Endothelial Cell Coculture Within Tissue-Engineered Cardiomyocyte Sheets Enhances Neovascularization and Improves Cardiac Function of Ischemic Hearts

Hidekazu Sekine, PhD; Tatsuya Shimizu, MD, PhD; Kyoko Hobo, MD, PhD; Sachiko Sekiya, PhD; Joseph Yang, ScB; Masayuki Yamato, PhD; Hiromi Kurosawa, MD, PhD; Eiji Kobayashi, MD, PhD; Teruo Okano, PhD

Background—Regenerative therapies, including myocardial tissue engineering, have been pursued as a new possibility to repair the damaged myocardium, and previously the transplantation of layered cardiomyocyte sheets has been shown to be able to improve cardiac function after myocardial infarction. We examined the effects of promoting neovascularization by controlling the densities of cocultured endothelial cells (ECs) within engineered myocardial tissues created using our cell sheet-based tissue engineering approach.

Methods and Results—Neonatal rat cardiomyocytes were cocultured with GFP-positive rat-derived ECs on temperature-responsive culture dishes. Cocultured ECs formed cell networks within the cardiomyocyte sheets, which were preserved during cell harvest from the dishes using simple temperature reduction. We also observed significantly increased in vitro production of vessel-forming cytokines by the EC-positive cardiac cell sheets. After layering of 3 cardiac cell sheets to create 3-dimensional myocardial tissues, these patch-like tissue grafts were transplanted onto infarcted rat hearts. Four weeks after transplantation, recovery of cardiac function could be significantly improved by increasing the EC densities within the engineered myocardial tissues. Additionally, when the EC-positive cardiac tissues were transplanted to myocardial infarction models, we observed significantly greater numbers of capillaries in the grafts as compared with the EC-negative cell sheets. Finally, blood vessels originating from the engineered EC-positive cardiac tissues bridged into the infarcted myocardium to connect with capillaries of the host heart.

Conclusions—in vitro engineering of 3-dimensional cardiac tissues with preformed EC networks that can be easily connected to host vessels can contribute to the reconstruction of myocardial tissue grafts with a high potential for cardiac function repair. These results indicate that neovascularization can contribute to improved cardiac function after the transplantation of engineered cardiac tissues. (Circulation. 2008;118[suppl 1]:S145–S152.)

Key Words: cell sheet n coculture n myocardial tissue engineering
myocardial tissues. After subcutaneous transplantation, these layered cardiomyocyte sheets have shown the development of elongated sarcomeres, gap junctions, and well-organized vascular networks as well as long-term survival of nearly 2 years.11–13 Importantly, when transplanted to infarcted hearts, the tissue-engineered cell sheets demonstrated morphological communication with the host myocardium and improved host heart function with increased ejection fraction and inhibition of left ventricular dilation.14,15

With the transplantation of engineered myocardial tissues, it has been expected that improved cardiac function can be attributed to mechanical support and inhibition of fibrosis by the transplanted tissues; recovery of damaged heart function by the secretion of various cytokines; or neovascularization within both the transplanted tissue grafts and host myocardium. In the present study, we examined the effects of promoting neovascularization by controlling the densities of endothelial cells (ECs) cocultured in 3-dimensional engineered myocardial tissues that were transplanted to treat severe heart failure after myocardial infarction.

Methods
Statement of Responsibility
The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

All animal experiments were performed according to the “Guidelines of Tokyo Women’s Medical University on Animal Use,” the “Principles of Laboratory Animal Care,” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals,” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (Publication No 85-23, revised 1996).

Preparation of Cardiac Cell Sheets Containing Specific Endothelial Cell Densities
Cardiomyocytes were isolated from the ventricles of 1-day-old Sprague-Dawley neonatal rats (SLC, Hamamatsu, Japan) according to previously described procedures.11 ECs were separated from primary myocardial cell suspensions of GFP-positive Sprague-Dawley neonatal rats (SD-TgN [Act-EGFP] OsbCZ-004, SLC) by magnetic cell sorting (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) using mouse monoclonal antirat CD31 antibody (MCA1334G, Serotec Ltd, Oxford, UK), as previously described.12 To fabricate cocultured cardiac cell sheets, GFP-negative cardiomyocytes (without ECs) and purified GFP-positive ECs were mixed at ratios of 12:1, 6:1, or 3:1 and were cultured on 35-mm temperature-responsive culture dishes (CellSeed, Inc, Tokyo, Japan) at 20°C for 1 hour to release the cultured cells as intact sheets. The cocultured cardiac cells were transferred to another incubator set at 37°C in a humidified atmosphere containing 5% CO2. After 4 days, the cocultured cardiac sheets were transferred to a temperature-responsive culture dishes (CellSeed, Inc, Tokyo, Japan) set at ratios of 12:1, 6:1, or 3:1 and were cultured on 35-mm temperature-responsive culture dishes (CellSeed, Inc, Tokyo, Japan) at 37°C in a humidified atmosphere containing 5% CO2. After 4 days, the cocultured cardiac sheets were assayed with 106 cells. The culture media from each sample was then collected and centrifuged at 800 rpm at 4°C for 10 minutes. Enzyme-linked immunosorbent assay kits for rat vascular endothelial growth factor (Quantikine, R&D, Minneapolis, Minn), human basic fibroblast growth factor (R&D), and rat hepatocyte growth factor (Institute of Immunology, Tokyo, Japan) were used according to the manufacturers’ suggested protocol. Values were calculated for total cell protein (n=12 per group).

Rat Myocardial Infarction Model and Cardiac Tissue Transplantation
F344 athymic rats (250 to 350 g, male; Charles River Japan, Tokyo, Japan) were anesthetized with 2% inhaled isoflurane and ventilated using a rodent mechanical ventilator. Myocardial infarction was produced by ligation of the proximal left anterior descending coronary artery with a 7-0 nylon suture, causing permanent coronary artery occlusion. The chest was then closed and the rats were weaned from the respirator.

The study design consisted of 5 experimental groups: Group 1, sham operations involving thoracotomy and cardiac exposure but without cardiac tissue transplantation; Group 2, transplantation of triple-layer EC-negative cardiac sheets (2.4×106 cardiomyocytes per sheet); Group 3, transplantation of triple-layer cardiac sheets cocultured with 2×105 ECs; Group 4, transplantation of triple-layer cardiac sheets cocultured with 4×105 ECs; and Group 5, transplantation of triple-layer cardiac sheets cocultured with 8×105 ECs (n=10 per group). Two weeks after myocardial infarction, the individual cardiac cell sheets were harvested from the temperature-responsive culture dishes and stacked into the triple-layer constructs. The 3-dimensional cardiac tissue grafts were then placed on a polypropylene support sheet and transplanted over the anterior cardiac wall including the infarcted area (n=10 per group). Skin incisions were then closed with 6-0 polydioxanone sutures.

Measurements of Left Ventricular Function
Cardiac function was assessed by echocardiography equipped with a 13-MHz linear array probe and electrocardiogram (EUB-8500; Hitachi Medical Corp, Tokyo, Japan) 4 weeks after the transplantation. Short-axis views were obtained and recorded at the level of midpapillary muscle. Measurements of left ventricular end systolic dimension, left ventricular end diastolic dimension, and end diastolic anterior wall thickness in motion mode were performed (n=10 per group). Fractional shortening was calculated as a measure of systolic function: fractional shortening=(left ventricular end diastolic dimension−left ventricular end systolic dimension)/left ventricular end diastolic dimension×100.

Blood Perfusion Analysis
Four weeks after transplantation, Indian ink was used to examine blood perfusion in the engineered myocardial tissues. Five hundred microliters of black Indian ink was injected into the inferior vena cava and the entire heart, including the transplanted myocardial tissues, and resected for further analysis (n=5).

Histological and Immunohistochemical Analyses
Four weeks after transplantation, rats were anesthetized and the thoracic cavity was opened. The hearts with the transplanted myocardial tissue grafts were then resected for histological analysis. Samples were fixed in 4% paraformaldehyde and routinely processed into 7-μm-thick paraffin-embedded sections. Azan staining was performed using conventional methods. For immunohistochemistry, deparaffinized sections were incubated with either a 1/200 dilution of anti-FGF rabbit polyclonal antibody (Molecular Probes, Eugene, Ore) or a 1/100 dilution of anti-troponin T antibody (Laboratory Vision, Fremont, Calif) overnight at 4°C. Samples were then treated with either a 1/200 dilution of Alexa-Fluor-488 conjugated anti-rabbit IgG antibodies (Invitrogen, Carlsbad, Calif) or Alexa-Fluor-568 conjugated anti-mouse IgG antibodies (Invitrogen) for 2 hours at room temperature. For the detection of ECs, sections were incubated with a 1/100 dilution of Alexa Fluor-568 conjugated isoclinet B4 (Molecular Probes) overnight at 4°C. Cell nuclei were then counterstained with a 1/500 dilution of Hoechst 33342 (Wako Pure Chemicals, Osaka, Japan) for 5 minutes. Sections were finally visualized using confocal microscopy (TCS-SP, Leica Microsystems).
tems, Wetzlar, Germany). The same concentrations of corresponding nonspecific immunoglobulins were used as negative controls.

Assessment of Fibrosis Within Cardiac Tissue Grafts
To quantify fibrotic tissue formation of EC-negative and EC-positive (4\times10^5 ECs per sheet) myocardial tissue grafts, 5 random locations were selected from each azan-stained tissue section and the percentage of connective tissue was determined from light microscopic photographs at a magnification of \times200 (n=5 per group) using winROOF image processing software (Version 5.7.2; Mitani Corp, Tokyo, Japan).

Measurements of Blood Capillary Density
For the detection of ECs, paraffin-embedded sections were incubated with a 1/200 dilution of Alexa Fluor-568 conjugated isolectin B4 overnight at 4°C (n=5 per group) and visualized using confocal microscopy (TCS-SP; Leica). To quantify blood vessel formation in EC-negative and EC-positive (4\times10^5 ECs per sheet) myocardial tissue grafts, 12 random locations were selected from each cross-section and the number of capillaries was counted in each field at a magnification of \times400.

Data Analysis
All data are expressed as mean±SD. An unpaired Student t test was performed to compare 2 groups. One-way analysis of variance was used for multiple group comparisons. If the F-distribution was significant, a Tukey’s test was used to specify differences between groups. For comparisons of multiple groups against sham transplantation controls, a Dunnett’s test was applied. A probability value of <0.05 was considered significant.

Results
Formation of Endothelial Cell Networks in the Cocultured Cardiac Cell Sheets
To distinguish ECs within the engineered myocardial tissues, CD31-positive cells from GFP-transgenic rats were isolated. Magnetic cell sorting of dissociated cardiac cell suspensions indicated that in the normal neonatal rat heart, cardiomyocytes and ECs were present at a ratio of approximately 6:1. By including 4\times10^5 GFP-positive ECs (equal to the EC density of normal isolated cardiac cell suspensions) in the cardiomyocyte cultures, the ECs formed cell networks that possessed an appearance similar to the well-established sprouting form of ECs cultured in Matrigel, throughout the pulsatile cardiac cell sheets at 4 days (see Supplemental Movie 1). In contrast, no EC network formation was observed in the cardiac cell sheets when ECs were removed by magnetic cell sorting. Furthermore, by varying the EC concentration within the cardiac cell sheets, the size and density of the sprouting GFP-positive cell networks increased, depending on the EC density (Figure 1A–D). When the secretion of angiogenic growth factors was examined, enzyme-linked immunosorbent assay measurements showed that the EC-positive cardiac cell sheets (4\times10^5 ECs) also secreted a significantly greater amount of vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor in comparison to the EC-negative cell sheets (Figure 2A–C). In contrast, when skin fibroblasts were cocultured with ECs, the EC-positive fibroblast sheets did not show increased secretion of any of the 3 angiogenic factors, demonstrating that specific interactions between the cardiomyocytes and ECs resulted in the increased angiogenic potential of the cocultured cardiomyocyte sheets. The presence of the EC networks along with the amplified secretion of vessel-forming cytokines after 4 days in culture indicated that by including ECs within the cardiac cell sheets, the angiogenic capability of the engineered myocardial tissues could be significantly enhanced.
Recovery of Cardiac Function by Controlling Endothelial Cell Densities Within the Tissue-Engineered Myocardial Grafts

To evaluate the effects of promoting neovascularization on the recovery of cardiac function, myocardial tissue grafts containing controlled densities of ECs were transplanted directly to the anterior cardiac wall in a rat myocardial infarction model (see Supplemental Movie 2). Four weeks after transplantation, echocardiography showed significant improvement in fractional shortening of the host hearts in comparison to the sham transplantation controls when EC densities were increased (sham: 14 ± 4%; EC - : 19 ± 7%; 2 x 10^5 ECs: 22 ± 4%; 8 x 10^5 ECs: 25 ± 5%; *P<0.05; Figure 3A). In addition, the end diastolic anterior wall thickness after the transplantation was significantly recovered in all groups receiving myocardial tissue transplantation at 4 weeks (sham: 0.6 ± 0.1 mm; EC - : 1.0 ± 0.3 mm; 2 x 10^5 ECs: 0.8 ± 0.2 mm; 8 x 10^5 ECs: 1.1 ± 0.3 mm; *P<0.05; Figure 3B).
transplantation of the engineered myocardial tissues was significantly recovered in all groups (sham: 0.6±0.1 mm; EC[−]: 1.0±0.3 mm*; 2×10⁵ ECs: 0.8±0.2 mm*; 4×10⁵ ECs: 1.0±0.3 mm*; 8×10⁵ ECs: 1.1±0.3 mm*; *P<0.05; Figure 3B).

**Endothelial Cell Cocultured Myocardial Tissue Grafts Inhibit Tissue Fibrosis in Ischemic Hearts After Transplantation**

To further examine the effects of neovascularization, we compared the transplantation of EC-negative and EC-positive myocardial tissue grafts in greater detail. We first analyzed the formation of fibrotic connective tissues after myocardial infarction and cardiac cell sheet transplantation. When ECs were included in the grafts, formation of collagenous connective tissue indicative of fibrosis was significantly reduced both within the grafts and in the surrounding host myocardium (Figure 4A–F). In comparison to the EC-negative cardiac cell sheets, the overall percentage of connective tissue within the heart cross-sections was significantly reduced in the EC-positive group (4×10⁵ ECs: 7±7% versus EC[−]: 17±10%*, n=5 per group; G). Error bars indicate the SD (*P<0.01).

**Figure 4.** Histological analysis of cardiac tissue grafts 4 weeks after transplantation. Azan staining demonstrates the presence of EC-negative myocardial tissues (A–C) and EC-positive (4×10⁵ ECs) myocardial tissues (D–F) on the ischemic heart surface. Arrowheads in A–B and D–E indicate the location of the transplanted myocardial tissue grafts. Bidirectional arrows in C and F represent the transplanted cardiac cell sheets. Quantitative analysis of connective tissue formation demonstrated significant reduction of fibrosis in the EC-positive group (4×10⁵ ECs: 7±7% versus EC[−]: 17±10%*, n=5 per group; G). Error bars indicate the SD (*P<0.01).

**Endothelial Cell Cocultured Myocardial Tissue Graft Transplantation Significantly Increases Capillary Densities in Ischemic Hearts and Directly Contributes to Newly Formed Blood Vessels**

Four weeks after transplantation, newly formed blood vessels were observed in both the EC-negative and EC-positive cardiac grafts. However, when the tissues were stained with isoelectin B4, densities of the capillaries derived from the
transplanted cell sheets were significantly higher in the EC-positive cardiac grafts compared with the EC-negative tissues (4 × 10^5 ECs: 124 ± 11 versus EC[-]: 70 ± 12*; *P<0.01; Figure 5).

In addition to the increased capillary densities in the EC-positive tissues, GFP-expressing blood vessels originating from the transplanted cardiac grafts also migrated into the host myocardium and appeared to connect to capillaries of the host heart (Figure 6A). Immunostaining for troponin T and GFP confirmed that ECs from the transplanted cardiac tissue grafts over the infarcted area were able to contribute directly to the neovascularization of the ischemic host myocardium (Figure 6B). Furthermore, staining with isoelectin B4 and anti-GFP antibodies also demonstrated that blood vessels originating from the transplanted EC-positive cardiac grafts bridged to connect with capillaries of the host heart, forming fused vessels containing ECs of both host and graft origin (Figure 6C). Interestingly, these fused vessels showed colocalization of GFP-positive and GFP-negative ECs were also observed in the fibrotic host tissues underlying the transplanted cocultured cardiac cell sheets (Figure 6D).

To determine whether the newly formed vascular networks in the transplanted cardiac cell sheets were able to form anastomoses with the host coronary vasculature, Indian ink was infused into the inferior vena cava and examined in the engineered myocardial tissues. After Indian ink infusion, blood vessels within the cardiac cell sheets were stained with the black ink indicating anastomotic connection between the host coronary vasculature and the blood vessels within the transplanted myocardial tissues (Figure 6E).

**Discussion**

Previous findings from the transplantation of tissue-engineered myocardial grafts have indicated that the recovery of damaged cardiac function can be mainly attributed to 4 key factors: mechanical support from transplanted myocytes; secretion of cytokines from the transplanted tissues, including angiogenic growth factors; inhibition of fibrosis in the host heart; and finally the formation of capillary networks at the site of myocardial infarction. Indeed, our previous experimental results with various cell types have indicated that cytokine secretion and cell integration with the host myocardium leads to the establishment of a critical environment for the improvement of cardiac function. Cardiomyocyte sheets transplanted to ischemic hearts were able improve cardiac function and also bridged to form morphological communication through functional gap junctions within intact areas of the damaged myocardium. Layered skeletal myoblast sheets also provided improved left ventricular contraction, reduced fibrosis, and prevented remodeling through recruitment of hematopoietic stem cells and the release of various growth factors. Most recently, mesenchymal stem cell sheets were able to reverse cardiac wall thinning and prolong survival after myocardial infarction, primarily due to growth factor-mediated paracrine effects and by decreasing left ventricle wall stress after transplantation of the cell sheets.

However, although neovascularization is believed to provide a key contribution to the recovery of damaged heart function, a direct correlation involving capillary formation in engineered myocardial tissues has not been previously established. In the present study, our results demonstrate that neovascularization in the transplanted myocardial tissue grafts leads to reduced fibrotic tissue formation and contributes to the improvement of cardiac function. When cardiomyocytes were cocultured with GFP-positive ECs on temperature-responsive culture dishes, the ECs developed a sprouting appearance rather than the classic cobblestone morphology within the cardiac cell sheets. In addition, due to the increased secretion of the angiogenic growth factors vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor, the cardiac cell sheets containing ECs appeared to possess a significant innate potential for neovascularization even before transplantation. After the formation of the EC networks, the cardiac cell
sheets could then be harvested without disruption from the temperature-responsive culture dishes. Using low-temperature cell sheet harvest without the use of enzymatic digestion, the previously established EC networks within the cardiac cell sheets could be maintained, allowing for the rapid development of graft-derived EC structures into mature microvessels upon transplantation to the ischemic heart surface.

Previous results have shown that the presence of EC networks profoundly improves cardiomyocyte survival and organization by maintaining a minimum intercapillary distance to provide oxygen and nutrients. Therefore, the presence of ECs may be directly correlated to cardiomyocyte function. Four weeks after transplantation of the engineered myocardial tissues, fractional shortening of the infarcted hearts could be significantly improved by increasing the EC densities within the cardiac cell sheets. Additionally, when EC networks were formed before transplantation to the infarcted hearts, we observed significantly greater numbers of capillaries in the grafts, resulting in decreased formation of fibrotic tissues compared with the EC-negative tissues. Taken together, these results strongly suggest that blood vessel formation within the engineered cardiac tissues contributed to improved cardiac function and repair of the damaged myo-

**Figure 6.** Contribution of endothelial cell networks to the neovascularization of ischemic hearts. A, Macroscopic view demonstrating capillary formation in the EC-positive myocardial tissues. GFP-expressing blood vessels originating from the transplanted cardiac grafts migrated into the host myocardium and connected to capillaries of the host heart. White circle in A indicates the position of the transplanted cardiac cell sheets. B, Immunostaining for troponin T and GFP showed that ECs from the transplanted cardiac tissue grafts over the infarcted area were able to contribute directly to neovascularization of the host myocardium. Arrows in B indicate vessels originating from the transplanted tissue grafts. C, Staining with isolectin B4 and anti-GFP antibodies demonstrated that fused blood vessels containing both host and graft-derived ECs were present in the engineered myocardial tissues. D, Additionally, GFP-positive ECs originating from the transplanted cardiac cell sheets migrated into the host myocardium and connected with host ECs to form capillaries in the host heart. White arrow in D indicates a fused vessel composed of both host and graft-derived ECs. E, Indian ink infusion revealed that black ink stained capillaries were present within the transplanted cardiac cell sheets.
cardium. In addition to the rapid establishment of a microvascular network within the engineered cardiac tissues and in the ischemic host myocardium, the newly formed blood vessels were also able to connect to the coronary vasculature through anastomoses, allowing for direct blood flow between the engineered tissues and the host circulation.

In the present study, we have shown that cardiac function of infarcted hearts could be improved by the transplantation of engineered cardiac tissues through controlled neovascularization as a virtue of coculture with ECs. These results indicate that in vitro engineering of 3-dimensional cardiac tissues with EC networks that can be easily connected to host vessels may likely contribute to the development of tissue grafts with a high potential for functional cardiac repair. Therefore, neovascularization can contribute to improved cardiac function after transplantation of engineered cardiac tissues. Overall, the novel approach of cell sheet engineering for myocardial tissue engineering applications provides a promising alternative for effective therapies in regenerative medicine.

Sources of Funding
The present work was supported by Grants for the Research and Development on Myocardial Regenerative Medicine Program from the New Energy Industrial Technology Development Organization (NEDO), Japan.

Disclosures
T.S. and M.Y. are consultants for CellSeed, Inc. T.O. is an inventor/designer designated on the patent for temperature-responsive culture surfaces.

References
Endothelial Cell Coculture Within Tissue-Engineered Cardiomyocyte Sheets Enhances Neovascularization and Improves Cardiac Function of Ischemic Hearts
Hidekazu Sekine, Tatsuya Shimizu, Kyoko Hobo, Sachiko Sekiya, Joseph Yang, Masayuki Yamato, Hiromi Kurosawa, Eiji Kobayashi and Teruo Okano

_Circulation_. 2008;118:S145-S152
doi: 10.1161/CIRCULATIONAHA.107.757286

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/118/14_suppl_1/S145

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/09/30/118.14_suppl_1.S145.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/