Pulsatile Cardiac Tissue Grafts Using a Novel Three-Dimensional Cell Sheet Manipulation Technique Functionally Integrates With the Host Heart, In Vivo

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Abstract—We devised a method of fabricating easily transplantable scaffoldless 3D heart tissue, made with a novel cell-sheet (CS) technology from cultured cardiomyocytes using a fibrin polymer coated dish. In the present study, we tested in vivo electrical communication which is essential for improving heart function between the host heart and the grafted CS. The epicardial surface of the ventricle of an anesthetized open-chest nude rat was ablated by applying a heated metal. Bilayered CS was obtained from neonatal rat primary culture. CS was transplanted onto the injured myocardial surface (sMI) (sMI+sheet group). The rats were allowed to recover for 1 to 4 weeks, to stabilize the grafts. Action potentials (APs) from the excised perfused heart were monitored by the fluorescence signal of di-4ANEPPS with a high speed charge-coupled device camera. The APs were observed under epicardial pacing of the host heart or the CS grafts. The pacing threshold of the current output was measured in the sMI+sheet group and in the nongrafted sMI group at the center of the sMI and in the normal zone (Nz). Bidirectional AP propagation between the sMI and Nz was observed in the sMI+sheet group (n=14), but was blocked at the marginal area of the sMI in the sMI group (n=9). The ratio of the pacing threshold (sMI/Nz) was significantly lower in the sMI+sheet than in the sMI group (3.0±0.7, 19.0±6.1 respectively P<0.05). There were neither spontaneous nor pacing-induced arrhythmias in these two groups. Bidirectional smooth AP propagation between the host heart and the grafted CS was observed. This finding suggested functional integration of this CS graft with the host heart without serious arrhythmia. 

Key Words: stem cell–based therapy ■ regenerative medicine ■ cardiac transplantation ■ electrophysiology ■ arrhythmia

Recently, there has been a major challenge in the field of cardiac stem cell based therapy to establish an alternative to donor hearts from living subjects. Cardiomyocytes from in vitro stem cells such as embryonic stem cells1,2 and marrow-derived stem cells3,4 were proposed as a possible cellular source for grafting. In fact, clinical trials involving myocardial injection of autologous myoblasts and mesenchymal stem cell have produced a limited but substantial recovery of impaired cardiac function.5,6 However, such direct delivery of isolated cells or delivery via coronary vessels induced aggregation and necrosis of the grafted cells7,8 and a small myocardial infarction in the host heart.9 Thus, it is difficult to transplant a sufficient number of cells to restore cardiac function.

Although in vitro cardiomyogenesis from human stem cells has been reported,10–12 the incidence of cardiomyogenic transdifferentiation from the engrafted stem cells was extremely low in vivo.13,14 Thus, the mechanism for recovery of cardiac function in the clinical trial was mainly angiogenesis into the infarcted myocardium.14,15 To restore impaired cardiac function, recruitment of cardiomyocytes by engrafted stem cells is essential. One possible strategy for expanding the efficiency of cardiomyocyte recruitment via stem cell based therapy is to purify regenerated cardiomyocytes from the population of undifferentiated cell types before cell transplantation. However, it would seem to be difficult to deliver differentiated cardiomyocyte graft cells diffusely into the host heart without aggregation or necrosis at the local injection site.7,8

Our novel cell-sheet technology may allow transplantation of condensed regenerated cardiomyocytes without necrosis.16–19 The electrical connection between the 2 cocultured cell sheets was established within a few days in vitro, suggesting potential electrical connections and functional...
integration between the host heart and grafted cell sheet,\textsuperscript{17,18} although no in vivo data were provided. Therefore, our aim in the present study is to confirm electrical communication between the grafted cell sheet and the host heart in vivo.

Materials and Methods

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University and conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Animal Model

Electrical synchronization is essential for achieving functional integration between the grafted cell sheet and host heart. However, it was difficult to distinguish the electrical activity of the cell sheet from that of the host myocardium, because the grafted cell-sheet layer was less than 0.1 mm in thickness. To suppress electrical signals from the host myocardium beneath the cell sheet, we ablated the host myocardium. The recipient male F344 nude rats (Clea, Tokyo, Japan) (8 weeks of age, n = 23) were anesthetized with 2% isoflurane gas. After left thoracotomy, the left ventricle was exposed and the anterior wall of the left ventricle was then ablated with 1 to 1.5 sec of pressure from a thermo-controlled (200°C) solder trowel tip (3 mm round shape) (Figure 1A, inset) that did not cause carbonization and usually produced only superficial myocardial necrosis of 0.15 to 0.3 mm in depth (Figure 1A).

We visualized the major coronary artery to avoid occlusion of this vessel with the epicardial ablation. In the present study, for convenience, we refer to this necrotic area as the simulated myocardial infarction area (sMI area). We marked the center of the sMI area by injecting carbon powder into the myocardium with a 27-gauge needle. In 14 rats, a bilayered myocardial cell-sheet graft (described later) was transplanted so as to cover the entire surface of the sMI area (sMI/sheet group) (Figure 1B). In 9 rats, we created the sMI area (sMI).
without cell-sheet transplantation (sMI group). The chests of the rats were then closed and the graft was allowed to stabilize for 1 to 4 weeks.

Creating of Myocardial Cell Sheets

Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old neonatal Wister rats (Clea), as described previously.18 We transplanted cell sheets, which were obtained by using a biodegradable polymerized-fibrin-coated dish as described previously.18 Cardiomyocytes were plated onto the dish at a cell density of $2.8 \times 10^5$ cells/cm², and myocardial cell sheets were obtained18 4 days thereafter. The 2 cell sheets thus obtained were overlaid and then preincubated, to allow stabilization, for at least 30 minutes before transplantation. The cell sheet becomes torn and damaged easily, especially during the transplantation procedure, such that it was mounted onto collagen film (CM-6 KOKEN, Tokyo, Japan) (Figure 2A) before the transplantation. The cell sheet mounted on the collagen film was easily delivered to the host heart. Immediately after transplantation, the collagen film base alone could be peeled off the grafted cell sheet immediately (Figure 2B and 2C; movie-2 in the online data supplement available at http://circres.ahajournals.org). A few minutes after cell-sheet transplantation, the cell sheet adheres so tightly to the surface of the host heart that it is impossible to remove even with the forceps. Any animal whose engrafted cell sheet was accidentally damaged and deformed on the recipient heart was excluded from the analysis.

Electrophysiological Study

Seven days after transplantation, recipient rats were anesthetized with pentobarbital sodium (0.5 g/kg IP injection), and the heart was then quickly excised. Immediately thereafter, the aorta was cannulated and perfused with cold Tyrode’s solution (Figure 2D) consisting of (in mmol/L) 129 NaCl, 4 KCl, 0.9 NaH₂PO₄, 20 NaHCO₃, 1.8 CaCl₂, 0.5 MgSO₄, and 5.5 d-glucose with oxygenation (95% O₂–5% CO₂). Connective tissue was trimmed for the physiological experiment and the sample was then mounted, in a thermo-controlled (37°C) bath (Figure 2E), on the ×0.5 objective microscope (Scime-dia THT-2, Tokyo, Japan). The heart was perfused with an oxygenated Tyrode’s solution via the aorta, toward the coronary artery, at a mean perfusion pressure of 70 to 100 mm Hg. Action potential (AP) propagation between the host heart and grafted cell sheet was monitored by fluorescent imaging of a voltage sensitive dye, di-4ANEPPS (Invitrogen). The dye stock solution (20 mmol/L) was freshly prepared with DMSO solution containing 20% F-127 (P-3000, Invitrogen) and added to a high-potassium Tyrode’s solution (KCl concentration of normal Tyrode’s solution was raised to 16 mmol/L) to produce a final dye concentration of $2 \times 10^{-6}$ mol/L. The sample was exposed to this dye containing the high-K Tyrode’s solution at 37°C for 30 minutes, then washed with normal Tyrode’s solution for more than 10 minutes to stabilize electrical and contraction activities. The sample was immobilized with $10^{-6}$ mol/L of cytochalasin-D (Sigma). The fluorescent signal was monitored through a high resolution charge-coupled device camera system (MiCAM01, Brain Vision, 192 × 128 points, 3.5-ms time resolution) at an emission wavelength greater than 610 nm and an excitation wavelength of 520 nm (Figure 1C).18,20 The ventricle was paced using an unipolar cathodal Ag-AgCl electrode located on the epicardial surface, at a rate slightly faster than the sinus rhythm, approximately 7 to 10 mm from the margin of the sMI area (Figure 1D). To ascertain the excitability of the cell sheet, in every experiment we measured the pacing current threshold (pacing duration=0.5 ms) (Model SS-202J, Kohden, Tokyo, Japan) of the normal ventricle and the sMI area using bipolar Ag-AgCl electrodes. The capture of the ventricle and cell sheet was monitored by bipolar electrography and optical imaging. Spontaneous arrhythmia or pro-

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Figure 3. A representative isochronal map for the sMI group. A, Optical image of the sample under visible light, with orientation of the sample denoted. The necrotic area (simulated myocardial infarction, designated sMI) is bordered by a white dotted line. A marker placed at the center of the sMI area can be seen (black arrow). Isochronal maps of AP propagation during RV (B), LV (C), base (D), and apex (E) pacing are shown. The time after stimulation is denoted on each isochronal line. The pacing site is denoted as p, electrode artifact as *.

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grammed premature stimulation-induced arrhythmia was monitored with the bipolar electrogram. The optical image thus obtained was processed using a program specially written by Igor Pro 4.0 (Wavemetrics), as described previously.20 A change in optical signal of less than 2% of the total fluorescent intensity was defined as indicating electrically unexcitable tissue.

**Histological Analysis**

Immunostaining was performed as described previously18 using anti–sarcomeric \( \alpha \)-actinin (Sigma) monoclonal antibodies and anti–connexin 43 (Sigma) polyclonal antibodies. The samples were incubated with either Alexa488-labeled anti-mouse IgG antibody (Invitrogen), TRITC-labeled anti-rabbit IgG antibody (Dako, Tokyo, Japan), Nuclei were stained with DAPI (Sigma), then observed under confocal laser microscopy (FV1000, Olympus Tokyo, Japan).

**Statistical Analysis**

Data were expressed as mean±SE. The difference between the 2 groups was determined with Student’s \( t \) test. Statistical significance was set at \( P<0.05 \).

**Results**

**Electrophysiological Study**

We observed AP propagation by epicardial ventricular pacing. In the sMI group, no AP propagation was observed in the sMI area, ie, the AP propagated away from the sMI area (Figure 3) (9/9). On the other hand, in the sMI+sheet group, there was AP propagation in the sMI area. When we paced the host heart of the sMI+sheet group (base, apex, right ventricle [RV], and left ventricle [LV]), AP propagation was observed within the sMI area and there was no delay (14/14) (Figure 4 and supplemental movie-1) at either 1 week and 4 weeks after the cell sheet transplantation. In 9 of 14 samples, small hematoma below the graft at 1 edge of the sMI area apparently suppressed the optical signal of the cell sheet. Therefore, in such cases we were able to observe AP propagation into the grafted cell sheet only from 3 directions (Figure 6D). Despite the fluorescent intensity being almost the same in the 2 groups (Figure 5C), the AP amplitude was significantly smaller in the sMI area in the sMI group (Figure 5A, 5B, and 5D). The AP duration in the grafted cell-sheet site did not differ from that of the host heart in sMI sheet group (Figure 5E). The pacing threshold in the sMI area of the control sMI group was significantly higher than that of sMI+sheet group, suggesting that the grafted cell sheet can be paced by electrical stimulation and that the AP elicited can propagate from the grafted to the host heart (Figure 6A). Anisotropic conduction was also observed in the grafted cell sheet (Figure 6B and 6C). In the normal zone, the conduction velocity along the fiber orientation (longitudinal [L]) was double that in the transverse (T) direction. In the sMI area of the sMI+sheet group, at 1 week after transplantation the conduction velocity (CV) along the L-axis had risen to the same value as the normal.
zone. There was neither spontaneous nor program stimulation protocol-induced ventricular arrhythmias in either group.

Histological Analysis
In the sMI group, we observed 0.15 to 0.3 mm in depth myocardial necrosis and fibroblast proliferation on the epicardial surface (Figure 7A and 7B). In the sMI+sheet group, a 0.05- to 0.1-mm thickness of the graft cell sheet was noted on the surface of the sMI region (Figure 7C, 7D, and 7G), where the cell sheet was attached directly to the host heart at the margin of the sMI area (Figure 7E). The transplanted myocardial cell sheets had rich neovascularization, not only capillary level but also vessels of 10 to 25 µm in diameter (Figure 7F). Four weeks after the transplantation a striation can be observed in the grafted cardiomyocyte of the cell sheet (inset). The immunohistochemical examination showed a clear striation pattern of α-actinin and diffuse connexin 43 staining of the cell sheet (Figure 7H) comparable to that seen in the host heart (Figure 7I). The fiber orientation of the cell sheet was almost the same as that of the host heart (Figure 7E and 7I), although in the grafted cell sheet the cardiomyocyte array was not as organized as in the host heart and connexin staining was relatively sparse as compared with that of the host heart.

Discussion

Electrical Synchronization of the Host Heart and Grafted Cell Sheet
In the present study, bidirectional electrical communication between the host heart and grafted cell sheet was observed. Thus, there is contraction of a grafted myocardial cell sheet which is functionally integrated and can contract simultaneously with the host heart. If we can further increase the thickness of the graft, we may be able to achieve increasing functional recovery of the heart. Further experiments are required.

In the present study, there was no difference in AP duration between the host heart and the grafted cell sheet. Tight electrical communication between the host and graft may also play a role in this homogeneous repolarization. Such tight electrical communication might facilitate suppression of inhomogeneity and arrhythmogeneity associated with transplantation. Thus in the present study, there was no induction of arrhythmia by the program stimulation protocol. However, our model would not be suitable for discussing arrhythmogenesis because the size of the heart is apparently too small to induce arrhythmias. Further examination is needed, on larger experimental animal, before clinical application.
Anisotropic Conduction

Anisotropic conduction is commonly observed in mature heart tissue. In ventricular tissue, the conduction velocity along the longitudinal axis of the fiber orientation was 2 to 3 times faster than that along the transverse axis. This anisotropic conduction was observed in the grafted cell sheet by optical mapping. Furthermore, the preferred direction of conduction is the same as that of the host heart. This suggests that myocytes in the grafted cell sheet are arrayed along the direction of those in the host heart. The establishment of anisotropic conduction may suppress inhomogeneous conduction, which promotes antiarrhythmia. As a function of maturation, the longitudinal versus transverse conduction velocity ratio (L/T ratio) increases. Therefore, the difference in the L/T ratio between the normal zone and grafted cell sheet depends on the maturity of the graft. Compared with L/T ratio in the mature host heart, L/T ratio was smaller in the graft cell sheet at 1 week after the transplantation and came to be the same as in the mature host heart at 4 weeks after the transplantation. In fact, the fiber orientation and connexin staining in the cell sheet were not as organized as those in the host heart. This may be attributable to the short time, since birth, for neonatal rat-derived cardiomyocytes in the cell sheet. Using electrophysiological data, we can show anisotropic conduction. However, histologically, it is quite difficult to show a clear myocyte array in the grafted cell sheet. This may also be attributable to immaturity of the grafted cell sheet.

Clinical Implications

Cardiomyogenic differentiation from human stem cells in vitro is quite rare and evidence of in vivo cardiomyogenesis from grafted stem cells in the host heart is also minimal. If the regenerated premature cardiomyocytes can be condensed in vitro in advance, transplanted back into the heart, we may be able to more efficiently increase the number of recruited cardiomyocytes. Hattan et al purified cardiomyocyte precursor cells from mouse marrow-derived mesenchymal stem cells using a recombinant plasmid containing enhanced green fluorescent protein cDNA under the control of the myosin light chain-2v promoter. Strong cardiomyogenic differentiation was observed. Clinically, however, it is difficult to use this feature. We cannot obtain adequate numbers of cardiomyocyte precursor cells from a limited

Figure 6. Measured parameters of conduction velocity and pacing threshold. A, The ratios of the pacing threshold between the sMI area and the normal zone (Nz) were averaged and are shown. The increased pacing threshold in the sMI area (sMI group) was significantly restored by cell-sheet transplantation (sMI+sheet group) at both 1 week (1W) and 4 weeks after transplantation (4W), suggesting the grafted cell sheet to be excited by pacing and that the elicited AP was successfully propagated from the cell sheet toward the host heart, via gap junctions. B, The conduction velocity (CV) ratios between the longitudinal axis along the fiber orientation of the host heart and the transverse axis (L/T) were measured and averaged. In the Nz, the CV along the longitudinal axis is approximately double that along the transverse axis. In the sMI area of the sMI+sheet group, which represents the electrical activity of the grafted cell sheet, the L/T CV ratio was significantly smaller than that in the Nz at 1W and the Nz had been restored by 4W. C, The CV in the longitudinal axis at 1W was significantly larger than that in the transverse axis of the cell sheet. D, Host to graft cell sheet (sMI area) AP propagation was observed in the entire sMI+sheet group, but not in the sMI group. However, hematomas below the grafted cell sheet at the edge of the sMI area can occasionally mask the fluorescent signal of the graft, such that we were unable to observe electrical communication from 1 of the 4 directions in the seven 1W samples and both 4W samples.
number of marrow-derived stem cells,\textsuperscript{25,26} because the transfection efficacy of such plasmids and cardiomyogenic differentiation efficacy are very poor.\textsuperscript{12}

Furthermore, our recent advancement in the technology enabled us to expand the cardiomyogenic transdifferentiation rate from human mesenchymal stem cells to the 60\% to 90\% in vitro.\textsuperscript{27} By this method, we can obtain enough transdifferentiated cardiomyocytes from a human sample in the near future. However, as mentioned previously, grafted stem cells may cause necrosis and apoptosis with the classic isolated cell injection type of transplantation maneuver.\textsuperscript{7,8} Our novel cell-sheet transplantation technique might overcome this problem. Previously, we showed that a cardiomyocyte grafted by means of the cell sheet was free of necrosis and apoptosis in vivo.\textsuperscript{16,18} Furthermore, in this study, we showed electrical communication between the host heart and grafted cell sheet, suggesting possible functional integration of the grafted cell sheet and host heart, with no arrhythmias. Thus, application of this cell-sheet technology has the potential to be a significant advancement in stem cell–based therapy for cardiac disease.

**Study Limitations**

Our aim was to evaluate electrical communication between the host and the graft. Therefore, we ablated the epicardial surface to simulate sMI instead of creating the MI itself in the present study. Ligation of the coronary artery caused MI, but it is known that several epicardial layers of myocytes in the area at risk usually survive. It is thus difficult to distinguish the electrical activity of the graft cell sheet from that of the residual epicardial host myocardium using a coronary ligation model. In other words, if we use a coronary ligation model to create MI, the electrical signal obtained from the MI region may not represent the electrical activity of the cell sheet but rather that of surviving host cardiomyocytes. Because the necrotic area was so small, we were unable to directly...
demonstrate mechanical improvement of cardiac function with this protocol.28

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Online Movie Legends

Movie-1
Action potential propagation between the host heart and engrafted cell-sheet graft observed by optical mapping.
Red color in the optical mapping denoted membrane potential depolarization and purple denoted hyperpolarization.
In the control (simulated) MI model, the propagation of action potential away from the simulated MI region. On the other hand, in the cell-sheet engrafted model, the action potential propagation was observed on the simulated MI region. This data suggest that the electrical signal of the cell-sheet graft on the MI region integrated with the host heart.

Movie-2
Making of simulated MI model and cell-sheet transplantation.
Suppl Fig 1

Action potential shape measured by optical mapping at 4 week after the cell sheet transplantation