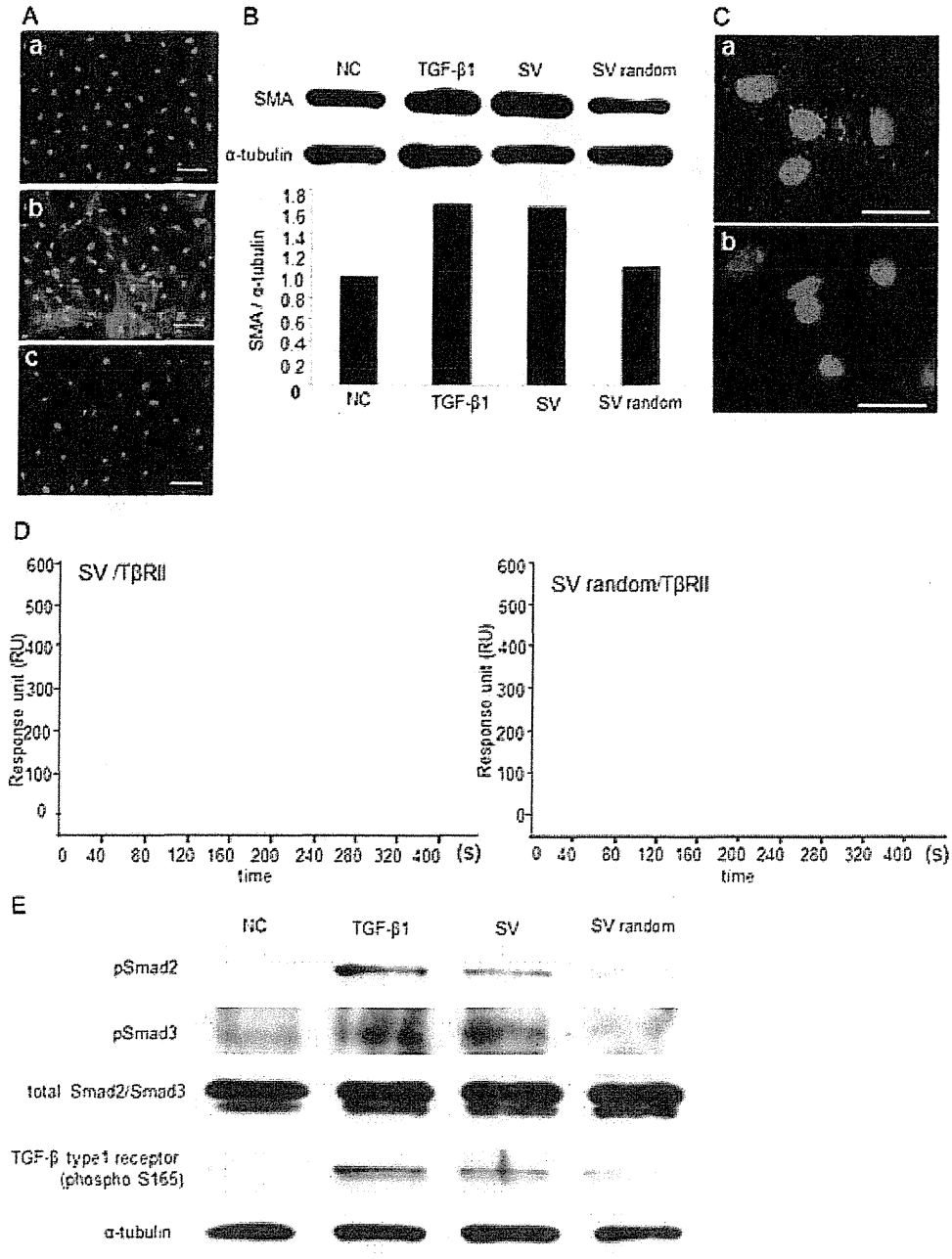

Figure 4 Myofibroblast differentiation induced by exposure of the isolated CFs to SV. (*A*) and (*B*) SMA expression induced by SV. (*A*) Immunofluorescence staining with an anti-SMA antibody: (*a*) non-stimulated CFs (NC); (*b*) CFs exposed to SV; and (*c*) CFs exposed to SV random peptide (×200 magnification, 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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Spatially Oriented, Temporally Sequential Smooth Muscle Cell-Endothelial Progenitor Cell Bi-Level Cell Sheet Neovascularizes Ischemic Myocardium

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Spatially Oriented, Temporally Sequential Smooth Muscle Cell-Endothelial Progenitor Cell Bi-Level Cell Sheet Neovascularizes Ischemic Myocardium

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Background—Endothelial progenitor cells (EPCs) possess robust therapeutic angiogenic potential, yet may be limited in the capacity to develop into fully mature vasculature. This problem might be exacerbated by the absence of a neovascular foundation, namely pericytes, with simple EPC injection. We hypothesized that coculturing EPCs with smooth muscle cells (SMCs), components of the surrounding vascular wall, in a cell sheet will mimic the native spatial orientation and interaction between EPCs and SMCs to create a supratherapeutic angiogenic construct in a model of ischemic cardiomyopathy.

Methods and Results—Primary EPCs and SMCs were isolated from Wistar rats. Confluent SMCs topped with confluent EPCs were spontaneously detached from the Upcell dish to create an SMC-EPC bi-level cell sheet. A rodent ischemic cardiomyopathy model was created by ligating the left anterior descending coronary artery. Rats were then immediately divided into 3 groups: cell-sheet transplantation (n=14), cell injection (n=12), and no treatment (n=13). Cocultured EPCs and SMCs stimulated an abundant release of multiple cytokines in vitro. Increased capillary density and improved blood perfusion in the borderzone elucidated the significant in vivo angiogenic potential of this technology. Most interestingly, however, cell fate–tracking experiments demonstrated that the cell-sheet EPCs and SMCs directly migrated into the myocardium and differentiated into elements of newly formed functional vasculature. The robust angiogenic effect of this cell sheet translated to enhanced ventricular function as demonstrated by echocardiography.

Conclusions—Spatially arranged EPC-SMC bi-level cell-sheet technology facilitated the natural interaction between EPCs and SMCs, thereby creating structurally mature, functional microvasculature in a rodent ischemic cardiomyopathy model, leading to improved myocardial function. (***Circulation.*** **2013;128[suppl 1]:S59–S68.)**

Key Words: angiogenesis ■ cardiovascular diseases ■ cells ■ endothelium ■ heart failure ■ tissue

Heart failure is the leading cause of death in the United States, with a 5-year mortality of 50%. Current treatment for heart failure entails medical optimization, along with limited revascularization and reconstructive techniques. These interventions do not address the microvascular deficiencies that develop in ischemic cardiomyopathy (ICM). Myocardial regenerative and cellular therapy is attracting growing interest as a means to improve left ventricular (LV) function in advanced heart failure. Among the many candidate cells, endothelial progenitor cells (EPCs), the precursor of blood vessels, have demonstrated excellent potential for therapeutic angiogenesis. Recent reports show beneficial effects of EPC transplantation therapy in several animal experimental models and patients with heart failure.[1–3]

The mechanism by which damaged myocardium is restored by transplanted EPCs is complex and involves many pathways. Recent large-scale clinical trials, in which EPCs were delivered using direct myocardial injection[4] or catheter-based intracoronary procedures,[5,6] reported only modest therapeutic benefits. The limited benefits are at least partially because of poor localized cell survival after transplantation, thereby greatly attenuating the angiogenic potential of EPC therapy. In addition, mature vasculature requires the presence of supporting elements, such as smooth muscle cells (SMCs), which are not delivered with simple EPC injection. In contrast, cell-sheet technology delivers cells more effectively with minimal cell dispersion and myocardial injury and improves microvascular structure, leading to better cardiac function than that attained

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by intracoronary injection or needle injection.[7–11] Specifically, the cell-sheet technology enables the construction of a cellular system that mimics the natural architecture of a desired tissue. Here, the proposed angiogenic therapy uses the cell sheet to optimize the spatial arrangement of EPCs and SMCs to maximally induce structurally mature vasculature. The cell sheet is generated on and removed from special dishes that are grafted with a temperature-responsive polymer that changes from hydrophobic to hydrophilic when the temperature is lowered. The greatest advantage of this technique is that the cell sheet consists of densely adherent cells without requiring an artificial scaffold, it is easily manipulated and has a high ability to integrate with native tissues without destroying the cell–cell or cell–extracellular matrix (ECM) adhesions in the cell sheet.[7] In addition, we focused on the concept that the natural endothelial–pericyte spatial relationship and interaction are crucial for vessel maturation and stabilization. Thus, we hypothesized that SMCs, which are components of vascular pericytes, would enhance EPC-mediated angiogenesis and facilitate blood vessel maturation. Neoangiogenesis should yield increased blood perfusion and restoration of cardiomyocyte viability. To demonstrate clear and direct contribution of the cell-sheet EPCs and SMCs to neovasculature, we constructed multiple fate-tracking experiments. A labeled cell sheet was created with EPCs from female rats ubiquitously expressing the enhanced green fluorescent protein (GFP), along with SMCs from male rats. This cell sheet with trackable elements was then implanted in female rats.

In short, this study examined the functional benefits of transplanting the bi-level cell sheet created from cocultured EPCs and SMCs in an ICM model, compared with direct myocardial needle injection.

Methods

Isolation of EPCs and SMCs

Wistar rats were administered pentobarbital (100 mg/kg, IP), and then the carotid artery was dissected and transected. Bone marrow mononuclear cells were isolated from the long bones of rats by density gradient centrifugation with Histopaque 1083 (Sigma-Aldrich) and cultured in endothelial basal medium-2 supplemented with EGM-2 SingleQuot (Lonza) containing human epidermal growth factor, 5% fetal bovine serum (Sigma-Aldrich), vascular endothelial growth factor (VEGF), basic human fibroblast growth factor, recombinant human long R3 insulin-like growth factor-1, ascorbic acid, gentamicin, and amphotericin B. The combination of endothelium-specific media and the removal of nonadherent bone marrow mononuclear cells were intended to select for the EPC phenotype. EPCs were cultured for 7 days in the same medium.[2] For EPC fate tracking, we used GFP transgenic female Wistar rats.

SMCs were isolated from the thoracic aorta of wild-type male Wister rats (3 weeks old; Charles River) by primary explant technique[12] and cultured in DMEM with 20% fetal bovine serum, gentamicin, and amphotericin B to confluency for 7 days at 37°C and 5% CO_2. For SMC fate tracking, we used male Wistar rats.

Bi-Level Cell-Sheet Preparation

The SMCs were plated at 1.5×10^5/cm^2 in a 35-mm Upcell dish, which is grafted with temperature-responsive polymers (CellSeed, Tokyo, Japan), and then cultured in EPC-specific medium. After 24 hours of culture at 37°C and 5% CO_2, EPCs were added at 1.5×10^5/cm^2 onto the Upcell dish, which was already confluent with SMCs. After 24 additional hours in culture, the dishes were transferred to another incubator, set at 20°C, for 1 hour to release the cultured cells as an intact cell sheet. Under this protocol, confluent SMCs topped with confluent EPCs were spontaneously detached from the plate as a sequentially cocultured and specifically spatially oriented SMC-EPC bi-level cell sheet (Figure 1A).[10,11]

Production and Release of Cytokines/Chemokines

To demonstrate proangiogenic biological activity, supernatant of the cocultured cells (EPCs: 1.5×10^5/cm^2, SMCs: 1.5×10^5/cm^2), EPCs (3.0×10^5/cm^2), or SMCs (3.0×10^5/cm^2), after being cultured for 24 hours, was centrifuged to remove debris and contaminating cells. Levels of VEGF, hepatocyte growth factor (HGF), transforming growth factor-β (TGFβ), and stromal cell–derived factor 1α (SDF1α) in the culture supernatants were analyzed by ELISA kit (Quantikine, R&D Minneapolis, MN; n=6 in each). ELISA was performed in duplicate.

Assessment of Cytokine Receptor Expressions by Flow Cytometry

To elucidate the biological impact of cocultured EPCs and SMCs on fetal liver kinase 1 (FLK1) and VEGF receptor 2 (VEGFR2) expression, flow cytometry was used in the EPC or SMC cultured with SMC or EPC using the transwell inserts, supplemented with recombinant VEGF, or media only for 24 hours (n=5 in each). The amount of VEGF was determined based on the results of ELISA. Test samples were incubated for 1 hour at room temperature with either mouse monoclonal anti-FLK1 (Santa Cruz Biotechnology) or rabbit anti-VEGFR2 (Abcam). After washing with cold fluorescence-activated cell sorter buffer, cells were incubated at room temperature

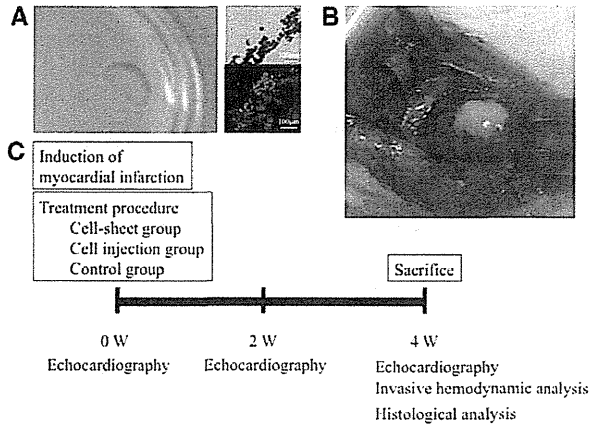

Figure 1. Preparation and transplantation of bi-level cocultured cell-sheet containing both endothelial progenitor cells (EPCs) and smooth muscle cells (SMCs). **A**, Confluent SMCs topped with confluent EPCs were spontaneously detached from an Upcell dish, which is grafted with temperature-responsive polymers (CellSeed, Tokyo, Japan), as a sequentially cocultured and specifically spatially oriented SMC-EPC bi-level cocultured cell-sheet. Hematoxylin-eosin staining; cross-sectional views of cell-sheet in vitro. Cocultured bi-level cell sheet maintained green fluorescent protein (GFP)-positive EPCs and Y chromosome–positive SMCs in separate layers in vitro. Red indicates rat Y chromosome; green, GFP. **B**, Bi-level cocultured cell-sheet, which consisted of 1.3×10^6 EPCs and 1.3×10^6 SMCs, was placed into the epicardium of the left ventricle covering the ischemic area. **C**, Study protocol used for assessment of cardiac function and histology. Wistar rats underwent induction of myocardial infarction by occluding the LAD permanently, followed by the concurrent treatment procedure. Cardiac function was assessed by echocardiography just before and at 2 and 4 weeks after the treatment procedure. Four weeks after the treatment procedure, invasive hemodynamic analysis and histological examination were performed after euthanasia.

for 30 minutes with Alexa 647 chicken anti-mouse IgG (Invitrogen) and Alexa 488 donkey anti-rabbit IgG (Invitrogen). The percentage of cells expressing each cell surface antigen was analyzed with a Becton Dickinson FACSCalibur flow cytometer. Data analysis was performed using FlowJo 8.8.3 (Tree Star Inc).[3,13]

Rat ICM Model and Cell-Sheet Transplantation

Female Wistar rats (8 weeks old, 250–300g; Charles River) were anesthetized with ketamine (75 mg/kg IP) and xylazine (7.5 mg/kg IP), intubated in an endotracheal manner with a 19-gauge catheter, and mechanically ventilated (Hallowell EMC). Anesthesia was maintained by inhalation of 2.0% isoflurane (Clipper Distributing Company Llc, Saint Joseph, MO).

The proximal left anterior descending coronary artery (LAD) of Wistar rats was permanently occluded using a left thoracotomy approach. This produced a consistent and reproducible myocardial infarction encompassing 35% to 40% of the left ventricle.[1–3] Within 5 minutes after LAD ligation, the rats were allocated into 3 groups by simple randomization, considering that there were no differences among the animals at this time point: those that underwent cocultured cell-sheet transplantation (cell-sheet group, n=14), those that underwent cocultured cell injection (cell injection group, n=12), and those that underwent no intervention (control group, n=13). The rats were allowed to recover under care.

In the cell-sheet group, the cocultured bi-level cell sheet, which consists of 1.3×10^6 EPCs and 1.3×10^6 SMCs, was placed on the epicardium covering the ischemic area (Figure 1B). The cell injection group received 1.3×10^6 EPCs and 1.3×10^6 SMCs, diluted in saline for a total volume of 200 μL by direct intramyocardial injection with a 30-gauge needle. Each rat received the same number of cells. Animals were then kept in temperature-controlled individual cages for 4 weeks.

The rats were euthanized at 4 weeks after surgery by intravenous injection of 200 mg/kg of pentobarbital and 2 mEq/kg of potassium chloride, under terminal anesthesia, and the heart was excised.

Histological and Immunohistochemical Analyses

Four weeks after treatment, the hearts were dissected and embedded in optimum cutting temperature compound for 10-μm-thick cryosections. The cryosections were used for routine hematoxylin-eosin staining to assess the myocardial structure. Masson trichrome staining was performed to assess cardiac fibrosis in the peri-infarct borderzone. The fibrotic region was calculated as the percentage of myocardial area. The data were collected from 5 individual views per heart at a magnification of ×200. The heart cryosections were also stained with an antibody to von Willebrand factor (vWF; 1:200 dilution; Abcam) to assess capillary density, which was calculated as the number of positively stained capillary vessels in 5 randomly selected fields in the peri-infarct borderzone area, per heart. The cryosections were also stained with an antibody to proliferating cell nuclear antigen (1:200 dilution; Abcam) to assess cellular proliferative activity in 5 randomly selected fields in the peri-infarct borderzone area. The cryosections were also stained with an antibody to integrin β1 (1:100 dilution; Abcam) to estimate cell–matrix attachment in 5 randomly selected fields in the peri-infarct borderzone area. Cell nuclei were counterstained with 6-diamidino-2-phenylindole (Invitrogen). The images were examined by fluorescence microscopy (Leica). Image J software was used for quantitative morphometric analysis.

EPC-SMC Fate Tracking

The cell sheet, which consisted of EPCs from GFP transgenic female Wistar rats and SMCs from non-GFP male Wistar rats, was transplanted into the female Wistar rat heart. To detect the fate of EPCs, cryosections were stained with an anti-vWF antibody (1:1000 dilution; Abcam), anti–smooth muscle actin (SMA) antibody (1:1000 dilution; Abcam), anti–vascular endothelial-cadherin antibody (1:1000 dilution; Santa Cruz), and anti-GFP antibody (1:1000 dilution; Abcam). The secondary antibodies were Alexa Fluor 555 donkey anti-rabbit IgG (1:1000 dilution; Invitrogen) and Alexa Fluor 555 donkey anti-mouse IgG (1:1000 dilution; Invitrogen). To detect the fate of SMCs, fluorescence in situ hybridization was performed on cryosections, which were then stained with anti-SMA antibody (1:500 dilution; Abcam). The secondary antibody was Alexa Fluor 555 donkey anti-rabbit IgG (1:500 dilution; Invitrogen). Cell nuclei were counterstained with 6-diamidino-2-phenylindole. GFP-positive cells and rat Y chromosome–positive cells were counted, respectively, and corrected by total number of tissue cells to estimate the survival cells quantitatively. GFP- and vWF-positive cells were counted and corrected by total number of GFP-positive cells to examine vascular regeneration. Rat Y chromosome– and SMA-positive cells were counted and corrected by total number of rat Y chromosome–positive cells to examine vascular regeneration.

Myocardial Perfusion Analysis

To quantify myocardial perfusion, at 4 weeks after treatment 200 μg of fluorescein-labeled *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories) was injected into the supradiaphragmatic inferior vena cava and allowed to circulate for 10 minutes. After lectin perfusion, the hearts were explanted and snap-frozen in liquid nitrogen. One-hundred twenty sequential images were obtained through 100-μm thick myocardial sections at the level of the papillary muscle using scanning laser confocal microscopy (z-series, ×20 air magnification, Zeiss LSM-510 Meta Confocal Microscope). Three-dimensional reconstructions of the image stacks were created using Velocity Software v.3.61 (Improvision). Fluorescein-labeled voxels were quantified as a percentage of total tissue section voxels, creating a quantifiable measurement of perfusion per unit of myocardial tissue volume.[2,3]

Echocardiographic Assessment

Echocardiography was performed under general anesthesia using 1.0% inhaled isoflurane just before and at 2 and 4 weeks after the treatment procedure (SONOS 7500, Philips Medical Systems, Andover, MA) with a 12-MHz transducer at an image depth of 2 cm (cell sheet, n=7; cell injection, n=8; control, n=9; Figure 1C). LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and end-diastolic anterior wall thickness at the level of the papillary muscles were measured for ≥3 consecutive cardiac cycles following the American Society for Echocardiology leading-edge method. Fractional shortening (FS) and ejection fraction (EF) were calculated as parameters of systolic function.[2,3,8] All analyses were performed by a single investigator in a group-blinded fashion.

Invasive Hemodynamic Assessment

Four weeks after the treatment procedure, animals (cell-sheet, n=6; cell injection, n=6; control, n=8) underwent invasive hemodynamic measurements with a pressure–volume conductance catheter (SPR-869; Millar Instruments, Inc; Figure 1C). The catheter was calibrated via 5-point cuvette linear interpolation with parallel conductance subtraction by the hypertonic saline method.[2,3] Rats were anesthetized using 1.0% inhaled isoflurane, and the catheter was introduced into the LV with a closed-chest approach via the right carotid artery. Measurements were obtained before and during inferior vena cava occlusion to produce static and dynamic pressure–volume loops under varying load conditions. Data were recorded and analyzed with LabChart version 6 software (AD Instruments) and ARIA Pressure Volume Analysis software (Millar Instruments, Inc). After hemodynamic assessment, the heart was removed for further histological analyses.

Statistical Analysis

Continuous variables are expressed as mean±SE. Comparisons between 2 groups were made using the Wilcoxon–Mann–Whitney *U* test because of small sample sizes. For comparisons among 3 groups, we used the Kruskal–Wallis test, followed by the post hoc pairwise Wilcoxon–Mann–Whitney *U* test. The multiplicity in pairwise comparisons was corrected by the Bonferroni procedure. A $P<0.05$ was

considered statistically significant. All statistical calculations were performed using SPSS software (version 11.0; SPSS Inc, Chicago, IL) and JMP 9.0 (SAS Institute Inc, Cary, NC).

Animal Care and Biosafety

Wistar rats were obtained from Charles River. Food and water were provided ad libitum. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol 803394).

Results

Production and Release of Cytokines/Chemokines by Coculturing EPC With SMC

VEGF was significantly higher in the coculture supernatant than the SMC-only group and tended to be higher than the EPC-only group (Figure 2A). The secretion of HGF was remarkably enhanced in the coculture supernatant, whereas HGF levels were not evident in either the EPC- or SMC-alone group (Figure 2B). The concentration of TGFβ was significantly higher in the coculture supernatant than both the EPC- and SMC-only groups (Figure 2C). The secretion of SDF1α was remarkably higher in the cocultured group compared with EPC and SMC alone (Figure 2D).

Upregulated Expressions of FLK1 and VEGFR2 on Either EPC or SMC Under Cytokines-Rich Medium of SMC or EPC

Flow cytometric analysis demonstrated that the percentage of FLK1$^+$ EPCs and VEGFR2$^+$ EPCs in total EPC population was 1.3±0.3% and 3.2±0.8%, respectively. Supplementation with VEGF significantly increased the percentage of FLK1$^+$ EPCs (17.2±3.2%) and VEGFR2$^+$ EPCs (32.0±5.4%). Furthermore, the percentage of FLK1$^+$ and VEGFR2$^+$ EPCs was significantly greater after coculturing with SMC (FLK1$^+$, 39.6±9.2%; VEGFR2$^+$, 52.5±9.8%; Figure 3A and 3B).

Flow cytometric analysis demonstrated a statistically significant increase in the percentage of FLK1$^+$ SMCs cocultured with EPC compared with SMC alone (75.7±5.4 versus 23.9±2.5%; P=0.02). Addition of VEGF significantly increased FLK1$^+$ SMCs compared with SMC (Figure 3C and 3D). There was no significant difference in the VEGFR2$^+$ expression on SMCs (P=0.14, Kruskal–Wallis test).

Enhanced Capillary Density and Microvascular Perfusion After Cocultured Cell-Sheet Transplantation

A large number of vWF-positive blood vessels were detected in the peri-infarct borderzone myocardium after cell-sheet therapy compared with injection alone (Figure 4A). This demonstrated a superior enhancement of capillary density in the cell-sheet group (Figure 4B).

Similarly, lectin microangiography of the peri-infarct borderzone myocardium sections revealed a more densely and well-developed capillary network in the cell-sheet group compared with injection alone (Figure 4C). Quantitative analysis showed significantly enhanced perfusion in the peri-infarct borderzone myocardium in the cell-sheet group (Figure 4D).

Enhanced Cell Proliferation Activity After Cocultured Cell-Sheet Transplantation

A large number of proliferating cell nuclear antigen-positive cells were identified in the peri-infarct borderzone myocardium after cell-sheet therapy compared with control (Figure 4E and 4F).

Migration of EPCs and SMCs to Myocardium Contributing to Neovasculature

Cocultured bi-level cell sheet contained GFP-positive EPCs and Y chromosome–positive SMCs in separate layers in vitro (Figure 1A).

Four weeks after transplantation, the GFP-positive EPCs were detected in the myocardium at the transplanted site at an appropriate depth of 650 μm (Figure 4G). Immunostaining for vWF and GFP showed that transplanted EPCs were able to contribute to neovascularization of the host myocardium (Figure 4H). This was further supported by immunostaining for vascular endothelial-cadherin and GFP (Figure 4I). In addition, staining with antibody to SMA and GFP indicated that GFP-positive EPCs originating from the transplanted cocultured bi-level cell sheet migrated into the engineered

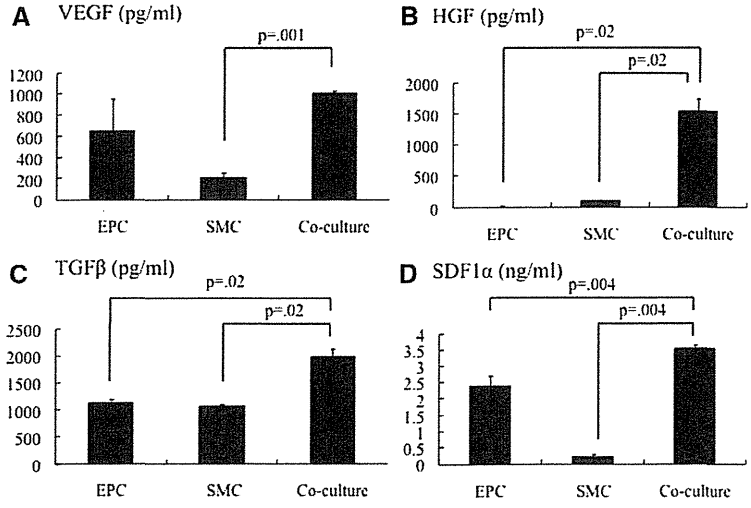

Figure 2. A, Vascular endothelial growth factor (VEGF), (**B**) hepatocyte growth factor (HGF), (**C**) transforming growth factor-β (TGFβ), and (**D**) stromal cell–derived factor 1α (SDF1α) in the culture supernatant, measured by ELISA. Cocultured endothelial progenitor cells (EPCs) with smooth muscle cells (SMCs) secreted abundant VEGF, HGF, TGFβ, and SDF1α compared with either EPC or SMC (n=6 in each; VEGF, P=0.002; HGF, P=0.01; TGFβ, P=0.01; SDF1α, P=0.001; Kruskal–Wallis test).

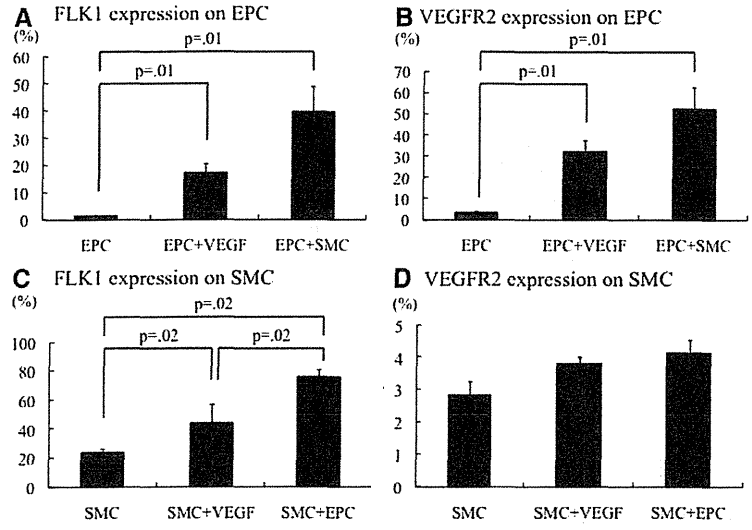

Figure 3. To elucidate the biological impact of cocultured endothelial progenitor cell (EPC)-smooth muscle cells (SMCs) on FLK1 and vascular endothelial growth factor receptor 2 (VEGFR2) expression, flow cytometry was used to study both EPC and SMC expression of these markers in the following settings: cocultured, cultured with VEGF, and cultured alone. The amount of VEGF used was determined based on the results of ELISA. **A** and **B**, The percentage of FLK1$^+$ EPC and VEGFR2$^+$ EPC was greatest in a cocultured setting (n=5 in each; FLK1 expression on EPC, P=0.01; VEGFR2 expression on EPC, P=0.01; Kruskal–Wallis test). **C** and **D**, Analysis of SMC FLK1$^+$ expression demonstrated a significant increase in the cocultured group. There was no significant difference in the VEGFR2$^+$ expression on SMC (n=4 in each; FLK1 expression on SMC, P=0.01; VEGFR2 expression on SMC, P=0.14; Kruskal–Wallis test).

myocardial tissues and were circumferentially surrounded by SMA-positive tissues (Figure 4J). Finally, to track SMCs from the cell sheet, we performed fluorescence in situ hybridization immediately to identify male SMCs in the female recipient. After the cell-sheet transplantation, GFP-positive EPCs and Y chromosome–positive SMCs were detected with a thickness of ≈50 μm into the epicardium (Figure 4K). Rat Y chromosome SMCs were partially able to differentiate into SMA-positive tissues (Figure 4L). Quantitative analysis showed a greater percentage of GFP-positive cells and rat Y chromosome–positive cells, respectively, in the cell-sheet group compared with cell injection (Figure 4M). Quantitative analysis of vascular regeneration showed that the number of both GFP- and vWF-positive cells is 18±3/hpf (60% of GFP-positive cells), which participated in new blood vessel formation. In addition, the number of both Y chromosome– and SMA-positive cells is 7±2/hpf (45% of rat Y chromosome cells), which participated in new blood vessel formation. One week after treatment, a large number of integrin β1–positive cells were observed in the peri-infarct borderzone myocardium after cell-sheet therapy compared with cell injection and control (Figure 4N and 4O).

LV Remodeling After Cell-Sheet Transplantation

The LV myocardial structure was superiorly maintained after cell-sheet transplantation compared with cell injection and control, as assessed by hematoxylin-eosin staining (Figure 5A). In addition, cell-sheet therapy significantly attenuated collagen accumulation in the infarct area compared with cell injection and control, as demonstrated by Masson trichrome staining (Figure 5B and 5C).

Cardiac Functional Recovery After Cell-Sheet Transplantation

The effects of cocultured bi-level cell-sheet transplantation on cardiac function were assessed in a rat ICM model. After permanent occlusion of the LAD, EF, FS, and anterior wall thickness (baseline, 1.7±0.1 mm; at 2 weeks, 0.8±0.1 mm, at 4 weeks, 0.8±0.1 mm; P=0.0001, Kruskal–Wallis test) showed steady reductions, whereas EDD/ESD showed steady increases (EDD, P=0.0002; ESD, P=0.0001; Kruskal–Wallis test), suggesting progressive LV remodeling. After cocultured cell injection, the heart showed mild recovery, including increases in FS and EF. At 4 weeks after treatment, EF and FS tended to be greater after cocultured cell injection than the control; however, an even greater recovery was observed after cell-sheet transplantation (Figure 6A and 6B). At 4 weeks, the bi-level cell-sheet group had a significantly greater EF and FS and significantly improved EDD and ESD compared with either cell injection or control (Figure 6C and 6D).

Assessment by pressure–volume catheter further confirmed the cell-sheet–induced functional enhancement demonstrated by the echocardiographic data. Four weeks after transplantation, the maximal rate of change in LV pressure (max. dP/dt) and end-systolic pressure–volume relationship were significantly enhanced in the cell-sheet group compared with cell injection and control (Figure 7). Mimimal rate of change in LV pressure (min. dP/dt) and cardiac output were higher in the cell-sheet group than the other 2 groups, but the difference was not significant.

Discussion

This study revealed a multifaceted mechanism by which the targeted implantation of an EPC-SMC bi-level cell-sheet enhances myocardial function in a rodent model of ICM. A significant chemokine effect was observed in vitro where cocultured EPC-SMCs stimulated an abundant release of SDF1α, VEGF, HGF, and TGFβ; this effect is a mechanistic component of the augmented angiogenesis demonstrated in vivo. More importantly, however, the data clearly established direct migration of the cell-sheet EPCs and SMCs into the myocardium and confirmed these cells to be some elements of newly formed functional vasculature. The observed increased capillary density and improved blood perfusion in the borderzone elucidated the significant in vivo angiogenic potential of this technology. Furthermore, cell fate–tracking experiments strongly suggested the cell-sheet EPCs and SMCs as components of newly assembled vasculature. With regard to cell engraftment, the cell-sheet group performed superiorly, demonstrating improved cell–matrix attachment compared

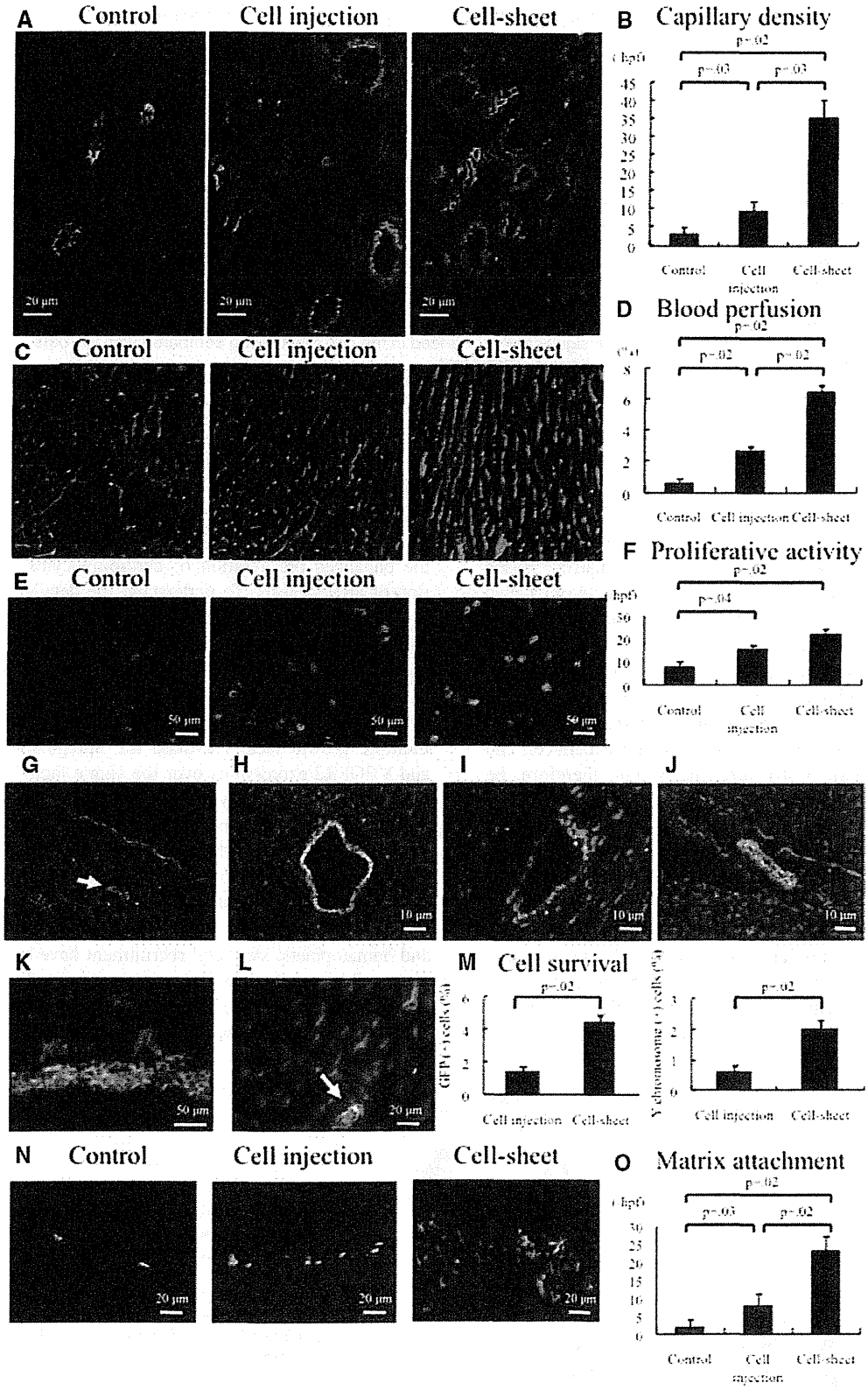

Figure 4. Effects on left ventricular remodeling, capillary density, and microvascular perfusion by bi-level cocultured cell-sheet transplantation (cell-sheet), cocultured cell injection (cell injection), and control (control) 4 weeks after the treatment procedure. **A**, Representative von Willebrand factor (vWF) staining of the borderzone myocardium. **B**, Quantification of capillary density. Capillary density was significantly enhanced in the cell-sheet groups compared with other groups (cell-sheet, n=4; cell injection, n=3; control, n=4; *P*=0.01, Kruskal–Wallis test). **C**, Representative lectin microangiographic imaging from the borderzone myocardium (×20 magnification). **D**, Quantitative analysis showed enhanced blood perfusion in the cell-sheet group compared with the other groups (cell sheet, n=4; cell injection, n=4; control, n=4; *P*=0.01, Kruskal–Wallis test). **E**, Representative antiproliferative cell nuclear

(*Continued*)

Figure 4. Continued antigen staining of the borderzone myocardium. **F**, Quantification of cell proliferative activity. Proliferative activity was significantly enhanced in the cell-sheet group compared with control (cell sheet, n=4; cell injection, n=4; control, n=4; P=0.02, Kruskal–Wallis test). **G**, Immunoconfocal microscopy demonstrated abundant green fluorescent protein (GFP)-positive cells in the myocardium. **H**, Cryosections were stained with an antibody to vWF and GFP to detect the fate of endothelial progenitor cells (EPCs) in the heart. Immunostaining for vWF and GFP showed that transplanted EPCs over the borderzone myocardium were able to contribute directly to neovascularization of the host myocardium. Green indicates GFP; red, vWF; blue, nuclei. **I**, Immunostaining for vascular endothelial (VE)-cadherin and GFP showed that transplanted EPCs were able to contribute to neovascularization of the host myocardium. Green indicates GFP; red, VE-cadherin; blue, nuclei. **J**, In addition, staining with antibody to smooth muscle actin (SMA) and GFP demonstrated that GFP-positive EPCs originating from the transplanted cocultured bi-level cell sheet migrated into the treated myocardial tissues and were circumferentially supported by SMA-positive tissues. Green indicates GFP; red, SMA; blue, nuclei. **K**, Furthermore, to track SMCs from the cell sheet, we performed fluorescence in situ hybridization to identify male SMCs in the female recipient. Immediately after the cell-sheet transplantation, GFP-positive EPCs and Y chromosome–positive SMCs were detected in the epicardium. Red indicates rat Y chromosome; green, GFP. **L**, Rat Y chromosome SMCs were able to differentiate into SMA-positive tissues (white arrow). Red indicates SMA; yellow, rat Y chromosome; blue, nuclei. **M**, Quantitative analysis of cell survival estimation. GFP-positive cells and rat Y chromosome–positive cells were counted, respectively, and corrected by total number of tissue cells to examine the survival cells quantitatively. **N**, Representative anti–integrin β1 staining of the borderzone myocardium. **O**, Quantification of cell–matrix attachment. Cell–matrix attachment was significantly enhanced in the cell-sheet group compared with the other groups (cell sheet, n=4; cell injection, n=4; control, n=4; P=0.01, Kruskal–Wallis test).

with injection alone. The robust angiogenic effect of bi-level cell-sheet translated to enhanced myocardial function of the ischemic heart.

Our group has investigated the effects of EPCs as a neovasculogenic therapy for ICM using EPC therapy alone,[14] with seeded EPCs,[13] and with a tissue-engineered matrix.[2] Based on these findings, we began to explore the effects of ex vivo expanded EPCs. Systemic and direct myocardial injection of EPCs, however, is fraught with complications, such as cell dispersion and high percentages of cell loss. In this study, we used cell-sheet technology, which allows efficient delivery of cells onto the ischemic area of myocardium with minimal myocardial injury and cell dispersion, preserves cell–cell and cell–ECM architectural structure, and might, therefore, be more applicable to human translation.[15]

Given our previous work and experience with cell-sheet technology, one possible mechanism is likely to include cytokine release and hematopoietic stem cell recruitment.[7–9] Previous studies have shown that EPCs acted as the natural supplier of SDF1α,[16] VEGF,[17] HGF,[18] and TGFβ.[19] Their roles and signaling pathways have been intensively investigated; SDF1α is related to cell migration, proliferation, and migration[2,13,16]; VEGF is critical to stimulate endothelial cell proliferation and migration to initiate neovascularization[19]; HGF is beneficial to an impaired heart and is associated with an antifibrotic effect.[7,20] Together with our findings, it is reasonable to conclude that coculturing EPCs with SMCs enhanced the secretion of cytokines, such as SDF1α, VEGF, HGF, and TGFβ, compared with either EPCs or SMCs, thus leading to the enhanced proliferation of cardiomyocytes and stimulation of angiogenesis. To understand the detailed mechanism by which coculturing enhances cytokine secretion, we performed additional investigations from a new perspective. We found that FLK1 and VEGFR2 were upregulated by additional VEGF, which were even more enhanced by numerous cytokines containing cell-culturing medium, suggesting that multiple growth factors evoked the upregulation of FLK1 and VEGFR2 expressions over the single factor (ie, VEGF), thereby possibly amplifying VEGF release. The understanding of our results may be translated into the emerging concept that SMCs support the biological aspects of EPCs via the endothelial–pericyte cytokine cross-communication.

The mechanism of restoration of damaged myocardium by EPC transplantation is complex.[2,3,13] Although cytokine release and hematopoietic stem cell recruitment have been proposed as possible mechanisms of regeneration, other important mechanisms are likely to be involved. The creation of mature, stable, and functional vessels is essential. It has been reported

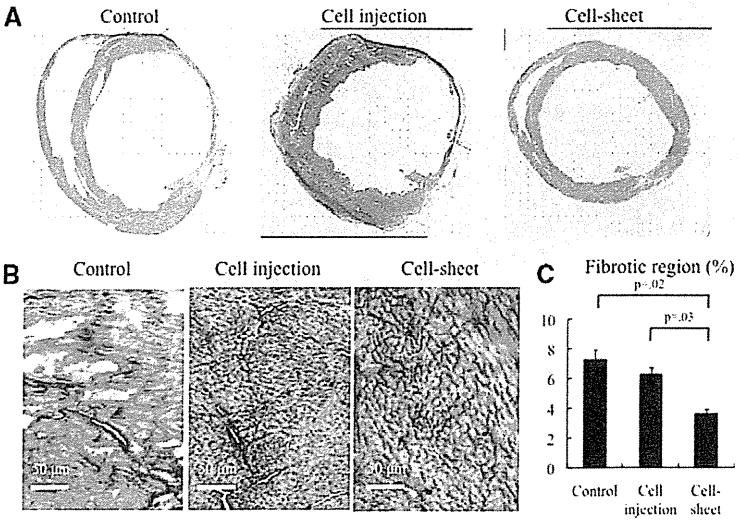

Figure 5. A, Representative macroscopic views of the heart (hematoxylin-eosin staining). The myocardial structure was superiorly maintained after cell-sheet transplantation compared with cell injection and control. **B**, Representative Masson trichrome staining at the borderzone myocardium. **C**, Quantification of fibrotic region. Fibrosis at the borderzone area was significantly suppressed in the cell-sheet group compared with the other groups (cell sheet, n=4; cell injection, n=3; control, n=4; P=0.02, Kruskal–Wallis test).

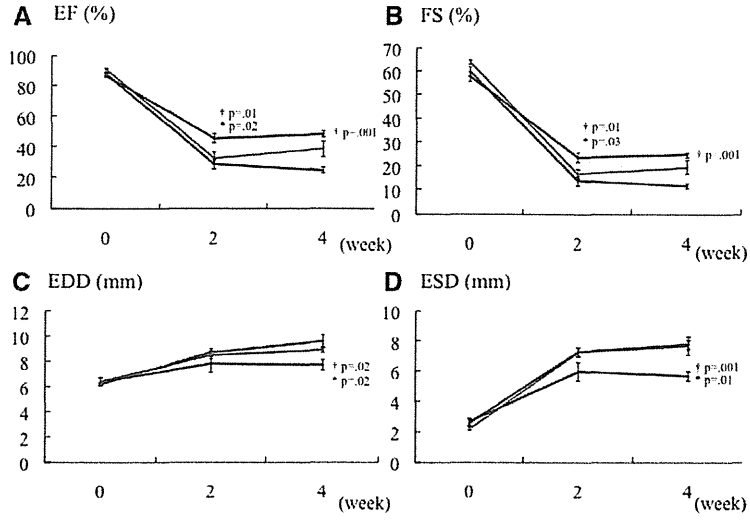

Figure 6. Serial changes in (**A**) ejection fraction (EF), (**B**) fractional shortening (FS), (**C**) end-diastolic diameter (EDD), and (**D**) end-systolic diameter (ESD) assessed by echocardiography (cell sheet, n=7, black line; cell injection, n=8, red line; control, n=9, blue line). Examinations were performed before (0) and at 2 and 4 weeks of follow-up after the operation. EF and FS were significantly higher at 2 and 4 weeks in the cell-sheet group compared with either cell injection or control (EF at 2 weeks, *P*=0.01; EF at 4 weeks, *P*=0.003; FS at 2 weeks, *P*=0.01; FS at 4 weeks, *P*=0.003; Kruskal–Wallis test). EDD and ESD were lowest at 4 weeks in the cell-sheet group (EDD, *P*=0.02; ESD, *P*=0.003; Kruskal–Wallis test). **P*<0.05 vs cell injection; †*P*<0.05 vs control, post hoc pairwise Wilcoxon–Mann–Whitney *U* test.

that capillary formation occurs via two basic vessel-constructing processes: angiogenesis (ie, the formation of new capillaries via sprouting or intussusception from preexisting vessels) and vasculogenesis (ie, de novo formation of vasculature as occurs in the developing embryo).[21] It has also been reported that angiogenesis requires a dynamic temporally and spatially regulated interaction among endothelial cells, pericytes, and angiogenic factors.[22] Given the natural relationship between endothelium and intima within mature vessels, we added SMCs, which are essentially vascular pericytes, to enhance the angiogenic performance of EPCs. Thus, it was hypothesized that coculturing EPCs with SMCs would promote a robust angiogenic response and induce formation of mature blood vessels. Our present study shows that in addition to the

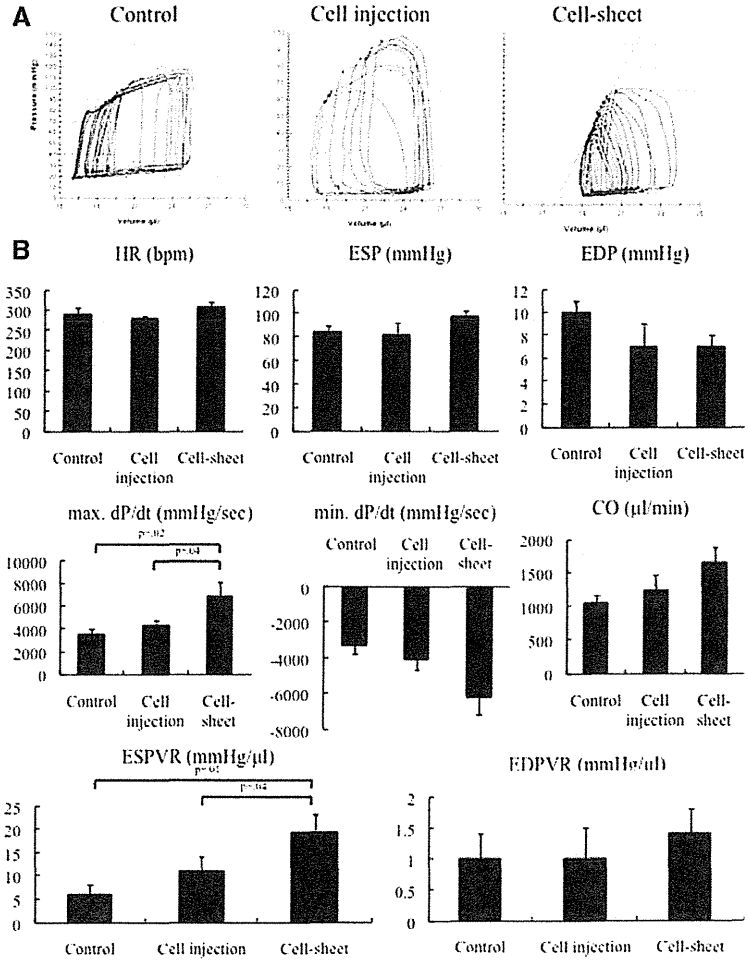

Figure 7. Hemodynamic measurements determined using cardiac catheterization after cocultured bi-level cell-sheet transplantation (cell-sheet, n=6), cocultured cell injection (cell injection, n=6), and control (control, n=8). Examinations were performed at 4 weeks of follow-up after the operation. **A**, Representative pressure–volume loops during inferior vena cava occlusion from cell-sheet, cell injection, and control groups. **B**, There was no significant difference in heart rate (HR), end-systolic pressure (ESP), end-diastolic pressure (EDP), mimimal rate of change in left ventricular (LV) pressure (min. d*P*/d*t*), cardiac output (CO), or end-diastolic pressure–volume relationship (EDPVR; HR, *P*=0.35; ESP, *P*=0.19; EDP, *P*=0.14; min. d*P*/d*t*, *P*=0.05; CO, *P*=0.07; EDPVR, *P*=0.70; Kruskal–Wallis test). The maximal rate of change in LV pressure (max. d*P*/d*t*) and end-systolic pressure–volume relationship (ESPVR) significantly improved in the cell-sheet group compared with the other 2 groups (max. d*P*/d*t*, *P*=0.04; ESPVR, *P*=0.03; Kruskal–Wallis test).

increased capillary density and organized capillary network in the engineered myocardial tissues, enhanced GFP-labeled EPCs originating from the transplanted cell sheet seemed to differentiate into an inner vWF- and vascular endothelial-cadherin–positive endothelial layer surrounded by an outer circumferential SMA-positive layer, partially derived from transplanted SMCs. The direct contribution of SMCs was confirmed by fluorescence in situ hybridization analysis of the myocardium, demonstrating new vasculature containing male SMCs in a female heart. Furthermore, the morphology of the vessel formation within myocardial tissues, including the diameter, composition, and stability of vessel walls, suggested that vessel maturation may occur under pathological stimuli. Furthermore, our data showed that coculturing EPCs with SMCs enhanced the secretion of TGFβ, which is thought to promote stabilization in multiple ways: the synthesis and deposition of ECM and contextual regulation of proliferation and differentiation.[17] Therefore, it is likely that the process of vessel maturation is a transition from an actively growing vessel to a quiescent fully functional mature vessel network via endothelial–pericyte interaction.

The mechanism by which the transplanted cocultured bi-level cell sheet attenuated ventricular remodeling and improved cardiac function, as shown in this study, seemed to depend on the cell sheet being placed over the scarred area of the myocardium and led to repair of the anterior wall thickness, reduction of LV wall stress, and the improvement of LV function. Previous studies indicated that the surviving myocardium and transplanted cell sheet attenuate complex cellular and molecular events, including hypertrophy, fibrosis, apoptosis of the myocardium, and the pathological accumulation of ECM.[7,23]

Cell engraftment is another critical aspect of myocardial regeneration. The potential advantages of the cell-sheet technology include the ability to deliver a larger number of transplanted cells that integrate with native tissues without destroying the cell–cell or cell–ECM adhesions in the cell-sheet.[7] Together with our significant findings of increased cell survival, integrin β1 upregulation, and the enhanced secretion of HGF in vitro in the cell-sheet group, it is likely that the cocultured bi-level cell-sheet prolonged cell survival by preventing anoikis mediated by the ECM receptors, in particular via integrin β1, or modulated by growth factor (eg, HGF).[24]

This treatment strategy for acute myocardial infarction is not yet directly applicable to the clinical arena because of the time required to isolate, cultivate, and manipulate cells in vitro However, the finding that this therapy yielded marked cardioprotective effects through angiogenesis should be beneficial for treating other types of cardiac pathologies, such as the chronic phase of myocardial infarction.

A potential limitation of this study is that the optimal number of transplanted cells was unknown in vivo. In addition, further studies are necessary to determine the optimal mixing ratio of transplanted EPCs and SMCs. We believe that this scaffold-free cell-sheet technique seems to be more transplantable to humans.[15] Although the cocultured bi-level cell sheet maintained different cell types in separate layers in vitro, our in vivo findings showed that the transplanted cell sheet could be a mixture of both cell types. This is probably because each cell type possessed different cell affinity, cell–matrix attachment, and migration ability.

In conclusion, we found that coculturing EPCs with SMCs in a bi-level cell-sheet delivery system enhanced the angiogenic effect by facilitating more architecturally mature microvascular formation. We also observed that bi-level cell-sheet technology initiated robust angiogenesis and regulated vessel maturation, thereby reducing fibrosis, attenuating ventricular remodeling, and improving cardiac function in ischemic cardiomyopathic rats. These findings suggest that novel bi-level cell-sheet technology creates an avenue of powerful cardiac repair. This concept may lead to new regeneration therapies in advanced cardiomyopathy.

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Disclosures

None.

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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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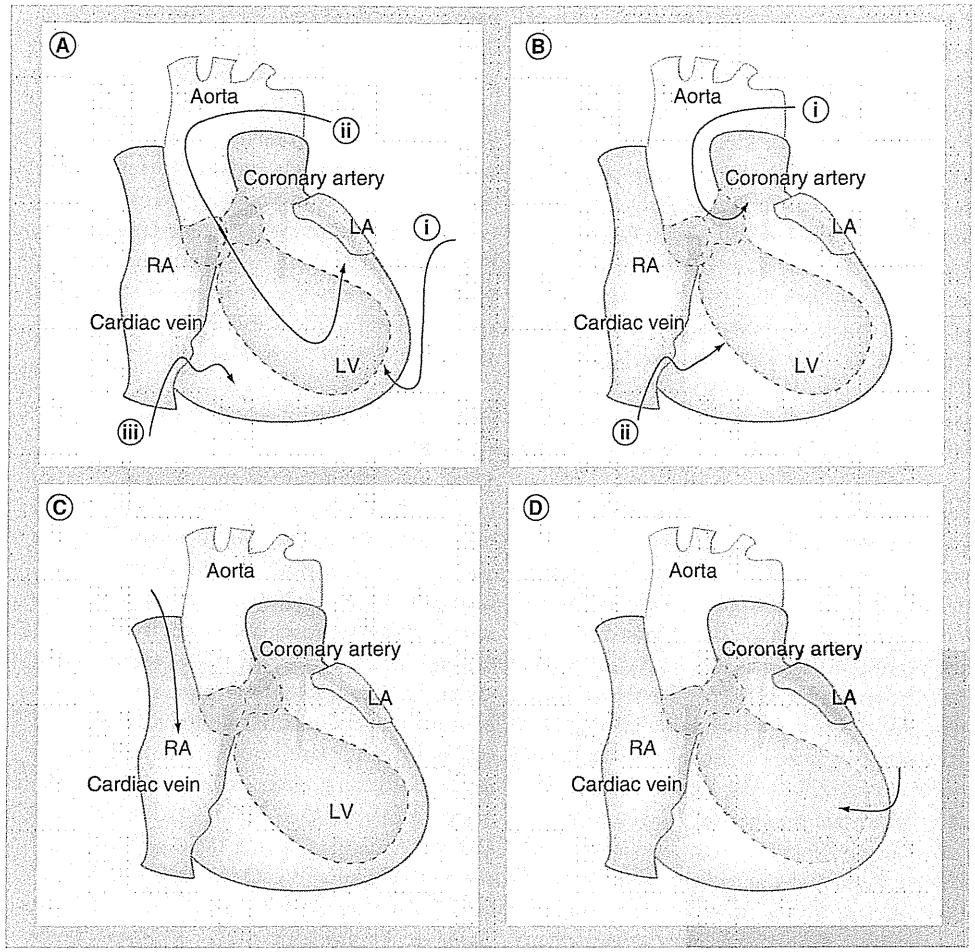

Figure 1. Possible cell-delivery methods into the heart. Major methods of cell delivery into the heart include: intramyocardial injection, intracoronary injection, intravenous injection and epicardial placement. **(A)** Intramyocardial cell injection can be carried out by **(i)** epicardial, **(ii)** 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