Spontaneously 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 injection4 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...
by intracoronary injection or needle injection.\textsuperscript{7,10} 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 temperature-responsive polymers that change 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.\textsuperscript{2} 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 neovascularization, 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.\textsuperscript{2} For EPC fate tracking, we used GFP transgenic female Wistar rats.

SMCs were isolated from the thoracic aorta of wild-type male Wistar rats (3 weeks old; Charles River) by primary explant technique\textsuperscript{11} and cultured in DMEM with 20% fetal bovine serum, gentamicin, and amphotericin B to confluency for 7 days at 37°C and 5% CO\textsubscript{2}. For SMC fate tracking, we used male Wistar rats.

Bi-Level Cell-Sheet Preparation

The SMCs were plated at 1.5×10\textsuperscript{5} cells/cm\textsuperscript{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\textsubscript{2}, EPCs were added at 1.5×10\textsuperscript{5} cells/cm\textsuperscript{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 oriented SMC-EPC bi-level cell sheet (Figure 1A).\textsuperscript{7,10}

Production and Release of Cytokines/Chemokines

To demonstrate proangiogenic biological activity, supernatant of the cocultured cells (EPCs: 1.5×10\textsuperscript{5} cells/cm\textsuperscript{2}, SMCs: 1.5×10\textsuperscript{5} cells/cm\textsuperscript{2}), EPCs (3.0×10\textsuperscript{5} cells/cm\textsuperscript{2}), or SMCs (3.0×10\textsuperscript{5} cells/cm\textsuperscript{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

![Cell-sheet](http://circ.ahajournals.org/)

**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 cell sheet. Hematoxylin-eosin staining; cross-sectional views of cell-sheet in vitro. B, 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\textsuperscript{5} EPCs and 1.3×10\textsuperscript{5} 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.
chloride, under terminal anesthesia, and the heart was excised. For 4 weeks.

with a 30-gauge needle. Each rat received the same number of cells.

infarction encompassing 35% to 40% of the left ventricle.1–3 Within

approach. This produced a consistent and reproducible myocardial

Wistar rats was permanently occluded using a left thoracotomy

maintained by inhalation of 2.0% isoflurane (Clipper Distributing

IP), intubated in an endotracheal manner with a 19-gauge catheter,

and mechanically ventilated (Hallowell EMC). Anesthesia was

went 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 1.3×10⁶ EPCs and 1.3×10⁶ SMCs, was placed on the epi-

cardium covering the ischemic area (Figure 1B). The cell injection

group received 1.3×10⁶ EPCs and 1.3×10⁶ 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 mL/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 structural integrity. 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 at 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. 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. 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 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.

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-systolic anterior wall thickness at the level of the papillary muscles were measured for ≥5 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. 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; Milarr Instruments, Inc; Figure 1C). The catheter was calibrated via 5-point cuvette linear interpolation with parallel conductance subtraction by the hypertonic saline method.19 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 Neovascularature
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=0.02; TGFβ, P=0.01; SDF1α, P=0.001; 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 \( \approx 50 \mu \text{m} \) 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 \( 18 \pm 3 \text{hpf} \) (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 \( 7 \pm 2 \text{hpf} \) (45% of rat Y chromosome cells), which participated in new blood vessel formation. One week after treatment, a large number of integrin \( \beta_1 \)-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. \( \frac{dP}{dt} \)) and end-systolic pressure–volume relationship were significantly enhanced in the cell-sheet group compared with cell injection and control (Figure 7). Minimal rate of change in LV pressure (min. \( \frac{dP}{dt} \)) 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 SDF1, 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)
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 in 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 amenable 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 migration2,13,14; VEGF is critical to stimulate endothelial cell proliferation and migration to initiate neovascularization19; 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 VEGRF2 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...
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). It has also been reported that angiogenesis requires a dynamic temporally and spatially regulated interaction among endothelial cells, pericytes, and angiogenic factors. 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 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.

**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), minimal rate of change in left ventricular (LV) pressure (min. dP/dt), cardiac output (CO), or end-diastolic pressure–volume relationship (EDPVR; HR, P=0.35; ESP, P=0.19; EDP, P=0.14; min. dP/dt, P=0.05; CO, P=0.07; EDPVR, P=0.70; Kruskal–Wallis test). The maximal rate of change in LV pressure (max. dP/dt) and end-systolic pressure–volume relationship (ESPVR) significantly improved in the cell-sheet group compared with the other 2 groups (max. dP/dt, 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. 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.

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. 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). 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. 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.

References


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

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