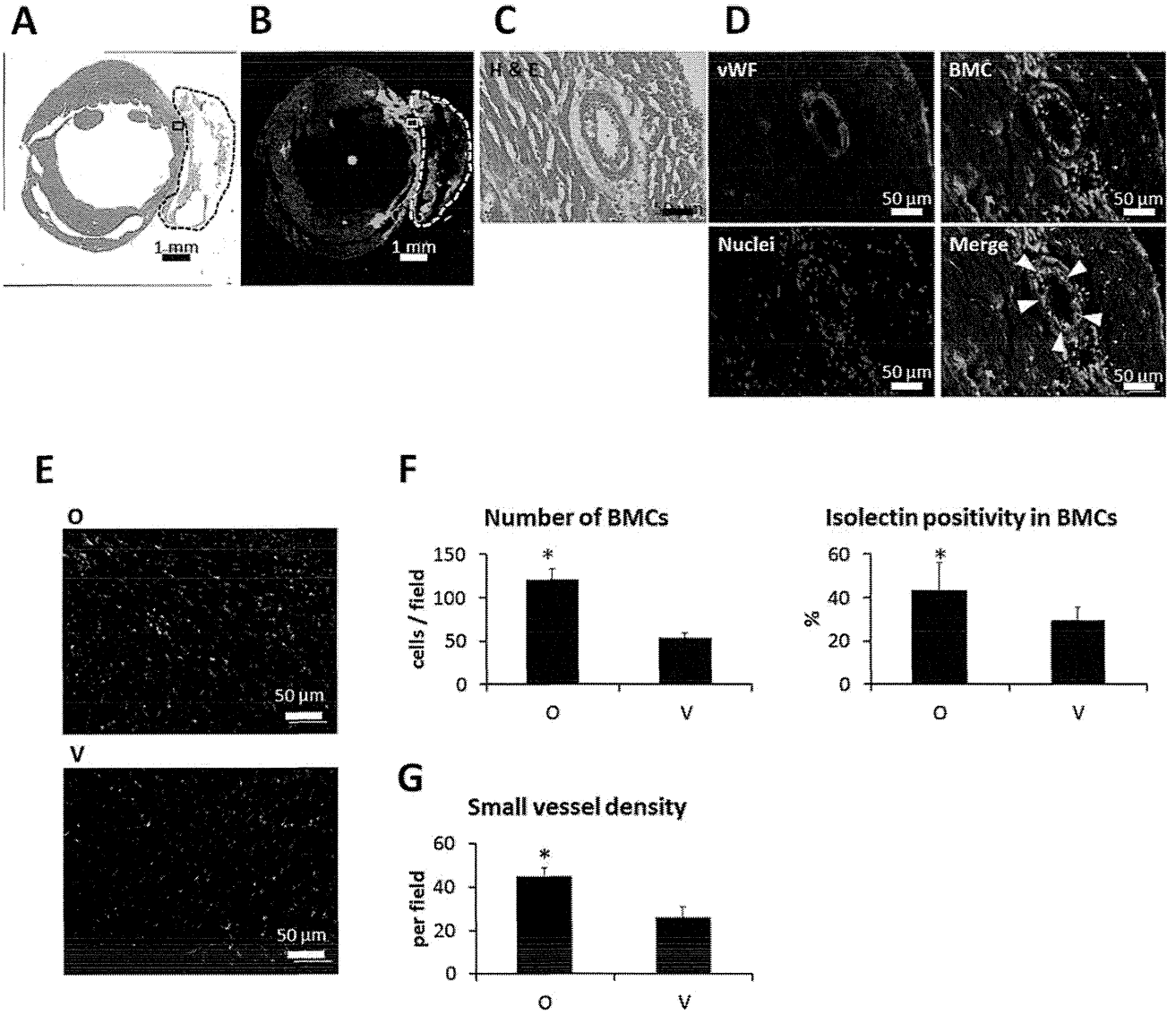

Figure 3. BMCs differentiated into capillary structures in the infarcted area after MI and ONO-1301 treatment. Representative macro image of H and E staining seven days after MI and ONO-1301 treatment. The transplanted sheet is enclosed by a dashed line. A) Serial section of A. The BMCs displayed GFP. B) High-magnification image of the boxed region in A. C) Serial section of C. Arrowheads indicate vWF-expressing BMCs. Red indicates vWF; green, BMCs; and blue, nuclei. D) Representative images of isolectin-stained BMCs seven days after MI and ONO-1301 treatment. E) BMC accumulation and percentages of isolectin-positive BMCs. The number of BMCs that accumulated in the infarcted myocardium was greater in the ONO-1301-treated (O) group than in the vehicle (V) group. The percentage of isolectin-positive BMCs was also greater in the O group than in the V group. *$P<0.05$ vs. V group. F) Small vessel density. Small vessels were detected by CD31 immunostaining. The density of small vessels in the O group was greater than in the V group. *$P<0.05$ vs. V group.
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It is difficult to understand the whole mechanism underlying the functional improvements induced by ONO-1301. It was already reported that ONO-1301 enhances the expression of angiogenic factors HGF and VEGF, leading to angiogenesis and the suppression of fibrosis progression [7,8,9]. In this study, we discovered an alternative mechanism for ONO-1301's therapeutic efficacy in the acute MI mouse, in which the upregulation of SDF-1 promotes BMC accumulation. Stem-cell recruitment and homing are regulated by the interplay of cytokines, chemokines, and proteases. In particular, the SDF-1/CXCR4 axis is central for the mobilization of stem cells from the bone marrow and their homing to ischemic tissues [12]. In the case of ischemic insult, SDF-1 is released by the injured tissue and stimulates the mobilization of progenitor cells from the bone marrow [1,13]. Furthermore, prostaglandins have been reported to facilitate BMC mobilization via upregulation of CXCR4 expression [14,15]. In our experimental setting, ONO-1301 was detected from peripheral blood samples 3 weeks after treatment (Fig. S4 in File S1), suggesting that ONO-1301 may similarly act on the bone marrow to promote the BMC mobilization. Thus, BMC recruitment in the injured myocardium may be enhanced by the upregulation of SDF-1 in cardiac fibroblasts and by the direct upregulation of CXCR4 in BMCs located in the bone marrow. In addition, recent reports show the possibility of endogenous regeneration in the injured heart, including proliferation of postnatal cardiomyocytes and cardiac stem cells [16,17,18,19]. While we were unable to

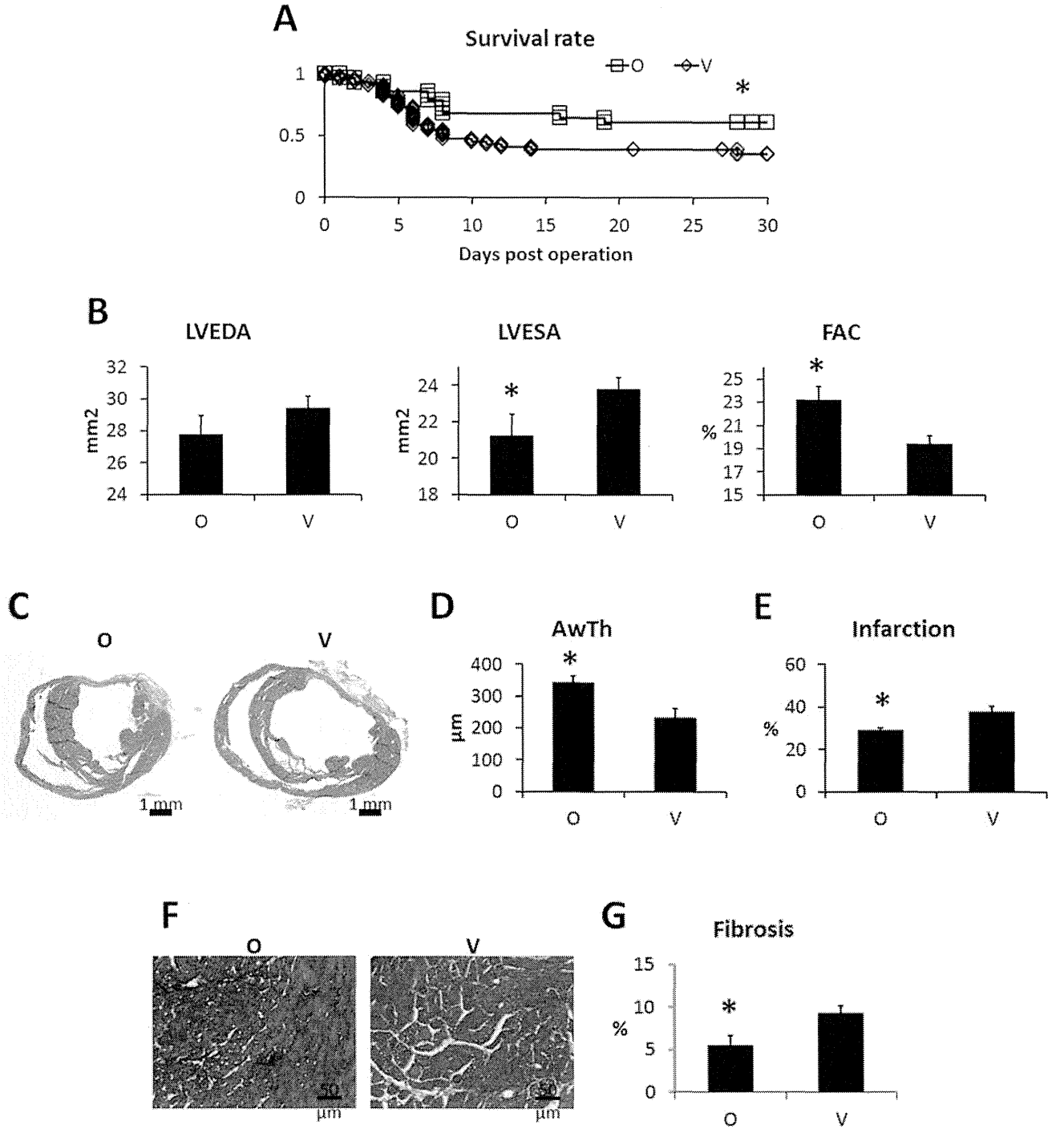

Figure 4. ONO-1301 treatment improved the cardiac performance and survival rate after MI. Survival rates after treatment. The ONO-1301-treated (O) group (n = 33) showed significantly better survival than the vehicle (V) group (n = 48). *$P<0.05$ vs. V group. A) Evaluation of cardiac performance 4 weeks after treatment. In the O group, the LVESA was smaller, and the FAC was significantly higher compared to the V group (O group, n = 22; V group, n = 20; *$P<0.05$ vs. V group). B) Representative macro images from each group. C) Quantification of anterior wall thickness. Anterior wall thickness was significantly thicker in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. D) Quantification of percent infarction. Infarction was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. E) Representative Masson trichrome staining images at the border zone. F) Quantification of fibrosis. Fibrosis at the border zone was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group.
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detect newly-generated cardiomyocytes derived from BMCs in this study, it would be interesting to evaluate the possibility of cardiomyogenesis involving other cell types.

We observed massive BMC accumulation 7 days after MI, including in the infarcted ventricular wall, where they provided structural support in place of the necrotic cardiomyocytes. The

BMCs recruited into the infarcted myocardium may contain various kinds of somatic stem cells, such as endothelial progenitor cells [20], bone marrow-derived stem cells [21], and bone marrow mononuclear cells [2], which have potent therapeutic effects in heart failure [22]. Furthermore, bone marrow-derived mesenchymal stem cells secrete prostaglandin [23], which may act like ONO-1301 and amplify the effects of the ONO-1301-mediated therapy. Kawabe et al. clearly showed that prostaglandin facilitates the recruitment of endothelial progenitor cells [24]. Although further analysis is needed, the enhanced accumulation of BMCs may predispose the damaged heart tissue to better restoration following MI.

Many reports have shown that granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) also induce BMC mobilization, with therapeutic effects in animal models [25]. However, G-CSF therapy in unselected patients with acute MI did not lead to functional improvements beyond those achieved with conventional therapy. In addition, the administration of GM-CSF in cancer patients has been shown to transiently increase the LV end-systolic dimensions and decrease cardiac contractility [25,26]. The lack of efficacy of G-CSF therapy in clinical trials may be due, at least in part, to its poor initiation and duration; such therapies are likely to be most beneficial during the early phase after acute MI. Although conventional prostacyclin and its analogs are chemically and biologically unstable, ONO-1301 is a long-acting prostacyclin agonist that exerts stable effects *in vivo*, because it lacks a prostanoid structure. Furthermore, we used a slow-release form of ONO-1301, made by polymerizing it with poly-lactic and glycolic acid; this ONO-1301 could still be detected in the blood 3 weeks after its administration (figure S4 in File S1).

Furthermore, in our *in vitro* analysis, although we used normal human dermal fibroblasts to examine the SDF-1/CXCR-4-dependent BMC migration, the reactivity to ONO-1301 stimulation will differ depending on the cell type. For example, the G-CSF expression was upregulated in some kinds of cells (unpublished data). Thus, together with the upregulation of multiple beneficial cytokines such as HGF and VEGF, because of the longer duration of its activity, ONO-1301 may be more potent than conventional protein-based therapies.

Our data showed that ONO-1301 treatment was a potent inducer of BMC homing. Of the BMCs that accumulated in the infarcted myocardium, 43 percent expressed isolectin, an endothelial cell marker, but the other BMCs had a fibroblastic morphology, and did not express cardiac-lineage or cardiofibroblast markers (Figure S3 in File S1). ONO-1301 administration resulted in the attenuation of cardiac dysfunction, with enhanced BMC accumulation. Further study is required to elucidate the mechanism, but we speculate that paracrine effects of factors released by the BMCs play pivotal roles in the therapeutic efficacy, rather than the transdifferentiation of the BMCs into the cardiac or vascular lineage. The effect of cardioprotective and angiogenic factors secreted by the accumulated BMCs and the direct stimulation of ONO-1301 itself may synergistically increase the angiogenesis and cardioprotection, leading to improved therapeutic results.

In summary, ONO-1301 may be a powerful, long-acting activator of multiple cytokines. In particular, SDF-1 may enhance the BMC accumulation in a SDF-1/CXCR4-signaling-dependent manner, leading to an attenuation of the cardiac dysfunction following MI. Our findings suggest that the method involving a sustained release of ONO-1301 may be adapted as a novel drug delivery system for treating heart failure.

Supporting Information

File S1.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: YI SM Y. Sawa. Performed the experiments: YI KI NS. Analyzed the data: YI AS. Contributed reagents/materials/analysis tools: Y. Sakai. Wrote the paper: YI SM SF Y. Sawa.

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Transplantation of myoblast sheets that secrete the novel peptide SVVYGLR improves cardiac function in failing hearts

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Aims Transplantation of myoblast sheets is a promising therapy for enhancing cardiac function after heart failure. We have previously demonstrated that a 7-amino-acid sequence (Ser-Val-Val-Tyr-Gly-Leu-Arg) derived from osteopontin (SV peptide) induces angiogenesis. In this study, we evaluated the long-term therapeutic effects of myoblast sheets secreting SV in a rat infarction model.

Methods and results Two weeks after ligation of the left anterior descending coronary artery, the animals were divided into the following three groups: a group transplanted with wild-type rat skeletal myoblast sheets (WT-rSkMs); a group transplanted with SV-secreting myoblast sheets (SV-rSkMs); and a control group (ligation only). We evaluated cardiac function, histological changes, and smooth muscle actin (SMA) expression through transforming growth factor-β (TGF-β) signalling. The ejection fraction and fractional shortening were significantly better, and the enlargement of end-systolic volume was also significantly attenuated in the SV-rSkM group. Left ventricular remodelling, including fibrosis and hypertrophy, was significantly attenuated in the SV-rSkM group, and SV secreted by the myoblast sheets promoted angiogenesis in the infarcted border area. Furthermore, many clusters of SMA-positive cells were observed in the infarcted areas in the SV-rSkM group. *In vitro* SMA expression was increased when SV was added to the isolated myocardial fibroblasts. Moreover, SV bound to the TGF-β receptor, and SV treatment activated TGF-β receptor–Smad signalling.

Conclusion The SV-secreting myoblast sheets facilitate a long-term improvement in cardiac function. The SV can induce differentiation of fibroblasts to myofibroblasts via TGF-β–Smad signalling. This peptide could possibly be used as a bridge to heart transplantation or as an ideal peptide drug for cardiac regeneration therapy.

Keywords Cell therapy • Peptides • Myocardial infarction • Myofibroblats • Transforming growth factor-β

1. Introduction

In heart failure, tissue damage processes caused by ischaemia, such as cell death, fibrosis, and hypertrophy, gradually progress until the cardiac tissue becomes dysfunctional.[1–3] Transplantation of myoblast sheets is a promising treatment for ischaemic heart failure, and can inhibit left ventricular (LV) remodelling and improve cardiac function via paracrine effectors.[4–8] The cell-sheet technique avoids the arrhythmogenicity associated with skeletal myoblast therapy by injection.[9]

However, this treatment has failed to achieve long-term therapeutic effects, because the transplanted sheets are exposed to blood and nutrient deprivation and drop out from the injured myocardium. Recent studies demonstrated that myoblast sheets that overexpress different cardioprotective agents display enhanced therapeutic effects.[10,11] Therefore, the combined application of gene therapy with angiogenic agents and myoblast sheet transplantation may achieve sustained therapeutic efficacy. Through the secretion of angiogenic factors from transplanted myoblasts, the newly formed blood vessels can supply blood flow to the surviving myocardium and the transplanted

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cells, and the functional deterioration of ischaemic cardiomyopathy should thus improve in the long term.

Osteopontin is a multifunctional cytokine expressed during healing and fibrotic processes.[12] We have previously reported that the osteopontin-derived peptide Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR; SV) exhibits angiogenic activity *in vitro* and *in vivo*,[13–17] and that its angiogenic activity is as potent as that of vascular endothelial growth factor (VEGF).[14] Owing to their high molecular weights, the most well-known angiogenesis-promoting factors, namely, VEGF and hepatocyte growth factor (HGF), are resistant to degradation. In contrast, peptides such as SV are more easily degraded by peptidase within an organism and show only a few adverse effects, such as oedema and pleural fluid accumulation.[18–20] This indicates the high biocompatibility of peptides.

In this study, we hypothesized that the augmentation of myoblast sheets by SV gene transfer could improve cardiac function in the long term.

2. Methods

2.1 Animal ethics

Animal care complied with the 'Guide for Care and Use of Laboratory Animals' (NIH publication no. 85-23, revised 1996). The Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine approved the experimental protocols.

2.2 Isolation of skeletal myoblasts and sheets

After induction of general anaesthesia with pentobarbital (300 mg/kg) and heparin (150 U) by intraperitoneal injection, myoblasts were isolated from the skeletal muscle of the tibialis anterior muscle of 3-week-old male Lewis rats. The muscles were minced and enzymatically dissociated with 0.2% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA) and trypsin at 37°C. The isolated cells were suspended in Dulbecco's modified Eagle's medium with 20% fetal bovine serum. After being pre-plated twice, non-adherent cells were then plated on a dish coated with Matrigel (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA) and incubated at 37°C in humidified air enriched with 5% CO_2. We maintained the cell densities at <70% confluence to prevent skeletal myoblast differentiation that would result in myotube formation. Myoblast sheets were formed by plating 3×10^6 infected myoblasts on a temperature-responsive culture dish (UpCell; CellSeed, Tokyo, Japan).

2.3 Animal model and myoblast sheet transplantation

The myocardial infarction (MI) models were generated via ligation of the left anterior descending (LAD) coronary artery in 8-week-old female F344/NJcl-rnu/rnu rats. The rats were anaesthetized by inhalation of isoflurane (2%, 0.2 mL/min), intubated, and placed on a respirator during surgery to maintain ventilation. The carrier gas for isoflurane is oxygen. The adequacy of anaesthesia was monitored by electrocardiography and pulse rate. Two weeks after ligation of the LAD coronary artery, the myoblast sheets were transplanted. The rats were randomly divided into the following three groups: (i) a WT-rat skeletal myoblast (rSkM) group (transplanted with three wild-type myoblast sheets, $n = 6$); (ii) an SV-rSkM group (transplanted with three SV-secreting myoblast sheets, $n = 8$); and (iii) a control group (sham operation, $n = 6$). Each sheet was individually applied to the infarcted area.

2.4 Overexpression and transfection of SV

A lentiviral vector containing the complementary DNA (cDNA) of SV (SV/pCS-CG) was constructed (*Figure 1A*). The cDNA of SV was synthesized using DNA oligonucleotides. The primer sequences were as follows: forward, 1,5′-GCGCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGT-3′; forward, 2,5′-TCCACTGGTGACGCGGCCCAGCCGGCCAGTGTGGTTTATGGACTGAGGCTCGAGTACCCATACGATGTTCCAGATTACGCTTAAC-3′; reverse, 1,5′-TCGAGTTAAGCGTAATCTGGAACATCGTATGGGTACTCGAGCCTCAGTCCATAAACCACACT-3′; and reverse, 2,5′-GGCCGGCTGGGCCGCGTCACCAGTGGAACCTGGAACCCAGAGCAGCAGTACCCATAGCAGGAGTGTGTCTGTCTCCATGGTGGCG-3′.

The synthesized DNA oligonucleotides were linked and ligated to pCS-CG, and the isolated rSkMs were infected via incubation for 48 h in the presence of SV/pCS-CG.

2.5 Dot blotting assay

The culture media were used for the assays. Each sample was coated onto a black 96-well microplate overnight. To evaluate the secretion SV volume, the serially diluted solution of SV-HA peptide was also coated onto the plate as a control. After blocking, the primary antibody against the HA tag (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to each well. After washing, anti-rabbit IgG-linked horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA) was added. After washing, the plate was exposed to an Enhanced Chemiluminescence (ECL) kit (GE Healthcare).

2.6 Measurement of cardiac function

The cardiac function of the treated rats was evaluated by echocardiography 2, 4, 6, and 8 weeks after sheet transplantation. Baseline measurements were

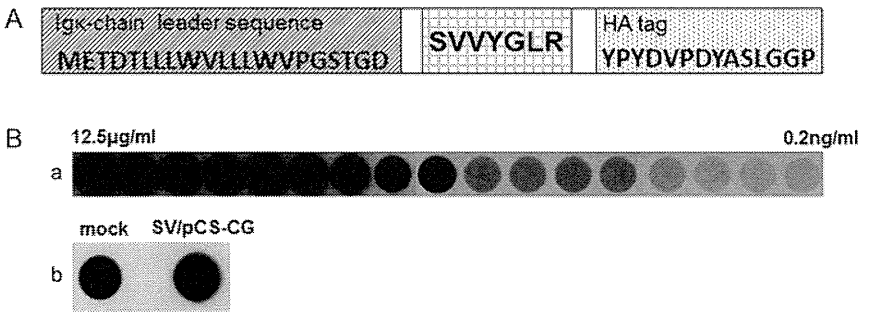

Figure 1 Assessment of SV expression. (*A*) View showing the frame format of the constructed SV gene. (*B*) Expression of SV in rSkMs infected with SV/pCS-CG by dot blotting: (*a*) the dilution series of SV-HA peptide; and (*b*) control cells infected with the empty vector (mock, left) and rSkMs infected with SV/pCS-CG (right).

made before sheet transplantation. The measurements were made using a SONOS 5500 sonograph (Philips Electronics, Tokyo, Japan) with a 12 MHz transducer under general anaesthesia induced and maintained by inhalation of isoflurane (2%, 0.2 mL/min) as mentioned above. The LV end-systolic area, LV end-diastolic area, and LV dimensions at end diastole and end systole (LVIDd and LVIDs, respectively) were determined. The ejection fraction (EF), fractional shortening (FS), end-diastolic volume (EDV), and end-systolic volume (ESV) were calculated as follows:

$$\text{LVEF}\ (\%) = (\text{LVDd}^3 - \text{LVDs}^3)/\text{LVDd}^3 \times 100\ (\%)$$
$$\text{LV\%FS} = [(\text{LVDd} - \text{LVDs})/\text{LVDd}] \times 100\ (\%)$$
$$\text{EDV} = \text{LVIDd}^3 \times (0.98 \times \text{LVIDd} + 5.90)\ (\text{mL})$$
$$\text{ESV} = \text{LVIDs}^3 \times (1.14 \times \text{LVIDs} + 4.18)\ (\text{mL})$$

2.7 Heart weight/body weight ratio

The body weights (BW; in grams) of the rats were measured 8 weeks after sheet transplantation, after which the rats were anaesthetized with pentobarbital (300 mg/kg) and heparin (150 U) by intraperitoneal injection, and their hearts were rapidly removed and weighed (in milligrams). The heart weight (HW)/BW ratio was then calculated.

2.8 Histological analyses

Myocardial specimens were obtained 8 weeks post-transplantation. The formalin-fixed samples were embedded in paraffin. The LV chamber diameter and the anterior wall thickness were measured from sections stained with haematoxylin and eosin. Infarcted wall thickness, posterior wall thickness, and LV chamber diameter were measured with the scale loupe. The sections were evaluated morphologically using the NIS Elements system (Nikon, Tokyo, Japan). Sirius Red staining was used to detect fibrosis. The percentage of fibrosis was calculated from the fibrotic ratio in the infarct border area. Periodic acid–Schiff staining for cardiomyocyte hypertrophy was also performed. We randomly selected 100 cardiomyocytes and measured the two-point shortest axes at the level of the nucleus.

Immunohistochemical staining for von Willebrand factor antigen was used to label vascular endothelial cells to permit the counting of blood vessels. The sections were incubated with primary antibody against von Willebrand factor (rabbit polyclonal; Dako, Glostrup, Denmark). The sections were incubated with a biotinylated anti-rabbit IgG antibody (Dako) and further incubated with peroxidase-conjugated streptavidin (SA; GE Healthcare). Visualization was performed with biphenyl-3,3′,4,4′-tetramine solution (Sigma, St Louis, MO, USA). The stained vascular endothelial cells were counted under a light microscope.

The distribution of myofibroblast-like cells was evaluated by immunohistochemical staining with anti-smooth muscle actin (SMA) antibody (Dako) and anti-smooth muscle myosin heavy chain (SM-MHC) type 2 antibody (Abcam Ltd, Cambridge, UK). The SMA-positive cell density was calculated as SMA positive area/infarcted area × 100 (%).

2.9 Primary culture of adult ventricular fibroblasts

Cardiac fibroblasts (CFs) were isolated from 8-week-old adult male Sprague–Dawley rats 4 weeks after the induction of MI by LAD ligation. The hearts were excised from anaesthetized rats and quickly transferred to Hank's buffered salt solution. The minced left ventricular tissues were digested using 100 U/mL type II collagenase and 0.1% trypsin at 37°C. The cells were centrifuged and suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and incubated at 37°C in humidified air enriched with 5% CO_2.

2.10 Immunofluorescence staining

The isolated fibroblasts were incubated with SV (10 μg/mL), SV random peptide (GYRVLSV; 10 μg/mL), or transforming growth factor-β1 (TGF-β1; 25 ng/mL) for 72 h. The cells were fixed with 4% paraformaldehyde and incubated with anti-SMA antibody followed by incubation with cyanine-3-conjugated anti-rabbit secondary antibody (GE Healthcare). The nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI; Invitrogen Life Technologies, Grand Island, NY, USA), and the fluorescent signals were detected by fluorescence microscopy (ECLIPSE E600, Nikon).

2.11 Western blotting assay

The isolated fibroblasts were incubated with SV (10 μg/mL), SV random peptide (10 μg/mL), or TGF-β1 (25 ng/mL) for 72 h. The cells were suspended in lysis buffer (50 mM Tris at pH 8.0, 120 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). Proteins in whole-cell lysates were separated by SDS–PAGE, transferred to a polyvinylidene fluoride transfer membrane (Millipore, Billerica, MA, USA), and probed sequentially with antibodies against SMA and α-tubulin (Sigma). The blots were developed using an ECL kit.

To examine the activity of TGF-β receptor–Smad signalling induced by SV, the phosphorylation of Smad2, Smad3, and TGF-β receptor I (TβRI) was studied by western blotting. The isolated fibroblasts were incubated with SV (10 μg/mL), SV random peptide (10 μg/mL), or TGF-β1 (25 ng/mL) for 1 h. Primary antibodies against phospho-Smad2, phospho-Smad3, Smad2/3 (Cell Signaling Technology, Inc., Danvers, MA, USA), TβRI (phospho S165; Abcam), and α-tubulin were used.

2.12 Construction of recombinant transforming growth factor-β receptorII

A pcDNA3.1 vector containing the cDNA encoding TβRII (pcDNA3.1-TβRII) was constructed. The recombinant TβRII was produced by transfecting HEK 293T cells with pcDNA3.1-TβRII. The culture media containing recombinant TβRII were harvested. The purification of recombinant TβRII was done using immunoprecipitation with anti-TβRII antibody.

2.13 Biacore analysis

The binding of SV to TβRII was assessed by Biacore analysis. Biotinylated SVs were captured on SA-coated BIAcore SA sensor chips (GE Healthcare, Piscataway, NJ, USA). Ligands were diluted to 10 μg/mL and injected at 10 μL/mL. To correct for refractive index change, non-specific binding, and instrument drift, a reference flow cell contained the SA-coated surface only. The recombinant TβRII was diluted to 10 μg/mL in Hank's buffered salt solution and injected during the association phase for 5 min (30 μL/min).

2.14 *In situ* proximity ligation assay

The Duolink *in situ* proximity ligation assay (PLA; Olink Biosciences, Uppsala, Sweden) was performed according to the manufacturer's protocol. The isolated fibroblasts were incubated in the presence of SV-HA peptide or SV-HA random peptide (GYRVLSV; 1 μg/mL) for 1 h. The fixed fibroblasts were incubated with the following primary antibodies: rabbit polyclonal anti-TβRII (Abcam) and mouse monoclonal anti-HA (Nacalai Tesque). The cells were then incubated with PLA probes consisting of two secondary anti-rabbit and anti-mouse antibodies, each tagged with an oligonucleotide. A hybridization solution consisting of two oligonucleotide linkers complementary to each PLA probe was added to the cells. The isolated cells were incubated with a Duolink Ligation stock containing ligase and Duolink polymerase. In addition, the cells were incubated with a detection solution consisting of fluorescently labelled oligonucleotides that hybridize to the rolling circle amplification product. The PLA signal was visualized using fluorescence microscopy.

2.15 Statistical analyses

Data are presented as the means ± SEM. Cardiac function was analysed by repeated-measures analysis of variance (ANOVA) for differences across the entire time course, as well as one-way ANOVA, whereas the Tukey–Kramer *post hoc* test was used to examine significant differences at each time point. To assess the significance of the differences between individual groups for

other data, statistical comparisons were performed using Student's unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Overexpression of SV in rat skeletal myoblasts

The signal strength of dots in wells coated with the culture medium of SV/pCS-CG-infected rSkMs was stronger than that of dots in wells coated with the culture medium of mock-infected rSkMs (*Figure 1B[b]*). The SV was synthesized and secreted by SV/pCS-CG-infected rSkMs. In addition, from the dilution series of SV-HA peptide, the secretion volume of SV was calculated to be approximately 3.125–6.25 ng/mL (*Figure 1B[a]*).

3.2 Effect of SV-secreting myoblast sheet on left ventricular function

Echocardiography revealed significantly better values of LVEF and %FS in the WT-rSkM and SV-rSkM groups compared with the control group at all time points after transplantation ($P < 0.01$). Although there were still significant differences between the control and WT-rSkM groups after the 4 week time point, the LVEF and %FS in the WT-rSkM group decreased dramatically. Furthermore, LVEF and %FS were significantly better in the SV-rSkM group at 2, 6, and 8 weeks after transplantation compared with the WT-rSkM group (2 and 6 weeks, $P < 0.05$; 8 weeks, $P < 0.01$; *Figure 2A* and *B*).

The evaluation of LVIDs illustrated the inhibition of dilatation in the SV-rSkM group in comparison with the control and WT-rSkM groups. In particular, at 6 and 8 weeks after transplantation LVIDs was significantly attenuated in the SV-rSkM group compared with the control group (6 weeks $P < 0.05$; 8 weeks $P < 0.01$; *Table 1*). The enlargement of ESV was also significantly attenuated in the SV-rSkM group compared with the control group at 6 and 8 weeks after transplantation (6 weeks, $P < 0.05$; 8 weeks, $P < 0.01$; *Table 1*). The increase in EDV was significantly inhibited in the SV-rSkM group compared with the control group only at 8 weeks after transplantation ($P < 0.05$; *Table 1*).

3.3 Heart weight/body weight ratio

We used the HW/BW ratio as an indicator of cardiac hypertrophy. The HW/BW ratio was significantly greater in the SV-rSkM group at 8 weeks after transplantation compared with the control and WT-rSkM groups ($P < 0.01$; *Figure 2C*).

3.4 Effect of SV-secreting myoblast sheet on left ventricular remodelling

Haematoxylin and eosin staining demonstrated thinning of the infarcted wall in the control and WT-rSkM groups, whereas the thickness of the infarcted wall was maintained in the SV-rSkM group (*Figure 2D* and *Table 2*). Statistical analysis demonstrated that the LV chamber of the SV-rSkM group was significantly less dilated that those of the control and WT-rSkM groups ($P < 0.05$; *Table 2*) and that the infarcted wall in the SV-rSkM group was significantly thicker than that in the control

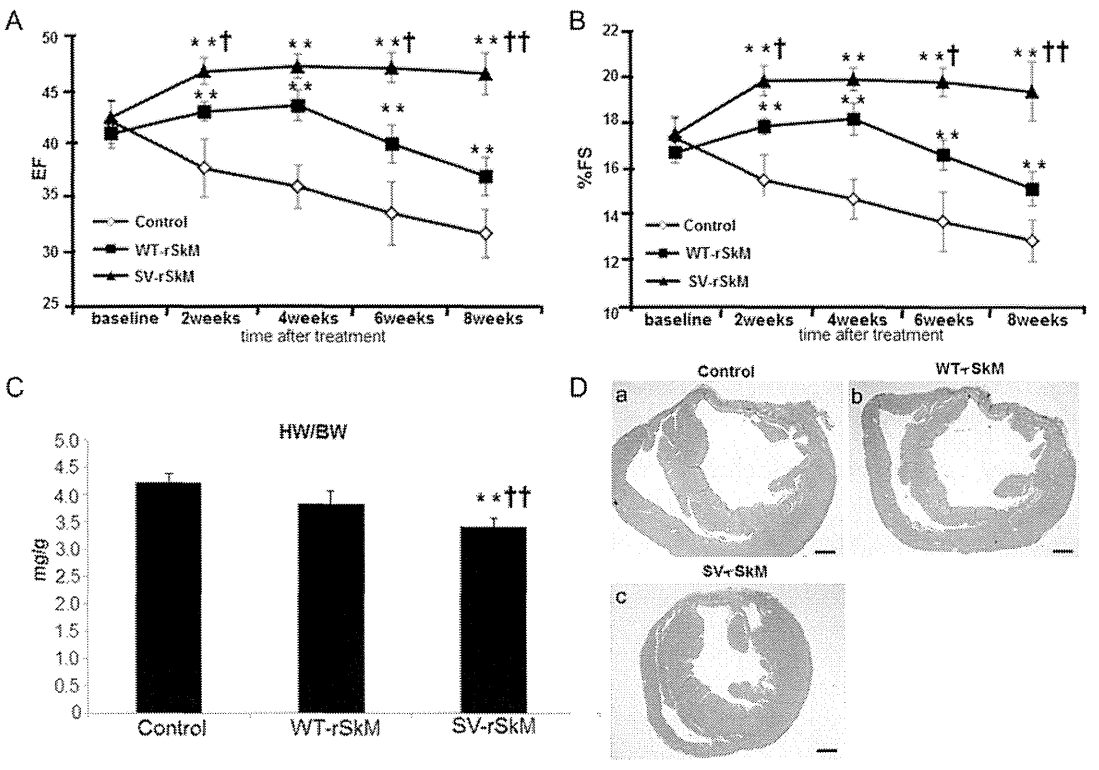

Figure 2 Echocardiographic evaluation of LV function after sheet transplantation (*A*, EF; *B*, %FS). $^{**}P < 0.01$ vs. Control group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. WT-rSkM group. Baseline is time of transplantation, which was 2 weeks after ligation of the LAD. Other times in weeks are post-transplantation. (*C*) Evaluation of HW/BW. $^{**}P < 0.01$ vs. Control group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. WT-rSkM group. (*D*) Haematoxylin- and eosin-stained section of the left ventricle: (*a*) control; (*b*) WT-rSkM; and (*c*) SV-rSkM (×10 magnification, scale bars represent 1000 μm).

Table 1 Assessment of LVIDd, LVIDs, EDV, and ESV over time by echocardiography

	Baseline	2 weeks	4 weeks	6 weeks	8 weeks
LVIDd (cm)					
Control	0.75 ± 0.04	0.77 ± 0.07	0.81 ± 0.05	0.87 ± 0.04	0.89 ± 0.01
WT-rSkM	0.76 ± 0.02	0.77 ± 0.04	0.80 ± 0.06	0.84 ± 0.06	0.85 ± 0.03
SV-rSkM	0.71 ± 0.05	0.78 ± 0.07	0.78 ± 0.07	0.83 ± 0.03	0.84 ± 0.03*
LVIDs (cm)					
Control	0.63 ± 0.02	0.67 ± 0.05	0.70 ± 0.05	0.75 ± 0.04	0.77 ± 0.02
WT-rSkM	0.63 ± 0.02	0.64 ± 0.03	0.67 ± 0.06	0.69 ± 0.05	0.72 ± 0.04
SV-rSkM	0.64 ± 0.04	0.65 ± 0.05	0.67 ± 0.04	0.67 ± 0.03*	0.69 ± 0.04**
EDV (ml)					
Control	2.87 ± 0.46	3.19 ± 0.93	3.65 ± 0.71	4.46 ± 0.63	4.79 ± 0.26
WT-rSkM	2.93 ± 0.24	3.04 ± 0.43	3.53 ± 0.77	4.05 ± 0.89	4.22 ± 0.54
SV-rSkM	2.77 ± 0.53	3.26 ± 0.70	3.37 ± 0.64	3.92 ± 0.43	4.08 ± 0.43*
ESV (ml)					
Control	1.20 ± 0.13	1.49 ± 0.36	1.71 ± 0.34	2.16 ± 0.40	2.34 ± 0.23
WT-rSkM	1.25 ± 0.14	1.28 ± 0.16	1.53 ± 0.37	1.69 ± 0.33	1.88 ± 0.22
SV-rSkM	1.29 ± 0.21	1.39 ± 0.27	1.48 ± 0.25	1.51 ± 0.23*	1.61 ± 0.25**

Abbreviations: EDV, end-diastolic volume; ESV; end-systolic volume; LVIDd, left ventricular dimensions at end diastole; LVIDs, left ventricular dimensions at end systole. Baseline is the time of transplantation, which was 2 weeks after ligation of the left anterior descending coronary artery; other times in weeks are post-transplantation. *$P < 0.05$, **$P < 0.01$ vs. control group at each time point.

and WT-rSkM groups ($P < 0.01$; *Table 2*). The values of the LV chamber diameter/posterior wall thickness were significantly lower in the SV-rSkM group compared with those in the control and WT-rSkM groups ($P < 0.01$; *Table 2*). There were no significant differences between the control and WT-rSkM groups regarding these indices.

The SV-rSkM group exhibited a significantly lower percentage of fibrosis than the control and WT-rSkM groups in the infarcted border area ($P < 0.01$; *Figure 3A*). The diameters of cardiomyocytes in the SV-rSkM group were significantly smaller than those in the control and WT-rSkM groups ($P < 0.01$; *Figure 3B*). There was no significant difference in the area remote from the transplant among the three groups.

3.5 The pro-angiogenic effects of SV

The capillary density 8 weeks after transplantation was significantly higher in the WT-rSkM and SV-rSkM groups than in the control group ($P < 0.01$). Furthermore, the capillary density in the SV-rSkM group was significantly higher than that in the WT-rSkM group ($P < 0.01$; *Figure 3C*[*a*]–*C*[*c*] and *D*). There was no significant difference in the area remote from the transplant among the three groups.

3.6 The accumulation of smooth muscle actin-positive and smooth muscle myosin heavy chain type2-positive cells by SV

Immunohistochemical staining with an anti-SMA antibody revealed that many clusters of SMA-positive cells were present in infarcted areas in the SV-rSkM group (*Figure 3E*). Statistical analysis indicated that the SMA-positive cell density was significantly higher in the WT-rSkM and SV-rSkM groups than in the control group (WT-rSkM , $P < 0.05$; SV-rSkM, $P < 0.01$; *Figure 3F*). Furthermore, the SMA-positive cell density was significantly higher in the SV-rSkM group than in the WT-rSkM group ($P < 0.05$; *Figure 3F*). Notably, SM-MHC type 2-positive cells were also detected in infarcted areas in the SV-rSkM group, whereas those cells were scarce in the control and WT-rSkM groups (*Figure 3G*).

Table 2 Thickness of the infarcted wall and posterior wall and left ventricular chamber diameter

	Control	WT-rSkM	SV-rSkM
Left ventricular chamber diameter (mm)	4.50 ± 0.46	4.48 ± 0.42	3.85 ± 0.29**††
Infarcted wall thickness (mm)	0.53 ± 0.05	0.51 ± 0.08	0.63 ± 0.06
Posterior wall thickness (mm)	2.04 ± 0.10	1.88 ± 0.25	1.96 ± 0.14
Percentage anterior wall thickness	25.98 ± 2.66	27.20 ± 2.63	32.29 ± 0.98**††
Left ventricular chamber diameter/ posterior wall thickness	2.36 ± 0.22	2.30 ± 0.15	1.98 ± 0.16**††

The percentage anterior wall thickness is the infarcted wall thickness/posterior wall thickness × 100. **$P < 0.01$ vs. control group. ††$P < 0.01$ vs. WT-rSkM group.

3.7 The induction of smooth muscle actin by SV

Expression of SMA was increased when SV was added to the isolated fibroblasts (*Figure 4A* and *B*). The expression level of SMA was similar to that of TGF-β1 (*Figure 4B*). Conversely, the expression level of SMA was unchanged by the addition of SV random peptide (*Figure 4A* and *B*).

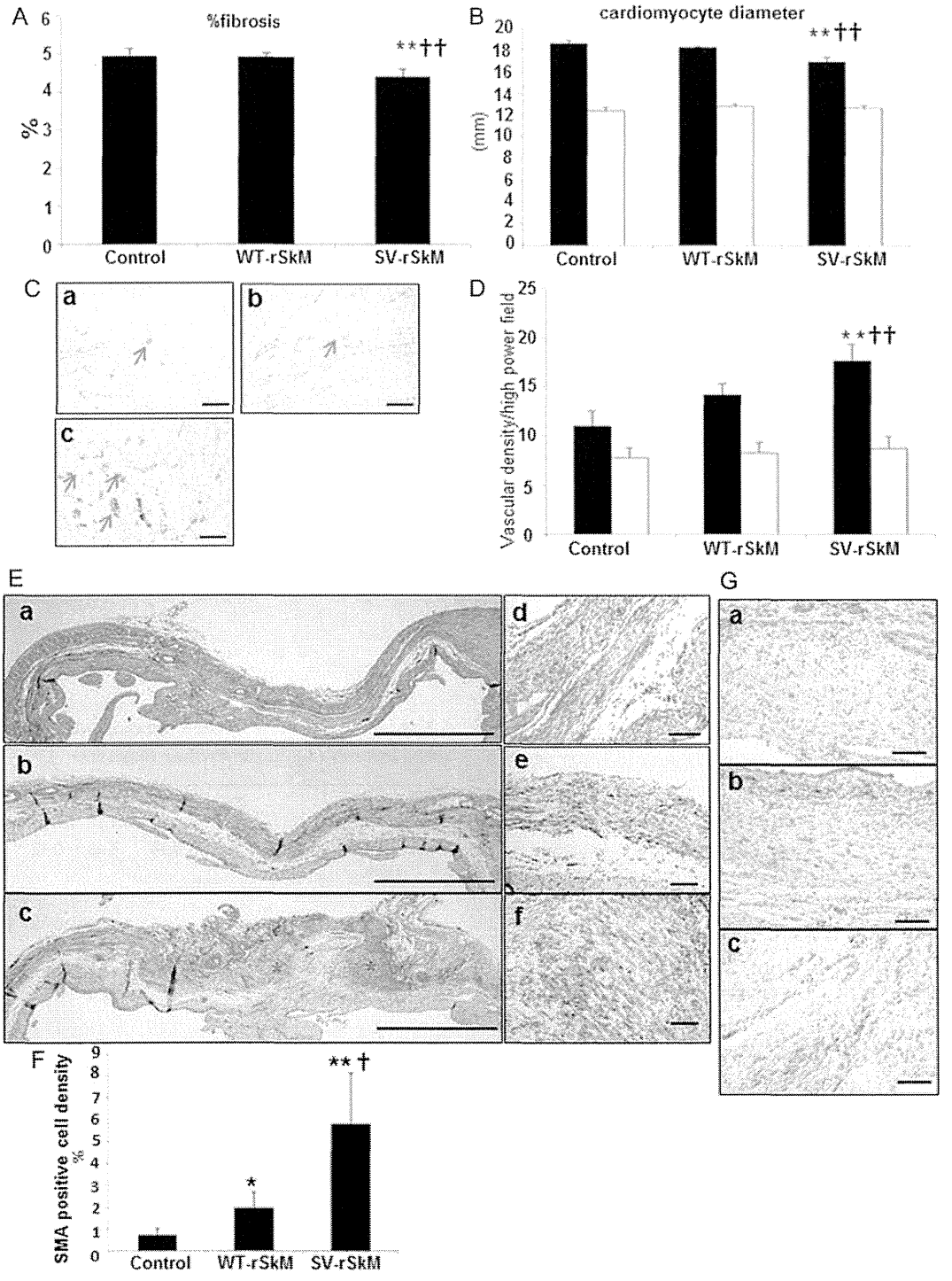

Figure 3 Histological evaluations of LV remodelling. (*A*) Percentage fibrosis. (*B*) Cardiomyocyte diameter. $^{**}P < 0.01$ vs. Control group. $^{\dagger\dagger}P < 0.01$ vs. WT-rSkM group. Filled bars, border area; open bars, remote area. Immunohistochemical staining. (*C*) A section of the infarcted border zone stained with an antibody against von Willebrand factor: (*a*) control; (*b*) WT-rSkM; and (*c*) SV-rSkM (×200 magnification, scale bars represent 100 μm). Newly formed vessel is stained brown. (*D*) Quantitative estimation of vascular density. $^{**}P < 0.01$ vs. Control group. $^{\dagger\dagger}P < 0.01$ vs. WT-rSkM group. Filled bars, border area; open bars, remote area. (*E*) The distribution of SMA-positive cells: (*a*) control; (*b*) WT-rSkM; (*c*) SV-rSkM (*a–c*, ×20 magnification, scale bars represent 1000 μm; e and *f*, ×200 magnification, scale bars represent 100 μm). Red asterisks denote SMA-positive cells. (*F*) Quantitative estimation of the SMA-positive cell density. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group. $^{\dagger}P < 0.05$ vs. WT-rSkM group. (*G*) The distribution of SM-MHC type 2-positive cells: (*a*) control; (*b*) WT-rSkM; and (*c*) SV-rSkM (*a–c*, ×200 magnification, scale bars represent 100 μm).

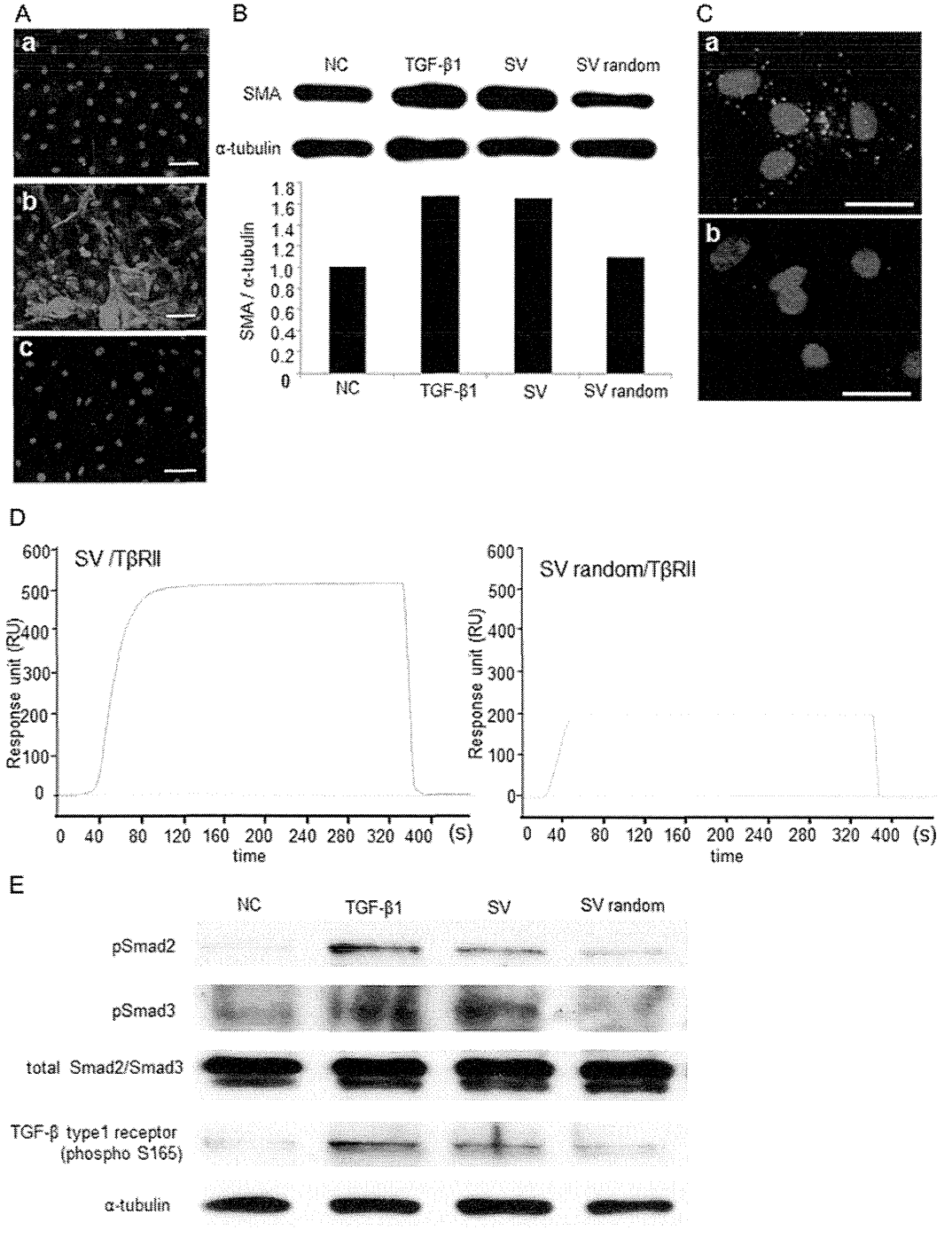

Figure 4 Myofibroblast differentiation induced by exposure of the isolated CFs to SV. (*A*) and (*B*) SMA expression induced by SV. (*A*) Immunofluorescence staining with an anti-SMA antibody: (*a*) non-stimulated CFs (NC); (*b*) CFs exposed to SV; and (*c*) CFs exposed to SV random peptide (×200 magnification, scale bars represent 100 μm). (*B*) Immunoblot of the myofibroblast differentiation marker SMA and its quantitative assessment. α-Tubulin was used as a loading control. (*C*) The examination of binding between TβRII and SV using an *in situ* PLA: (*a*) CFs exposed to SV; (*b*) CFs exposed to SV random peptide (scale bars represent 50 μm). The binding between TβRII and SV and the TGF-β–Smad signalling induced by SV. (*D*) Biacore analysis of the interaction of TβRII with SV. (*E*) Assessment of TGF-β–Smad signalling in CFs exposed to SV by western blotting. α-Tubulin was used as a loading control.

3.8 Binding of SV to transforming growth factor-β receptor II

When PLA was performed using rabbit polyclonal anti-TβRII and mouse monoclonal anti-HA antibodies for the isolated fibroblasts treated with SV-HA peptide, PLA-positive red signals were found (*Figure 4C[a]*). In contrast, PLA-positive red signals were not detected in the isolated fibroblasts treated with SV-HA random peptide (*Figure 4C[b]*). We assessed the ability of SV to bind to TβRII, using a sensor chip immobilized with biotinylated SV (K_D = 13.5 nM), and this peptide bound to TβRII with high affinity (500 Resonance Unit; *Figure 4D*). However, SV random peptides (K_D = 16 nM) had a much lower R_{max} (R_{max} = analyte molecular weight (MW)/ligand MW × the immobilization level × the stoichiometric ratio) value (200 Resonance Unit).

3.9 The effects of SV on Smad activation

Treatment with TGF-β1 or SV induced the phosphorylation of TβRI, Smad2, and Smad3 to similar degrees (*Figure 4E*). Conversely, treatment with SV random peptide had no effect on TβRI, Smad2, and Smad3 phosphorylation.

4. Discussion

In this study, we transplanted myoblast sheets to the myocardium in an infarcted rat model. The cell sheets are removed from special temperature-responsive dishes without destroying the cell–cell or cell–extracellular matrix adhesions in the cell sheet. The myoblast sheet does not require an artificial scaffold, because it has a great ability to integrate with the infarcted area via an adhesion factor, such as integrin-$\alpha_7\beta_1$ and α-dystroglycan, which are expressed on the surface of myoblasts; thus, the sheets do not fall off after the chest is closed.[5–7]

The effect of myoblast sheet transplantation is mediated mainly by paracrine growth factors that stimulate the injured myocardium.[6,7] The paracrine effectors include HGF, VEGF, and stromal-derived factor 1. These factors can promote angiogenesis in the ischaemic myocardium. Hepatocyte growth factor is also associated with anti-fibrosis and anti-apoptosis. The grafted myoblasts beneficially attract haematopoietic stem cells to home in on the infarcted heart area for heart regeneration and angiogenesis by stromal-derived factor 1.[6] These paracrine activities induce angiogenesis and reduce fibrosis and hypertrophy; as a result, the depressed cardiac function improves. Therefore, we hypothesized that functional modification of myoblast sheet properties by overexpressing a factor associated with angiogenesis, anti-fibrosis, and anti-apoptosis could further promote and maintain the therapeutic effects of the sheet. Our previous results demonstrated that SV has a much stronger pro-angiogenic action than VEGF.[14] Given that SV has a straight-chain sequence, rather than a complicated conformation, we can speculate that this peptide would be degraded by peptidase within an organism. Our previous research has shown that synthetic SV has no effect on the proliferation of endothelial and muscle cells.[13,14] The degradation rate and function for the proliferation of SV could have high biocompatibility with peptides. In this study, we investigated the effects of SV-secreting myoblast sheets in infarcted rat hearts.

Most of the transplanted myoblasts drop out at 4 weeks after sheet transplantation.[21] As a result, cardiac function in the WT-rSkM group at 4 weeks after sheet transplantation was markedly decreased. In contrast, in the SV-rSkM group the functional improvements were maintained for 8 weeks after sheet transplantation. The capillary density 8 weeks after transplantation was significantly higher in the SV-rSkM group than in the control and WT-rSkM groups. The vessels newly formed by the secreted SVs from the myoblast sheets remained until 8 weeks post-transplantation, after the drop-out of the transplanted cells. The paracrine factors from transplanted myoblasts also promoted angiogenesis. Thus, in this study, the secreted SV showed an enhanced angiogenic action after myoblast transplantation. It is possible that SV induced angiogenesis in both the surviving cardiomyocytes and the transplanted cells; as a result, the survival time of the transplanted cells would have been extended. However, there are no data concerning the effect of SV-rSkM on the endogenous mobilization/proliferation/apoptosis and differentiation of cardiac resident cardiac stem/progenitor cells. More research is needed to define the effects of SV on these cells.

Siltanen *et al.*[11] reported the efficacy of a heart failure treatment involving the transplantation of myoblasts genetically modified to overexpress HGF. Hepatocyte growth factor is a cardioprotective factor associated with angiogenesis, anti-fibrosis, and anti-apoptosis.[22,23] Hepatocyte growth factor-overexpressing myoblast sheets stimulated angiogenesis and inhibited myocardial fibrosis in a rat chronic heart failure model. However, cardiac function was not improved by the transplantation of HGF-overexpressing sheets.[16] In contrast, SV-expressing sheets, which also have a pro-angiogenic action, enhanced cardiac function and angiogenesis. Transplantation of SV-secreting sheets enhanced the functional recovery of ischaemic myocardium compared with the findings in the control and WT-rSkM groups. In particular, systolic parameters, such as LVIDs and ESV, were significantly improved in the SV-rSkM group.

Myofibroblasts share morphological features with fibroblasts and smooth muscle cells. Differentiated myofibroblasts are characterized by increased α-SMA and the morphological features of well-developed stress fibres.[24] Although myofibroblasts in normal tissue, granulation tissue, and pathological tissue exhibit phenotypic α-SMA expression, SM-MHC, vimentin, and desmin, myofibroblasts more commonly express α-SMA.[25] Myofibroblasts have a greater contractile capability than undifferentiated CFs, and this property is believed to be important in maintaining the structural integrity of healing scars.[26] Expression of α-SMA in stress fibres is instrumental in force generation by myofibroblasts.[27] Additionally, myofibroblasts confer mechanical tension to remodelling matrix via anchoring and contracting.[24] In this study, many clusters of SMA-positive and SM-MHC type 2-positive cells were observed in infarcted areas in the SV-rSkM group. These cells differentiated from CFs into myofibroblasts in the infarcted area after the addition of SV, and the myocardial contractile performance of the infarcted wall in the SV-rSkM group was improved by the accumulation of myofibroblasts. Our previous study indicated that, when skeletal myoblast sheets were transplanted into a swine acute MI model, well-developed smooth muscle cells accumulated in the centre of the scar.[28] In our study, more SMA-positive cells accumulated in the infarcted area in the SV-rSkM group than in the WT-rSkM-group, and the secreted SV enhanced the effect of SMA expression by CFs. Furthermore, owing to the accumulation of myofibroblasts in the infarcted area, adverse effects on the uninjured myocardium and its exercise endurance were decreased; consequently, cardiac remodelling processes, such as fibrosis and cardiomyocyte hypertrophy, were attenuated. The fibroblasts in scar tissue of the infarcted area are differentiated into SMA-positive and SM-MHC type 2-positive cells by SV. There is no cell–cell connectivity between these cells and the recipient's cardiomyocytes, and it is possible that they have not been synchronized with the cardiomyocytes. However, they do have a contractile capability, and SV could have transferred the contractility to the infarcted wall via the

accumulation of these cells, improving the motion of the scared left ventricular wall and inhibiting the dilatation of the LV chamber in the SV-rSkM group.

Our previous research has shown that synthetic SVVYGLR peptides *in vitro* activate the adhesion and migration of endothelial cells and smooth muscle cells, and stimulate tube formation by vascular endothelial cells.[13,14] In contrast, SV has no effect on the proliferation of these cells, whereas it enhances the adhesion and proliferation of several types of human mesenchymal cells.[17] Although the effects of SV on apoptosis in these cells have not been evaluated, the results regarding proliferation suggest that SV has no effect on apoptosis. According to these data, SV should have no impact on the proliferation and apoptosis of myoblasts, while stimulating the proliferation of fibroblasts and myofibroblasts.

Osteopontin is highly expressed during the differentiation of fibroblasts into myofibroblasts, and could have an effect on fibroblast differentiation and a role in myofibroblast function during tissue remodelling.[29] Transforming growth factor-β plays an important role in the activation of fibroblasts in wound repair, and it induces myofibroblast differentiation via Smad signalling.[30] Osteopontin is required for the differentiation and activation of myofibroblasts formed in response to TGF-β1.[31] This study illustrated that, in isolated CFs, SV had a great degree of affinity for TβRII and activated Smad signalling via TβRs. The secreted SV bound TβRII and induced the differentiation of fibroblasts into myofibroblasts through TGF-β receptor–Smad signalling.

Transforming growth factor-β participates in vascular development and the maintenance of vascular homeostasis, and it induces angiogenesis at low levels.[32] Transforming growth factor-β regulates angiogenesis by acting on both vascular endothelial and smooth muscle cells.[31] SV also stimulates angiogenesis at low levels, but this effect plateaus at high levels.[14] Thus, SV induces angiogenesis via the same mechanism as TGF-β. However, we believed that SV could also bind receptors other than TβRII and exhibit myocardium-protecting actions, such as promoting angiogenesis and inhibiting hypertrophy. To explain the effect of SV in improving cardiac function, SV receptors in myocardial tissue will have to be identified, and the details of its mechanism will need to be examined.

Functional SV peptide-secreting myoblast sheets facilitate long-term improvement in cardiac function and inhibition of cardiac remodelling. The SVs secreted from myoblast sheets effectively stimulated angiogenesis in the failing myocardium. The accumulation of SMA-positive cells induced by SV confers a contractile property on the infarcted wall. The early therapeutic effects after SV-secreting myoblast sheet transplantation were due to the paracrine effects of the transplanted myoblasts, and the late effects were caused by the pro-angiogenic effects of SV and its induction of myofibroblast accumulation via TGF-β–Smad signalling. These results suggest that SV could change CFs to muscle-like cells, allowing it to be used as a bridge to heart transplantation or as an ideal peptide drug for cardiac regeneration therapy.

Conflict of interest: none declared.

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Review
Future Cardiology

Choice of cell-delivery route for successful cell transplantation therapy for the heart

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The cell-delivery route is one of the major factors influencing the therapeutic effect and complications of cell transplantation therapy for cardiac diseases. There are four major clinically practical routes, with each method having its own advantages and disadvantages. First, intramyocardial injection allows targeted cell delivery into the areas of interest, although this induces mechanical injury, inflammation and islet-like donor cell clusters, leading to limited donor cell survival and arrhythmogenicity. Second, intracoronary injection is less likely to induce inflammation, whereas poor initial cell retention in the heart is a concern. Third, intravenous injection is easy and economical, but cell recruitment into the heart is not frequent. Finally, epicardial placement of 'cell sheets' enables higher efficiency of cell engraftment, but poor integration into the myocardium may be an issue. This review summarizes up-to-date clinical and preclinical knowledge regarding these cell-delivery methods. We further discuss the ways to refine these methods towards optimizing cell transplantation therapy for the heart.

A number of basic and clinical studies have shown that cell transplantation therapy elicits functional and structural recovery of the failing heart in relation to reverse remodeling of the left ventricle (LV) [1,2]. The role of various types of pluripotent stem, progenitor and precursor cells as donors for this innovative treatment has been investigated. However, to date, the large-scale randomized trials have reported that this treatment is associated with only modest efficacy to achieve sufficient functional or prognostic benefits [3–12]. Physicians and scientists are thereby prompted to further investigate fundamental mechanisms of this therapy with the aim of optimization of the practical protocol so that we would be able to draw the maximum benefit, without causing complications, from this treatment. Major factors to influence the degree of the therapeutic effects and the complications of cell transplantation therapy include donor cell type and cell-delivery route [13–15]. While the former has been extensively discussed on other occasions, the latter was less considered and is therefore focused on in this review.

It is known that the cell-delivery method affects fundamental behaviors of the transplanted donor cells in the myocardium, such as retention, distribution, survival/death, secretion, proliferation, differentiation and anatomical/functional integration. In addition, the cell-delivery method may affect the response of the host myocardium to the transplanted cells. As a consequence, the choice of cell-delivery method would affect therapeutic efficacy and complications, and may thus determine the success of this treatment.

There are four major routes of cell delivery into the heart that are currently practiced in clinical settings: intramyocardial (IM) injection, intracoronary (IC) injection, intravenous (iv.) injection and epicardial placement (Figure 1). IM cell injection is when donor cells are injected directly into the heart using a needle, while IC cell injection injects donor cells via the cardiac circulatory system. Intravenous cell injection is to inject donor cells via the peripheral vein into the systemic circulation, in the hope that some cells are recruited into the heart. On the other hand, epicardial placement applies the cells onto the epicardial surface of the heart, typically by placement of 'cell sheets' on the heart. Each method has its own advantages and disadvantages, and none are perfect. This review summarizes the up-to-date knowledge regarding these cell-delivery methods, with a particular focus on the therapeutic efficacy in treating heart disease, possible complications and underlying mechanisms. We further discuss the ways to refine these methods to optimize cell transplantation therapy to the heart.

IM cell injection

IM cell injection is a common and versatile cell-delivery method into the heart, and has actually

Keywords
■ cell-delivery route ■ cell transplantation ■ heart failure ■ intracoronary injection ■ intramyocardial injection ■ regenerative medicine ■ stem cells ■ tissue engineering

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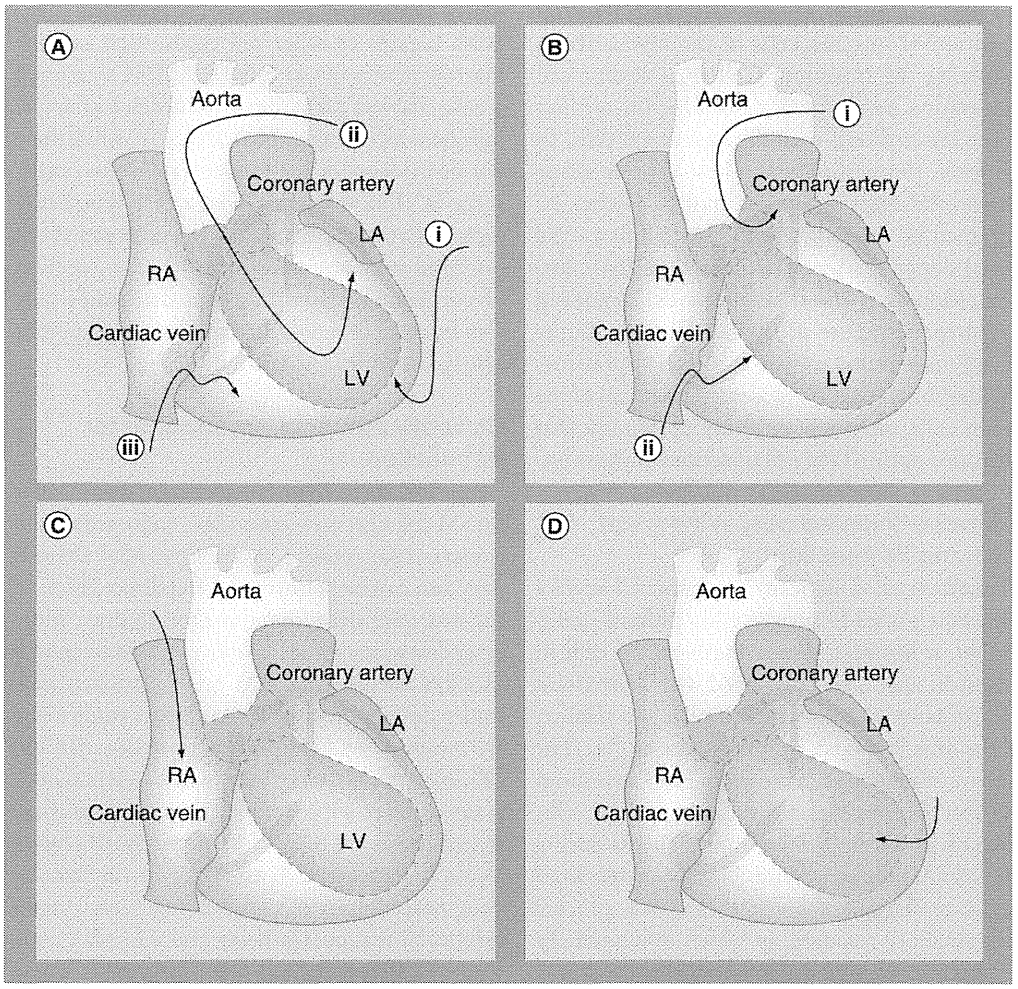

Figure 1. Possible cell-delivery methods into the heart. Major methods of cell delivery into the heart include: intramyocardial injection, intracoronary injection, intravenous injection and epicardial placement. **(A)** Intramyocardial cell injection can be carried out by **(i)** epicardial, **(ii)** endocardial or **(iii)** transvascular approaches. **(B)** Intracoronary cell injection can be carried out by **(i)** antegrade injection into the coronary arteries or **(ii)** retrograde injection into the cardiac vein approaches. **(C)** Intravenous cell injection can be performed by central or peripheral vein approaches. **(D)** Epicardial cell placement can be carried out by attaching cells on the epicardial surface, for example by the cell-sheet technique.
LA: Left atrium; LV: Left ventricle; RA: Right atrium.

been utilized in a large number of basic and clinical studies [16]. At the initial period of clinical application of cell transplantation therapy, this method was preferably used by surgeons to transplant autologous skeletal myoblasts (SMB) in conjunction with cardiac surgery, such as coronary artery bypass grafting [17,18]. This is now used more widely for transplanting many other cell types. This method allows targeted delivery of the cells directly into the specific myocardial area of interest.

Initial donor cell retention following IM injection

The cells that are injected via the IM route will retain in the myocardial interstitium via interaction with the existing extracellular matrices (ECMs) and/or the cellular membrane of host cardiac cells. However, it has been reported that a considerable number of injected cells escape from the injection sites or into the systemic circulation [19,20]. This results in a limited rate of retention of the injected cells in the heart. Hou *et al.* injected radiolabeled peripheral blood mononuclear cells via the epicardial IM route into the infarcted swine heart and measured distribution of the injected cells in the whole body at 1 h after the injection by γ-counter [21]. This study showed that only 11% of the total injected cells retained in the heart, 26% retained in the lung, less than 3% retained in other organs and 12% retained in the syringe-needle device [21].

There are many factors that affect initial retention of injected cells in the myocardium following IM injection. Cell type and/or cell preparation method will determine the affinity of the injected cells with the host tissue/cells. In addition, the nature of the host cardiac tissue around the injection site (i.e., normal, ischemic, inflamed or scarred) would also influence the initial donor cell retention following IM injection. Moreover, injection methods, including size/shape/structure of the needle and volume of cell suspensions, as well as injection pressure, will have an impact on successful retention of the donor cells, although there are no reports that identified optimal IM cell injection methods [19].

Survival of injected cells in the heart following IM injection

The cells retained in the myocardium will interact with host tissues to survive with/without proliferation. It has been shown that retained cells in the myocardium are confronted with mechanical and oxidative insults, which inhibit the viability of the cells immediately after injection [22]. The reactive oxygen species are thought to stem from the dead donor cells and/or damaged host cells, both of which are mechanically injured during the cell injection procedure. This mechanical and biochemical insult subsequently provokes an acute inflammatory response, in which inflammatory cells are accumulated and inflammatory cytokines, such as IL-1β or TNF-α, are upregulated, leading to the inhibition of viability and functionality of the transplanted donor cells [22–24].

Distribution & integration of injected cells in the heart following IM injection

One of the important features of IM cell injection is that, after this method, transplanted, retained and surviving cells form islet-like cell clusters around the site of cell injection [25,26]. The clusters include not only donor cells, but also accumulated host inflammatory cells. Also, the islet-like cell clusters are often surrounded by fibrous components [25]. These make the most of donor cells isolated from host cardiac cells or existing ECM, limiting the interaction and integration of the donor cells to the host myocardium [25,27]. Although it is common that cell clusters become smaller and smaller with time after cell transplantation, the isolated clusters within the myocardium will interfere with electrical propagation of the heart, causing re-entry circuits and subsequent ventricular arrhythmias [27,28]. This complication has been extensively reported when SMBs, which rarely form gap junctions with native cardiomyocytes, were transplanted [17,29]. With regards to other cell types, such as bone marrow mononuclear cells (BMMNCs), animal studies showed arrhythmia occurrence after IM injection, including in BMMNCs [27], while there was no evidence of ventricular arrhythmia occurrence in clinical studies. Given that ventricular tachyarrhythmias in the early period post-IM cell injection were attenuated by systemic injection of corticosteroids [29], these arrhythmias may also be related to acute inflammatory responses associated with IM injection.

Clinically practical approaches for IM injection

There are three reported technical approaches for IM cell injection: epicardial, endocardial and transvascular routes [1,30–32]. Epicardial IM cell injection can be performed using direct vision of the heart via sternotomy or thoracotomy, while an endoscopic approach from a small subxyphoid incision for epicardial IM cell injection is now under development in large animal studies [33]. By contrast, endocardial or transvascular IM cell injection is carried out by the percutaneous transcatheter approach, such as the electrical mapping-based NOGA® Map system (Biologics Delivery Systems, CA, USA) [34] or the fluoroscopy-based Helix™ catheter injection system (Helical Infusion Catheter and Morph Guide Catheter, Biocardia Inc., CA, USA) [35,36]. These systems are useful to ensure cell injection into the myocardial area of interest. Transvascular IM cell injection also requires a catheterization laboratory and can be performed by using a percutaneously inserted catheter into the lumen of the cardiac vein or coronary artery, which is then inserted into the myocardial interstitium by penetrating the vacsulcar wall [37,38]. It was recently reported that cells can be injected into the perivascular space and myocardial interstitium through the vascular wall by using a microneedle catheter (Cricket™, MercatorMedSystems, Inc., CA, USA) [39]. There are no studies comparing the retention, survival and functionality of the transplanted cells among these different technical approaches. Percutaneous catheter-based IM cell injection procedures carry a risk of injecting the cells into the coronary circulation or the perivascular space. This risk might be avoided by concomitant guidance by transesophageal echocardiography.

Clinical studies of IM cell injection to the heart

A number of basic and clinical studies have proven that IM injection of adult stem/progenitor

cells, including mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), BMMNCs and SMBs, among others, is feasible, and induces functional recovery in the failing heart [40–43]. The major mechanism underlying the functional recovery is now believed to be the paracrine effect, in which the transplanted cells release a variety of cardioprotecive factors, such as growth factors, cytokines and chemokines, to attenuate adverse ventricular remodeling, including suppression of inflammation, attenuation of cardiomyocyte apoptosis, reduction of fibrosis, improvement of neovascular formation, enhancement of cardiomyocyte cellular function and activation of endogenous stem/progenitor cells. There is accumulating evidence that these adult stem cells do not differentiate into cardiomyocytes to a significant extent *in vivo* [44].

Since the first-in-man report of this method using SMBs [45], there are many, but relatively small-scale, clinical reports utilizing this route. Menasche *et al.* reported 6-month results of the placebo-controlled, randomized trial (MAGIC study), in which cultured, autologous SMBs were injected into the infarct myocardium via the epicardial IM approach in conjunction with coronary artery bypass grafting surgery [12]. This study revealed no statistically significant functional benefits with potential arrhythmogenicity by this treatment [12]. van Ramshorst *et al.* reported 6-month results of the placebo-controlled, randomized trial, in which fresh, autologous BMMNCs were injected into the infarct myocardium via the endocardial IM approach using the NOGA system without any additional interventions [10]. This study has proven the feasibility and safety of the method, while also revealing significant, but modest benefits in symptom and coronary perfusion of this treatment [10]. Losordo *et al.* reported 6-month results of the placebo-controlled, double-blinded, randomized trial in which $CD34^+$ circulating mononuclear cells were endocardially injected for intractable angina, suggesting positive effects of this treatment [46]. More recently, Perin *et al.* reported the 6-month results of the placebo-controlled, double-blinded, randomized trial in which autologous BMMNCs were endocardially injected for no-option patients with chronic myocardial infarction (MI), failing to show positive effects of this treatment [47,48]. In addition, there are currently many ongoing trials of IM injection of MSCs [36].

Enhancing direct IM cell injection

Strategies to enhance the effects of direct IM cell injection may be primarily designed to target the reduction in invasiveness, increase in donor cell retention, improvement of donor cell survival and/or attenuation of host myocardial damage.

Epicardial IM cell injection has been extensively investigated and used in a number of clinical studies, but this requires invasive surgical procedures, such as sternotomy or thoracotomy. More recently, the percutaneous transcatheter routes are more frequently used to achieve less invasiveness. However, sternotomy or thoracotomy is not disadvantageous when cell transplantation therapy is carried out in conjunction with other surgical treatments, such as coronary artery bypass grafting or left ventricular assist device implantation. However, for this therapy to be performed as a sole therapy, reduction of invasiveness is important. For this purpose, new technologies, including the thoracoscopy-guided approach, would be useful [49]. Transvascular IM injection, either via coronary artery or cardiac vein, is also less invasive to surgical epicardial IM injection. This method has already been applied to patients [37,38]; however, further investigation to confirm the efficiency and safety of this method is warranted.

A variety of treatments of donor cells – chemical, physical and genetic – prior to injection has been shown to improve retention and/or survival of donor cells. Our laboratory reported that pretreatment of the cells with superoxide dismutase or anti-IL-1β antibody was effective in reducing superacute (within 24 h) and acute (within 72 h) donor cell attrition, respectively [22,23]. Other pharmacological pretreatment with prosurvival factors, including cell-permeate peptide from Bcl-XL that blocks mitochondrial death pathways, cyclosporine A that attenuates cyclophilin D-dependent mitochondrial death pathways, pinacidil that opens ATP-dependent K^+ channels to mimic ischemic preconditioning, IGF-1 that activates Akt pathways and a caspase inhibitor ZVAD-fmk, have been suggested to be useful in enhancing survival of the transplanted cells in the myocardium [16,50]. We have reported that physical treatment of donor cells with heat shock prior to injection improved tolerance of the cells to hypoxia–reoxygenation *in vitro* and enhanced their survival after epicardial IM injection *in vivo* in rats [51]. In addition, the use of biomaterials, such as Matrigel™ (Becton Dickinson & Co., NJ, USA), gelatin hydrogel or fibrin polymer, as a vehicle of the cells has been shown to improve retention for the cells and protect the cells from mechanical injury and chemical stress, so that cell survival and integration are improved [52–55]. Furthermore, injection of donor cells as cell

clusters, in which intercellular connections and ECM are preserved, may enhance the retention, viability and functionality of the cells, compared with injection of cell suspensions [20]. Genetic manipulation of donor cells will also enhance survival of donor cells after transplantation. It has been suggested that transduction of IL-1 receptor antagonist [24], IGF-1 [56,57], VEGF-1 [58,59] or Akt [60] were effective in protecting donor cells from necrosis or apoptosis following injection into the heart. More simply, increasing the number of injection sites and reducing the cell number injected in each site might be useful in enhancing the retention efficiency [19].

Treatment of the host myocardium before and/or after cell transplantation is another possible option to protect the transplanted cells and/or the myocardium itself from the inflammatory insult related to IM cell injection. Clinical studies have suggested that systemic administration of amiodarone, which stabilizes electrical conductance, and corticosteroids, which inhibits accumulation of inflammatory cells, are effective in reducing cell injection-related inflammation and arrhythmogenicity [40,61].

IC cell injection

IC cell injection can be conducted by two different methods: injection into the coronary artery (antegrade IC injection) or injection into the cardiac vein (retrograde IC injection). Since the first clinical report in 2001 [62], an increasing number of small-to-large scale clinical studies have confirmed the feasibility and safety of antegrade IC cell injection [3–5,11]. By contrast, there is a lack of a reproducible experimental model for this method in small animals, limiting our indepth understanding of this method. Reproducible antegrade IC cell injection requires appropriate insertion of the catheter into a target coronary artery; however, such a catheter or technique in small animals has not been established. This is simply because of the small size of the animals.

Initial donor cell retention following IC cell injection

Our recent study using an original *ex vivo* system in mice showed that only 15% of injected BMMNCs were retained in the normal heart at 10 min after antegrade IC injection [63]. This poor retention rate is consistent with that in large animal and clinical studies [21], and is one of the most important issues associated with IC cell injection.

Successful retention of donor cells that are injected into the heart by the IC route requires at least two biological steps, which are likely to be similar to the accumulation of inflammatory cells in the myocardium when the heart is injured or inflamed. First, the cells injected via either the antegrade or retrograde IC route need to attach to the coronary endothelium to be retained in/migrate into the heart, otherwise injected cells will just be flushed out into the systemic circulation. This initial attachment of the donor cells to the host endothelium can be established by active intercellular connections mediated by a variety of adhesion molecules or by passive contact between donor and endothelial cells. Then, the attached donor cells must establish firm adhesion to the host endothelial cells to carry out extravasation to migrate into the host myocardial interstitium or integrate into the vascular walls. Types and levels of adhesion molecules expressed in the donor cells are different between different cell types and changeable by the donor cell preparation method. This will affect the efficiency of the active attachment of the donor cells to the host endothelium. Expression of intercellular adhesion-related molecules on the endothelium is also an important determinant to active retention. Size, shape or concentration of donor cells, volume of cell suspensions and cell-injection pressure would affect the rate of passive attachment. Therefore, conditions of both donor cells and the host myocardium are important determinants of the initial retention of donor cells injected via the IC route.

Subsequently, adhered donor cells to the endothelium need to undergo transendothelial migration or integration in the vascular walls for long-term survival and functional integration. This would again be affected by the communication between injected cells and host endothelial cells, and the ECM in the myocardium. The mechanism underlying this event is largely unknown. Further investigation is warranted.

Donor cell survival following IC cell injection

It has been shown that IC cell injection results in less mechanical injury or biochemical stress to donor cells than IM cell injection does. In addition, the retained/migrated cells after IC injection usually stay adjacent to the vessels, suggesting that they are less likely to be confronted with ischemic insult. However, previous studies have shown that survival of the cells that were injected via the IC route is comparable with or lower than that of those injected via the IM route [27]. Little is known about the mechanism or determinants of survival of the retained cells after IC injection, which need to be identified.

Clinically practical approaches for IC cell injection

Antegrade IC cell injection is performed using the same approach with similar equipment/materials as the routine percutaneous coronary intervention [5,64,65]. This method has been widely used in clinical studies of BMMNC injection with negligible complication, whereas SMBs or MSCs, which are larger in cell size than BMMNCs, are suggested to carry a risk of coronary embolism when they are injected via this route [66,67]. Therefore, to assure the feasibility and safety of antegrade IC cell injection, donor cell type-specific consideration for potential risk of coronary embolism will be needed. On the other hand, retrograde IC injection requires a specific catheter with an occlusion balloon, which is percutaneously inserted into the great cardiac vein via the coronary sinus. It has been shown that the risk of coronary embolism is not substantial by retrograde IC cell injection even when injecting large cell, such as SMBs [68,69]. Retention, survival and integration of the transplanted cells are likely to be similar between the two IC cell delivery methods [21].

Clinical studies of IC cell injection into the heart

A number of clinical studies have been completed or are ongoing to assess the feasibility, safety and efficacy of IC injection of adult stem/progenitor cells into the heart. Four major placebo-controlled randomized clinical studies, in which autologous BMMNCs were injected via the antegrade IC route to treat acute MI, were published in 2006 [3–5,11]. While Lunde *et al.* [3] and Janssens *et al.* [11] reported no therapeutic effects by this treatment, Schachinger *et al.* [4] and Meyer *et al.* [5] reported significant but modest improvement in left ventricular ejection fraction post-treatment. Following these studies, a number of randomized controlled studies have been performed using a similar study design, consistently showing some modest therapeutic effects of antegrade IC injection of autologous BMMNCs on acute and chronic MI, as summarized in two recently published meta-analysis studies [70,71].

Clifford *et al.* demonstrated a preserved ejection fraction and reduced infarct size by this treatment for acute MI compared with those by placebo control [70]. On the other hand, Jeevanantham *et al.* did not identify significant differences in the magnitude of functional benefits between the IM and the IC cell-delivery routes in treating chronic MI [71]. More recently, antegrade IC injection is used in patients with other donor cell types including cardiac progenitor cells or cardiosphere-derived cells, providing encouraging, although preliminary, data on the safety and efficiency of the treatment [72,73].

Enhancing IC cell injection

One of the major issues of IC cell injection is the lack of appropriate small animal models, which largely limit our knowledge of the mechanism underlying donor cell retention, survival and integration after this injection method. Although we have recently reported a useful mouse model using a modified Langendorff isolated heart perfusion [63], further development is needed. There are at least two major strategies to refine the efficacy of IC cell injection; enhancing initial retention of donor cells and enhancing survival of retained donor cells.

Donor cell retention following IC injection would be improved by pretreatment of donor cells, modulation of the condition of the host myocardium and/or optimization of injection method. Physical or chemical treatment of donor cells prior to injection into the heart using brief hypoxia, heat shock or cytokine administration would modulate expression of the adhesion molecules and might enhance active attachment of the cells to the endothelium and/or transendothelial migration [74,75]. In addition, treatment of the host myocardium prior to cell injection, such as ischemic preconditioning or drug administration, could upregulate expression of the adhesion molecules, such as P-selectin, ICAM-1 and/or VCAM-1 [76,77], which possibly enhances the active attachment of the donor cells to the endothelium and/or transendothelial migration. In fact, temporary balloon occlusion of the coronary arteries, for which the cells are injected, is the standard procedure in antegrade IC cell injection in clinical studies [3,11,78]. This procedure aims to increase pressure and contact time of donor cells with the endothelium and, in addition, it may upregulate expression of cell retention-related adhesion molecules. Balloon occlusion of the coronary artery into which donor cells are injected is, therefore, a potential method to enhance the retention of donor cells in IC cell injection. However, there is a negative report on the effect, in which 3-min balloon occlusion did not alter retention of the donor cells compared with no occlusion in a porcine model [79]. Further studies are warranted on the effects of balloon occlusion [79].

Moreover, it has been suggested that expression of SDF-1 in the myocardium enhances

recruitment of the circulating cells expressing CXCR4 into the injured myocardial territory to contribute to healing and regeneration of the tissue. This CXCR4–SDF-1 axis may also be a target to enhance IC cell injection [80]. Further preclinical optimization is essential for these treatments to be applicable in the clinical arena.

Enhancing survival of retained donor cells is also a strategy to enhance donor cell engraftment after IC cell injection. For this aim, the strategies to improve donor cell survival after IM injection, which are discussed in the section of IM cell injection, are similarly applicable, although these have not been studied in the scenario of IC cell injection.

Use of the retrograde IC route is theoretically advantageous in donor cell retention compared with the antegrade IC route, as retrograde IC injection would allow a longer time for the injected cells to interact with coronary vasculatures, in particular at postcapillary venules, by occluding the stem of the cardiac vein, compared with antegrade IC injection. In addition, retrograde IC injection enables a widespread cell delivery regardless of occluded arteries (i.e., into the normal, ischemic and infarcted areas) [68,69]. By contrast, antegrade IC cell injection after reopening the responsible coronary artery for treating acute MI might result in limited cell delivery due to impairment of coronary endothelial function, such as 'no-reflow' phenomena [81]. Although it was reported that cell retention is not significantly different between the antegrade and the retrograde cell injection [21], the protocol was much less optimized in retrograde IC injection. Appropriate optimization of the injection protocol, such as injection pressure, cell density or occlusion time, might yield different results.

iv. cell injection

Other possible routes for cell delivery into the heart include systemic iv. injection [82,83]. This method is much less invasive and more economical than other methods, and thus draws the interest of many scientists and clinicians. However, this systemic route via either peripheral vein, central vein or right/left atrium is highly likely to result in severely limited efficiency in delivering donor cells into the heart, as cells injected via this route are largely entrapped in other organs, such as the lung, prior to reaching the heart. Aicher *et al.* reported that iv. injection of radiolabeled EPCs of human origin into athymic nude rats following MI yielded only 2% of the total radioactivity retained in the heart, with 70% being retained in the liver and the spleen after 24–96 h [84]. This finding has been replicated by other studies using BMMNCs [85] or purified $CD34^+$ cells [86].

Success of this route is, therefore, dependent on the improvement of cell delivery efficiency to the heart. This may be achieved by amplifying the homing signals that prompt donor cells to effectively recruit/migrate to the host myocardium. Interestingly, it is reported that MSCs are preferentially accumulated in the failing heart after iv. injection [86–88], suggesting that efficacy of homing to the heart is donor-cell-type specific. In addition, the condition of the host myocardium, particularly the coronary endothelium, will be important for effective homing of donor cells into the heart. Importantly, Hare *et al.* reported 6-month results of a double-blind, placebo-controlled clinical study, in which allogeneic MSCs were intravenously injected, on treating acute MI postreperfusion, showing improvement in symptom and ejection fractions of the LV by this treatment [89]. Further investigations to understand and enhance homing signals in both donor cells and the host myocardium, which should be donor-cell-type specific, are essential for the future success of iv. cell injection.

Epicardial cell placement: the cell-sheet technique

Epicardial cell placement is an emerging method for cell delivery to the heart. This became practical since the development of the 'cell sheet' technique by Okano *et al.* [90] and Sawa *et al.* [91].

Principal concept of the cell-sheet method

It is now possible to generate scaffold-free cell sheets by using the thermoresponsive culture dishes (Cell Seed Inc., Tokyo, Japan), which are coated with a temperature-responsive polymer that is hydrophilic at 37°C, while hydrophobic below 25°C [92]. These dishes allow cells to attach to the bottom and grow at 37°C as usual. However, by decreasing the culture temperature to 25°C or below, the confluent cells are spontaneously detached from the dish, generating a scaffold-free cell sheet.

Donor cells contained in the cell sheet are advantageous as donors for cell transplantation over those in cell suspensions. Due to cell dissociation procedures, such as trypsinization, the cells prepared as cell suspensions will have suffered damages before transplantation; cell surface proteins, intercellular connections and underlying ECM can be damaged. By contrast, the donor cells prepared as the cell sheet preserved