-treatment with the Holter system (Unique Medical Co, Tokyo, Japan) for swines sacrificed at 8 weeks after cell transplantation (n=6 each group). The heart rate and arrhythmia events during the first 24 hours were analyzed using software (Softron Co, Tokyo, Japan).

Multi-detector CT and conductance catheterization

Global LV function was assessed by multi-detector CT at pre-treatment and 8 weeks post-treatment (n=6 each), and by cardiac catheterization at 8 weeks post-treatment (n=5 each).

After infusing 45 mL of nonionic contrast agent (Iomeron 350, Eisai, Co, Tokyo, Japan) *via* the ear vein, 5-mm slice images of the entire heart were obtained in the cranio-caudal direction using a 16-slice CT scanner (Emotion 16, Siemens, Tokyo, Japan). Every 10% of the R-R interval was reconstructed to calculate the LVEF and end-diastolic/systolic volume (EDV and ESV, respectively) using software (Lexus, Aze Inc, Tokyo, Japan).

Pressure-volume (P-V) cardiac catheterization was performed after median sternotomy by inserting a conductance catheter (Unique Medical) and a Micro Tip catheter transducer (SPR-671; Millar Instruments, Inc, Houston, Tex) into the LV cavity. The P-V loop data under stable hemodynamics or inferior vena cava occlusion were analyzed with Integral 3 software (Unique Medical).

Speckle-tracking echocardiography and myocardial contrast echocardiography

Short-axis echocardiographic images, obtained using the Artida 4D Echocardiography System (Toshiba Medical Systems Co, Tochigi, Japan) and PST-30SBT transducer, were analyzed by the speckle-tracking method using wall motion tracking software (Toshiba Medical Systems) (2,4). End-systolic radial strain (RS) values at the mid and apical levels were averaged in a layer-specific manner to measure the endocardial and epicardial wall motion index (WMI), respectively.

Myocardial contrast echocardiography was performed using real-time contrast pulse sequencing operating on the Aplio ultrasound system (Toshiba Medical Systems) (8). Briefly, after an intravenous injection of 20 mL of ultrasound contrast agent (Sonazoid, Daiichi Sankyo Inc, Parsippany, NJ), images in the apical 2-chamber view were acquired to score the myocardial opacification using Volmac software (YD Ltd, Nara, Japan).

Histology

Cultured CSCs on 8-well Lab-Tec chamber slides were fixed with 4% paraformaldehyde, labeled, and examined by confocal microscopy (FV300, Olympus, Tokyo, Japan). AlexaFluor-488 Phalloidin (Molecular Probes) was used to enhance the background actin filaments. Paraffin-embedded transverse sections at the papillary muscle level was stained with Masson's trichrome, and the amount of interstitial collagen at the entire LV wall was semi-quantified by MetaMorph software (Molecular Devices, Sunnyvale, CA) (n=6, in each). In addition, the thickness of the ventricular wall was measured at two points from the LV posterior area and two points from the interventricular septum, and results were expressed as the average of the four points. Five-micrometer cryo-sections were subjected to either periodic acid-Schiff (PAS) staining or immunohistolabeling. The following primary antibodies were used: rabbit anti-c-kit (Dako, Co, Glostrup, Denmark), mouse anti-smooth muscle actin (Dako), rabbit anti-von Willebrand factor (vWF) (Dako), mouse anti-ki67 (Dako), rabbit anti-connexin 43 (Sigma Aldrich), mouse anti-cardiac troponin I (cTn-I) (Abcam, Co, Cambridge, UK), mouse anti-stromal cell-derived factor 1 (SDF-1) (Abcam), rabbit anti-vascular endothelial growth factor (VEGF) (Thermo Scientific), rabbit anti-insulin-like growth factor 1 (IGF-1) (Abcam). Capillary density was expressed as the average number of vWF-positive circular structures in five randomly selected sections, corrected for the total area of the tissue section measured. PAS-stained sections were used to determine the cell diameter of the cardiomyocytes at the remote zone. Dil-red positive cells were traced by MetaMorph software to quantify the area of engrafted clusters of CSC sheet.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the CSCs and the swine heart tissues post-treatment using an RNeasy Kit (Qiagen, Hilden, Germany), then reverse-transcribed using Omniscript Reverse Transcriptase (Qiagen) and amplified using the Gene Amp (R) PCR System 9700 (Life Technologies, Tokyo, Japan). The primer pairs were as follows: human-specific KDR primer sequence, sense CCT CTA CTC CAG TAA ACC TGA TTG GG, antisense TGT TCC CAG CAT TTC ACA CTA TGG; humam-specific CXCR4 primer sequence, sense ACG TCA GTG AGG CAG ATG, antisense GAT GAC TGT GGT CTT GAG; human GAPDH primer sequence, sense AAT GGG CAG CCG TTA GGA AA, antisense GCG CCC AAT ACG ACC AAA TC; swine-specific SDF-1 primer sequence, sense CCG AAC TGT GCC CTT CAG AT, antisense ATA AAC ATC CCG CCG TCC TC; swine-specific CXCR-4 primer sequence, sense GCG CAA AGC TCT CAA AAC CA, antisense CAG TGG AAA AAG GCA AGG GC; swine-specific VEGF primer sequence, sense GAC GTC TAC CAG CGC AGC TAC T, antisense TTT GAT CCG CAT AAT CTG CAT G; swine-specific IGF-1 primer sequence, sense ACA TCC TCT TCG CAT CTC TTC TAC TT, antisense CCA GCT CAG CCC CAC AGA; swine GAPDH primer sequence, sense CTG CAC CAC CAA CTG CTT AGC, antisense GCC ATG CCA GTG AGC TTC C. The transcript level of gapdh was used as an endogenous reference. The products from the cultured CSCs were stained with ethidium bromide, separated by electrophoresis on an agarose gel, and quantified.

Fluorescence in-situ hybridization (FISH)

Paraffin-embedded sections were predenatured, dehydrated, and then labeled with deoxyribonucleic acid probes, a Cy3-conjugated probe for human-specific genome, Cy5-

conjugated probe for swine-specific genome (Chromosome Science Laboratory, Hokkaido, Japan), and mouse anti-cTn-I or rabbit anti-vWF. The sections were visualized with secondary antibodies conjugated to Alexa fluorochromes. Nuclei were labeled with 4,6-diamino-2-phenylindole (DAPI, Molecular Probes).

Statistical analysis

Continuous data are summarized as medians with ranges (minimums to maximams) or means ± SEM (standard error of mean) and are plotted in figures with raw values or barplots of the means with symmetric SEM bars, as appropriate. Distributions of the continous data were checked for normality with the Shapiro-Wilk test and for equality of between-group variances with the Levene test. Normally distributed data were compared between four groups using the analysis of variance (ANOVA), followed by the Tukey multiple comparison for equal variances, or Welch's ANOVA, followed by the Games-Howell multiple comparison for unequal variances. Non-normally distributed data were compared using the Kruskal-Wallis test, followed by the Steel-Dwass multiple comparison. Normally and Non-normally distributed data before and after treatment were compared using the paired t-test and the Wilcoxon signed rank-sum test, respectively. All P values are 2-sided, and values of P less than 0.05 were considered to indicate statistical significance. All analyses was performed with the SPSS 11.0J for Windows (SPSS, Chicago, IL) and the R program (10).

RESULTS

Human atrium-derived c-kit-positive cells showed CSC characteristics *in vitro*

The isolated right-atrium-derived cells were characterized *in-vitro* by FACS, immunohistolabeling, and RT-PCR analyses. The proportion of C-kit-positive cells at the second passage was 99±4% (Figure 1A and 1B). However, as the cells expanded, they lost the primitive phenotype, and more than half of them had the potential to differentiate to the endothelial rather than the cardiomyocyte or smooth muscle cell lineage. The proportion of c-kit-positive cells at the fifth passage was 20±10%, while 34%, 71%, and 99% of them expressed CD34, CD31, and CD105, respectively (Figure 1C). Immunohistochemistry revealed that approximately 5% of the cells expressed cTn-I in the cytoplasm (Figure 1D). In addition, RT-PCR clearly revealed the expression of CXCR4 and KDR in the cells (Figure 1E).

After incubating the cells on temperature-responsive dishes at 37°C for 12 hours, CSC sheets were generated by lowering the temperature. Each cell sheet was approximately 42 mm in diameter, and the gap-junction protein connexin 43 was expressed between the cells (Figure 1F). Most of the cells expressed the proliferation marker Ki67 in their nucleus (Figure 1G).

Successful cell transplantation with minimal arrhythmogenicity

A total of 44 pigs underwent treatment, which was performed without any procedure-related mortalities (n=11 each). Fatal arrhythmias, such as ventricular tachycardia and fibrillation, or composite ventricular arrhythmias (grades 3-5 in Lown's classification) were not detected in

any group during the first 24 hours post-treatment, as assessed by Holter ECG monitoring (n=6 each) (Table 1). There were no significant differences in the heart rate or number of unifocal premature ventricular/atrial contractions among the groups.

Global functional recovery after CSC-sheet transplantation was enhanced by EPC injection

Multi-detector CT measured LVESV, LVEDV, and LVEF, and cardiac catheterization measured the systolic parameters dP/dt max, end-systolic pressure-volume relation (ESPVR), and diastolic parameters: τ, end-diastolic pressure (EDP), and dP/dt min. There were no significant differences in LVESV, LVEDV, or LVEF among the groups pre-treatment (Figure 2A to 2C). The pigs treated with the sham operation developed increases in LVESV ($P<0.001$) and LVEDV ($P<0.001$) but not in LVEF (34±5 to 32±6 %, $P=0.44$) in 8 weeks. In contrast, the pigs in the EPC-only or CSC-only group showed preserved LVESV (EPC-only, $P=0.23$; CSC-only, $P=0.89$), LVEDV (EPC-only, $P=0.15$; CSC-only, $P=0.13$), and LVEF (EPC-only, 37±5 to 37±4, $P=0.91$; CSC-only, 36±4 to 43±5 %, $P=0.12$). Moreover, the combined treatment induced a significant decrease in LVESV ($P=0.04$) and a significant increase in LVEF (36±5 to 53±3 %, $P=0.001$) in the 8 weeks following cell transplantation.

At 8 weeks post-treatment, the LVESV of the CSC-only and EPC-only groups was significantly smaller than that of the sham group, and the LVESV of the CSC-EPC group was even smaller than that of the EPC-only group ($P<0.001$ for CSC-EPC, CSC-only, and EPC-only vs. sham, and for CSC-EPC vs. EPC-only). The LVEDV of the CSC-EPC, CSC-only, and EPC-only groups was significantly smaller than that of the sham group ($P=0.001$ for CSC-EPC, CSC-only, and EPC-only vs. sham). The LVEF of the CSC-only group was

significantly greater than that of the sham group, while the LVEF of the CSC-EPC group was even greater than that of the other groups ($P<0.001$ for CSC-EPC vs. CSC-only, EPC-only, and sham, and for CSC-only vs. sham). Moreover, the 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 (Figure 2D).

The dP/dt max, τ, EDP, and absolute value of dP/dt min were significantly greater in the CSC-EPC group than in the CSC-only and EPC-only groups, and these values except for the absolute value of dP/dt min were significantly greater in the CSC-only and EPC-only groups than in the sham group (Figure 2E).

Differential region- or layer-specific effects by CSC and EPC transplantation

Region- or layer-specific systolic LV function was assessed using speckle-tracking echocardiography, which was carried out at pre-treatment and at 4 and 8 weeks post-treatment (n=6). There was no significant difference in the endocardial or epicardial wall motion index in the ischemic anterior area among the groups at pre-treatment (Figure 3A and 3B).

The pigs given the sham operation developed a significant decrease in the epicardial wall motion index (10±2 to 7±1 %, P=0.01) and no change in the endocardial wall motion index (10±1 to 9±1 %, P=0.40) in 8 weeks. In contrast, cell transplantation induced a significant increase in epicardial (CSC-only, 10±2 to 18(14-28), P=0.03; CSC-EPC, 10±1 to 20±5 %, P=0.01) and endocardial (EPC-only, 10±1 to 12±1, P=0.01; CSC-EPC, 10±1 to 14±1 %, P=0.001) indices at 8 weeks.

At 8 weeks post-treatment, the epicardial wall motion index of the CSC-EPC and CSC-only groups was significantly greater than that of the EPC-only or sham group ($P<0.001$ for CSC-EPC and CSC-only vs. EPC-only and sham) (Figure 3A and 3C). In contrast, the endocardial wall motion index of the CSC-EPC group was the greatest followed by that of the EPC-only group, and both were significantly greater than that of the CSC-only or sham group ($P<0.001$ for CSC-EPC vs. EPC-only vs. CSC-only and sham) (Figure 3B and 3D).

Consistent with these findings, the myocardial perfusion score in the ischemic zone at 8 weeks post-treatment was significantly greater in the CSC-EPC (36±1 dB) than in the sham group (19±2 dB, $P<0.001$) (Figure 3E and 3F).

Engraftment of transplanted CSCs and EPCs, and neovascularization of the infarcted wall

The engraftment of transplanted DiI-red-labeled CSCs and DiI-blue-labeled EPCs was examined, and the capillary density was assessed by fluorescence-based immunohistolabeling for vWF. At 3 weeks after cell transplantation, most of the transplanted CSCs were present over the ischemic area in the sheet shape, although no DiI-red- or DiI-blue-positive cells could be seen in any heart slice at 8 weeks post-treatment (Figure 4A and 4B). 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 (CSC-EPC, 251±84; CSC-only, 257±43; EPC-only, 105±20; sham, 112±23 /mm^2; $P=0.001$ for CSC-EPC and CSC-only vs. EPC-only and sham) (Figure 4C). In contrast, 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 or sham group (CSC-EPC, 269±11; EPC-only, 193±14; CSC-only, 108±30; sham, 74±38 /mm^2; P<0.001 for CSC-EPC vs. EPC-only vs. CSC-only and sham). In association with these findings, some of the transplanted CSCs in both the CSC-EPC and CSC-only groups were observed in native epicardial tissues, while some had migrated into the endocardial tissues only in the CSC-EPC group (Figure 4D). On the other hand, transplanted EPCs in both the CSC-EPC and EPC groups were observed in the ischemic and peri-ischemic area of the endocardium, especially near the vascular wall.

To elucidate the possible mechanism for the transplanted CSC migration, RT-PCR and immunostaining for angiogenic growth factors were performed (n=4 each). The mRNA levels of swine-specific SDF-1, VEGF, and IGF-1 were up-regulated in all the treatment groups (Figure 5A). In particular, the mRNA levels of SDF-1 and the SDF-1 receptor CXCR4, were markedly greater in the CSC-EPC group than in the other groups (SDF-1 or CXCR4: P<0.001 for CSC-EPC vs. CSC-only, EPC-only, and sham). In addition, SDF-1, but not VEGF or IGF-1, was expressed in the cytoplasm of the transplanted CSCs that were present in the native myocardial tissue in the CSC-EPC group (Figure 5B).

Preserved myocardial integrity 8 weeks after cell transplantation

Interstitial fibrosis and capillary density in the heart at 8 weeks post-treatment were assessed by MT (Figure 6A) and PAS (Figure 6B) staining and immunohistolabeling for vWF (Figure 6C), respectively (n=6 each). Excluding the sham group, there were no significant difference in infarct area among the other groups. On the other hand, the accumulation of collagen or area of chronic ischemic injury was the smallest in the CSC-EPC group followed by the CSC-only and EPC-only groups, and then by the sham group (CSC-EPC, 7±1; CSC-only,

15±3; EPC-only, 20±3; sham 28±5 %; P<0.001 for CSC-EPC vs. CSC-only and EPC-only vs. sham) (Figure 6D). The LV wall thickness was significantly larger in the CSC-EPC group than in the sham group (CSC-EPC, 9±1; sham 7±1 mm; P<0.01 for CSC-EPC vs. sham) (Figure 6E).

The myocyte cell diameter at 8 weeks post-treatment was assessed in the 5-mm heart sections of the remote zone stained by PAS (n=6 each). The myocyte diameter was significantly smaller in the CSC-EPC group than in the CSC-only, the EPC-only, or the sham group (CSC-EPC, 33±9; CSC-only, 49±10; EPC-only, 55±8; sham 61±8 mm; P<0.001 for CSC-EPC vs. CSC-only, EPC-only, and sham) (Figure 6F).

The number of vWF-positive capillaries in the peri-ischemic zone of the CSC-only or EPC only group was greater than that of the sham group, and the number of the CSC-EPC group was even greater than that of the EPC-only or sham group. (CSC-EPC, 83±11; CSC-only, 74±6; EPC-only, 62±8; sham 32±10 /mm^2; P<0.001 for CSC-only and EPC-only vs. sham, and for CSC-EPC vs. EPC-only and sham) (Figure 6G). In addition, greater vessel formation with vascular lumen was induced in the CSC-EPC group compared with the other groups.

Phenotypic fate of transplanted CSCs and EPCs post-transplantation

The phenotype of the transplanted CSCs and EPCs in the native myocardium at 8 weeks post-transplantation was histologically assessed by human- and/or swine-specific genome-based FISH analysis. Small numbers of cardiomyocytes and endothelial cells with a human genome in the nucleus were present in the native myocardium of both the CSC-EPC and CSC-only groups, but not in the EPC-only group (Figure 7A and 7B). However, there was no

difference in the number of cells with a human genome between the CSC-EPC and CSC-only groups. Of note, all the cells with a human genome in the nucleus also carried a swine genome in the nucleus, which thus had chimeric nuclei (Figure 7C and 7D).

DISCUSSION

We here demonstrated that primary c-kit-positive CSCs were successfully isolated from the human right atrium and expanded, and showed that they had a differentiation potential for endothelial rather than cardiomyogenic lineages, *in vitro*. Human CSC-sheets were successfully transplanted into the swine chronic ischemic injury heart with minimal arrhythmogenicity, and elicited global functional recovery in 8 weeks. CSC-sheet transplantation concomitant with intramyocardial EPC injection showed enhanced global functional recovery compared with the CSC sheet-only therapy. Transplantation of the CSC sheet alone induced histological reverse LV remodeling, including attenuated interstitial fibrosis and increased vessel numbers, and concomitant EPC injection induced greater reverse LV remodeling effects than the CSC sheet-only therapy. Most of the transplanted CSCs were engrafted onto the surface over the ischemic area, while a small number migrated into the epicardium following the transplantation of the CSC sheet only. In contrast,

concomitant EPC injection enhanced the migration of the transplanted CSCs into the myocardium, in association with local up-regulations of SDF-1, VEGF, and IGF-1. While the CSC of human origin in this study rarely differentiated into the cardiomyocytes in a porcine heart, transplantation of the CSC induced functional and pathological recovery, suggesting that paracrine effects are the major therapeutic mechanisms in this study. Importantly, speckle-tracking echocardiography and histological data indicate that the CSC and the EPC produced different paracrine effects in the failing heart, suggesting synergic effects of these two cell-types as shown in this study.

Clinical cell therapy has been reported to yield only modest functional recovery, as assessed by standard echocardiography (19,22). However, the latest echocardiographic methods, such as strain and contrast echocardiography may better dissect layer-specific regional cardiac functions or myocardial perfusion, leading to a clearer understanding of the functional benefits of cell therapies. The adult mammalian heart is formed in three layers: contractile myocardium, inner endocardium, and outer epicardium. The layers differ in their contribution to cardiac performance and biological function (18). Myocardial infarction affects these myocardial layers to different extents, and ischemic change occurs in a wavefront pattern from the endocardium to the epicardium. In particular, the epicardium is thought to have a rich cardiac progenitor cell niche and to play an important role in cardiac repair (18,24,34). Of note, it has been shown that cell-sheet implantation into the epicardium induces the expression of multiple cardioprotective factors in the heart, and activates host epicardial cells crucial for cardiac repair through 'cross-talk' between the implanted cell sheet and the native epicardium (20,33). This delivery method has also been demonstrated to maximize the retention and survival of the transplanted cells and to minimize the risks of

cell-delivery-related myocardial damage (23,27). Thus, we believed that the transplantation of CSCs by the cell-sheet technique might have greater therapeutic effects in a swine chronic ischemic injury model than other delivery methods.

Our layer-specific analysis by strain echocardiography revealed that the CSC-sheet transplantation induced significant functional improvement in myocardial function only in the ischemic epicardium. Consistent with this result, epicardial CSC-sheet implantation induced neovascularization and reduced fibrosis in a paracrine manner in this area. We also observed the migration of transplanted CSCs into the native ischemic epicardium, which suggested that these functional and morphological benefits might be associated with the location of transplanted CSCs in the host myocardium. Previous reports demonstrated that the SDF-1-CXCR4 axis plays an important role in the migration of bone-marrow and cardiac stem cells from the cell sheet to the infarct myocardium (17,28,29). Our data suggested that since migrated CSCs were located in the vicinity of SDF-1 in the host tissue, SDF-1 present in the ischemic tissue might promote the cell migration from the cell sheet to the native ischemic tissues. The strong expression of CXCR4 in the CSC sheet *in vitro* might support this scenario. In fact, the expressions of angiogenic growth factors, including SDF-1, in the chronic ischemic tissues were markedly diminished in the sham group, although these cytokines and adhesion molecules, which play important roles in cardiac repair after injury, are known to be abundant in ischemic tissues at the acute stage of myocardial infarction (1). Thus, these microenvironmental factors in the host cardiac tissue might not greatly contribute to the therapeutic efficacy of the CSC sheet for the chronic ischemic injury model.

In contrast to CSC-sheet transplantation, intramyocardial EPC injection significantly enhanced the improvement in myocardial function only in the ischemic and peri-ischemic

endocardium, and not in the epicardium, in a paracrine manner. Urbich et. al. reported that soluble factors released by EPCs promote the migration of cardiac-resident progenitor cells, using an *in-vitro* migration assay (31). Thus, we considered that EPCs intramyocardially injected into the ischmic endocardium might promote the host tissue's expression of the angiogenic cytokine SDF-1, consequently enhancing the therapeutic efficacy of CSC sheet transplantation by improving the migration of the transplanted CSCs into the native ischemic myocardium.

The graft rate of transplanted EPCs by intramyocardial injection was lower than that of transplanted CSCs by the cell-sheet technique. Transplanted CSCs were uniformly identified in the ischemic epicardium, whereas transplanted EPCs were densely located within the vascular wall of the ischemic and peri-ischemic endocardium. These different graft rates and patterns of transplanted cells might be related to the greater improvement in ESPVR and lower frequency (although not significant) of premature ventricular contraction seen in the animals receiving CSC-sheet implantation alone compared with intramyocardial EPC injection alone, as previously described (20,26). One might think that EPCs could be mixed into the CSC sheet to enhance the CSCs' function; however, we have not been able to generate cell sheets from such mixed cultures. In addition, the injection of EPCs into the endocardial area might be important for attracting the migration of CSCs from the surface to the endocardial area.

In this study, contrast echocardiography dissected the improved myocardial perfusion that was seen 8 weeks after cell transplantation in all the cell therapy groups, but not the sham group. It has been shown that myocardial perfusion is impaired in chronic ischemic cardiomyopathy, and that improved myocardial perfusion is associated with a suppression of

LV remodeling (8). These findings indicate that the therapeutic efficacy in all the cell therapy groups might have been mainly attributable to the suppressive effects on LV remodeling. Consistent with this idea, multi-detector CT showed that the increased rate of EDV between before and 8 weeks after treatment was less than 20% in all the cell therapy groups, but not in the sham group. On the other hand, invasive hemodynamic assessment using a conductance catheter also showed that CSC-sheet implantation or the intramyocardial injection of EPCs significantly improved measures of LV diastolic functions, such as τ and EDP, compared with the sham operation. Previous reports demonstrated that the stiffness of the infarcted myocardium plays an important role in the postinfarction remodeling process (25). Thus, the different types of cell therapy we used may have had a softening effect on the scar tissue by increasing the cellularity, leading to a more pliable scar and less global cardiac remodeling.

In this study, *in-vivo* CSC-sheet implantation or concomitant EPC injection rarely induced the differentiation of transplanted CSCs into cardiomyogenic or vasculogenic lineages. Instead, all of the cardiac protein-expressing transplanted cells arose from fusion with existing myocytes or endothelial cells. Several lines of evidence support the idea that differentiation potential can be altered by differences in the severity of the pretreated infarct, as well as the timing of cell transplantation (20,21). In particular, Matsuura et al. reported that in viable ischemic tissues with existing cardiomyocytes, CSCs are likely to differentiate into myocytes or vascular cells *via* a cell fusion-dependent mechanism (20). Our layer-specific regional functional analysis by strain echocardiography identified residual viability in the ischemic area just before the cell therapies, and these conditions might have limited the differentiation potential of the CSC sheet *in vivo* and the ability of additional EPC injection to promote the differentiation of CSCs into the cardiomyogenic or vasculogenic lineages.

A potential limitation of this study is that the human c-kit-positive CSCs were obtained from a single donor. However, the isolation and culture methods for the primary c-kit-positive CSCs used in this study were previously established to yield CSCs of consistent functionality regardless of the donor (30). The findings of this study are therefore likely to be consistent with those obtained using CSCs from a different donor. In addition, although the swine were immunosuppressed by a previously reported regimen (15), xenogeneic cell transplantation might limit or exaggerate the therapeutic efficacy of the treatment. However, our analysis of layer-specific regional function clearly demonstrated the previously undescribed therapeutic benefit that additional EPC injection improves the CSC-sheet therapy for the swine ischemic injury model.

In summary, CSC transplantation by the cell-sheet technique prevented LV remodeling, through increased neovascularization and reduced fibrosis in a paracrine manner, and consequently improved the global LV function, and these effects were further enhanced by combination therapy with EPC injection. Our layer-specific strain analysis revealed that CSC-sheet implantation improved regional wall motion in the epicardium, but not the endocardium. However, concomitant EPC transplantation significantly enhanced the therapeutic potential of CSC-sheet therapy in endocardium. Concomitant EPC injection promoted migration of transplanted CSCs into the host myocardium, which might contribute to enhancement of functional recovery in the endocardium by the combination therapy. The combination of CSC-sheet implantation and intramyocardial EPC injection may represent a promising strategy for ischemic cardiomyopathy.

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Mr. Akima Harada is a full-time employee of CellSeed Inc, Japan. Mr. Tatsuya Shimizu and Mr. Teruo Okano are members of the scientific advisory board of CellSeed Inc, Japan.

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