

# Long-Term Survival and Growth of Pulsatile Myocardial Tissue Grafts Engineered by the Layering of Cardiomyocyte Sheets

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## ABSTRACT

Recently researchers have attempted to bioengineer three-dimensional (3-D) myocardial tissues using cultured cells in order to repair damaged hearts. In contrast to the conventional approach of seeding cells onto 3-D biodegradable scaffolds, we have explored a novel technology called *cell sheet engineering*, which layers cell sheets to construct functional tissue grafts. In this study, *in vivo* survival, function, and morphology of myocardial tissue grafts were examined. Neonatal rat cardiomyocytes were noninvasively harvested as contiguous cell sheets from temperature-responsive culture dishes simply by reducing the culture temperature. Cardiomyocyte sheets were then layered and transplanted into the subcutaneous tissues of athymic rats. The microvasculature of the grafts was rapidly organized within a few days with macroscopic graft beatings observed 3 days after transplantation and preserved up to one year. Size, conduction velocity, and contractile force of transplanted grafts increased in proportion to the host growth. Histological studies showed characteristic structures of heart tissue, including elongated cardiomyocytes, well-differentiated sarcomeres, and gap junctions within the grafts. In conclusion, long-term survival and growth of pulsatile myocardial tissue grafts fabricated by layering cell sheets were confirmed, demonstrating that myocardial tissue regeneration based on cell sheet engineering may prove useful for permanent myocardial tissue repair.

## INTRODUCTION

ALTERNATIVE THERAPIES TO cardiac transplantation, due to donor shortages, are strongly desired to repair impaired hearts. Cell-based therapies are currently considered to be one of the most promising methods to treat damaged myocardial tissue.<sup>1,2</sup> Although further studies are necessary to identify procedures for the isolation and expansion of transplantable human cardiomyocytes, alternative cell sources, such as autologous myoblasts or bone marrow cells, have already been clinically transplanted to treat ischemic hearts.<sup>3,4</sup>

In these methods, cell suspensions are usually injected into damaged myocardial tissues via thoracotomy, coronary artery infusion, or intraventricular approaches. However,

with the direct injection of dissociated cells, various factors, such as size, shape, and location of transplanted cells, can be difficult to control, severely limiting efficacy. Moreover, injection of cell suspensions cannot be applied to treat patients with congenital heart defects. To overcome these limitations, conventional tissue engineering approaches have been applied to create three-dimensional (3-D) myocardial tissue grafts.<sup>5</sup> In general, these approaches involve the seeding of dissociated cells into 3-D biodegradable scaffolds, which act as alternatives to extracellular matrix (ECM). It is hoped that as the scaffolds degrade over time, cells can proliferate and fill the space formerly occupied by the polymer, resulting in tissue formation.<sup>6</sup> Recently, several studies using poly(glycolic acid) (PGA), collagen, gelatin, and alginate as biodegradable scaffolds have been reported, with

the intention of constructing 3-D myocardial grafts to restore impaired heart function after transplantation.<sup>7–10</sup>

In contrast to conventional methods, we have fabricated 3-D pulsatile myocardial tissue using our novel technology–cell sheet engineering–which reconstructs 3-D functional tissue by layering confluent cell sheets without the use of biodegradable scaffolds. To obtain viable cell sheets, we use intelligent culture surfaces, from which confluent cultured cells spontaneously detach as intact sheets simply by reducing the culture temperature.<sup>11</sup> These surfaces, grafted with the temperature-responsive polymer poly(*N*-isopropylacrylamide)(PIPAAm) are slightly hydrophobic and cell adhesive under normal culture conditions at 37°C, but become hydrophilic and non-cell adhesive, due to rapid hydration and swelling of grafted polymer, when the temperature is reduced below 32°C. This intelligent surface property allows for cultured cells to be noninvasively harvested by lowering the incubation temperature. While vital cell-to-cell junctions and adhesive proteins are severely disrupted by enzymatic treatments, such as trypsinization, they can be perfectly preserved with low-temperature treatment, resulting in production of contiguous cell sheets.<sup>12–16</sup> Furthermore, ECM preserved on the basal surface of the cell sheets can act as an adhesive agent for attachment to other cell sheets or even host tissues,<sup>17</sup> allowing for the re-creation of various tissues by the layering of intact cell sheets.<sup>18,19</sup> We have previously confirmed electrical and morphological communication between layered cardiomyocyte sheets and successfully fabricated electrically communicative pulsatile 3-D cardiac constructs both *in vitro* and *in vivo*.<sup>20</sup>

In terms of clinical application, long-term survival of functional myocardial tissue is a strict requirement, but previous results have not presented sufficient evaluation over extended periods of time. Additionally, in order to be applicable for the repair of congenital heart diseases, appropriate growth of the engineered constructs is required. In the present study, we therefore performed physiological and morphological analyses of layered neonatal rat cardiomyocyte sheets transplanted into dorsal subcutaneous tissues of nude rats and examined their long-term survival and growth for up to one year.

## MATERIALS AND METHODS

All animal experiments were performed in accordance with the Guidelines of Tokyo Women's Medical University on Animal Use.

### *Preparation of square-geometry PIPAAm-grafted cell culture dishes*

Specific procedures for the preparation of square-geometry PIPAAm-grafted cell culture dishes have been

previously described.<sup>21</sup> Briefly, IPAAm monomer (kindly provided by Kohjin, Tokyo, Japan) in 2-propanol solution was spread onto tissue culture polystyrene dishes. The dishes were then subjected to irradiation (0.25 MGy electron beam dose) using an area beam electron processing system (Nisshin High Voltage, Kyoto, Japan), resulting in polymerization and covalent bonding of IPAAm to the dish surface. The PIPAAm-grafted dishes were then rinsed with cold distilled water to remove ungrafted monomer and dried under nitrogen gas. Next, the PIPAAm-grafted surface was masked with a square glass coverslip (24 × 24 mm, Matsunami, Osaka, Japan), and acrylamide (AAm) monomer (Wako Pure Chemicals, Tokyo, Japan) solution in 2-propanol was spread onto the masked dish surface. The dish surface was then irradiated in the same fashion. Resulting culture dishes had center square areas grafted with temperature-responsive PIPAAm surrounded by non-cell adhesive poly-AAm.

### *Primary culture of neonatal rat ventricular myocytes*

Ventricles from 1-day-old Wistar rats (Nisseizai, Tokyo, Japan) were digested at 37°C in Hank's solution (Sigma-Aldrich, St. Louis, MO) containing collagenase (class II, Worthington Biochemical, Lakewood, NJ). Isolated cells were suspended in the culture medium composed of 6% FBS (Moregate Biotech, Bulimba, QLD, Australia), 40% Medium 199 (Invitrogen, Carlsbad, CA), 0.8% penicillin-streptomycin solution (Wako Pure Chemicals, Tokyo, Japan), 2.7 mmol/L glucose, and 54% balanced salt solution containing 116 mmol/L NaCl, 1.0 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 0.8 mmol/L MgSO<sub>4</sub>, 1.18 mmol/L KCl, 0.87 mmol/L CaCl<sub>2</sub>, and 26.2 mmol/L NaHCO<sub>3</sub>. The cell suspension was plated at a density of  $4.8 \times 10^6$  cells per square-geometry PIPAAm grafted dish and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### *Manipulation of cardiomyocyte sheets*

Neonatal rat cardiomyocytes were cultured for 4 days at 37°C on square-geometry temperature-responsive cell culture surfaces. To release confluent cells as intact sheets, culture dishes were placed in a separate CO<sub>2</sub> incubator set at 20°C. Cardiomyocyte sheets detached spontaneously within 1 h and isotropically shrank to approximately 1cm<sup>2</sup> square cell sheets due to cytoskeletal reorganization, as described previously.<sup>20</sup> The entire cell sheet with culture media was gently aspirated into the tip of a pipette. The cell sheet was then transferred onto new temperature-responsive culture surfaces, with media gently dropped onto the sheet to spread folded regions. After sheet spreading, culture media was aspirated and the dish was incubated at 37°C to allow the cell sheet to adhere to the culture surface. For cell sheet layering, additional cardiomyocyte sheets detached from temperature-

responsive dishes were transferred and attached onto the initial sheets in the same manner.

### *Myocardial tissue graft transplantation into nude rats*

Male F344 nude rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30mg/kg). A  $3 \times 2$  cm L-shaped incision was made in the right dorsal skin and then opened. Layered cardiomyocyte sheet constructs on PIPAAm-grafted surfaces were detached by low-temperature treatment prior to surgery and then washed with Hank's solution. The cell constructs were lifted on a polypropylene supporting sheet ( $2 \times 1.5$  cm) and transplanted onto dorsal subcutaneous tissue via sliding from the slick polypropylene sheet. Skin incisions were then closed with 7-0 nylon sutures. Triple-layer constructs were transplanted into 4 ~ 6-week-old rats, and grafts were examined at day 1 ( $n = 1$ ), day 2 ( $n = 1$ ), day 3 ( $n = 2$ ), day 7 ( $n = 5$ ), 4 weeks ( $n = 3$ ), and 24 weeks ( $n = 3$ ) after the transplantation. A four-layer construct and a six-layer construct were transplanted into 8 ~ 9-week-old rats and examined 1 year after implantation. At the appropriate time periods, rats were anesthetized, as previously described, and physiological and morphological analyses were performed. Experimental animals were then euthanized with overdoses of pentobarbital.

### *Macroscopic observations of transplanted myocardial tissue grafts*

Macroscopic images of pulsatile myocardial tissue grafts were recorded using a digital video camera (DCR-TRV900, Sony, Tokyo, Japan). Graft lengths and areas were measured from still images using NIH Image software. The areas in contracted (C) and relaxed (R) states were measured to evaluate graft function. Contractile area ratio was calculated as  $(R-C)/R \times 100\%$ .

### *Electrophysiological analysis*

To measure conduction velocity of the transplanted myocardial tissues *in vivo*, the skin of the transplantation site was opened, and a recording microelectrode (100  $\mu$ m in diameter, Unique Medical, Tokyo, Japan) was positioned over the edge of the grafts. Then a stimulation bipolar electrode (TF200-029, Unique Medical) was set at the opposite edge of the graft, and monophasic pacing pulses (2.5 Hz, 10 ms, 3 V) were applied by an electrical stimulator (UPS-801, Unique Medical). Electrical potentials were amplified by bioelectric amplifiers (UA102, Unique Medical) and recorded by a data acquisition system (NR-2000, Keyence, Osaka, Japan). The stimulus-activation time intervals (conduction times) were measured from electrical potential tracings. Conduction velocity was calculated as electrode distance (cm)/conduction time(s).

### *Contractile force measurements*

Graft contractile force was measured as described previously.<sup>20</sup> Briefly, a commercial strain gauge (AE-801, SensoNor, Horten, Norway) was impaled into the edge of triple-sheet grafts at 1 week, 4 weeks, and 24 weeks following operations ( $n = 3$  for each time period). Contractile force was measured under 150 bpm (2.5 Hz) pacing using a force transducer (UPB-400, Unique Medical) connected to the gauge and recorded by the same data acquisition system previously described.

### *Histological analysis*

For cross-sectional observations, resected grafts were fixed with formalin, embedded in paraffin, and sagittally sectioned into 10  $\mu$ m slices. Azan staining was performed by conventional methods. For actinin staining, samples were incubated with 1:500 diluted anti- $\alpha$ -sarcomeric actinin antibody (Sigma-Aldrich) overnight and then incubated with a 1:100 dilution of FITC-labeled anti-mouse IgG antibody (Wako Pure Chemicals, Tokyo, Japan). For vascular endothelial cell staining, samples were incubated with an anti-factor VIII-related antigen antibody conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) overnight and then treated with chromogenic substrate solution for 5 min. Nuclei were simultaneously stained with hematoxylin. For gap junction staining, the grafts were frozen sectioned, fixed with 100% acetone, and then incubated with a 1:100 dilution of anti-connexin 43 antibody (Chemicon, Temecula, CA) for 3 h. Samples were then incubated with 1:200 diluted FITC-labeled anti-mouse IgG antibody. Fluorescent images of the grafts were observed under a fluorescence microscope (ECLIPSE TE2000-U, Nikon, Tokyo, Japan) or a confocal laser-scanning microscope (TCS, Leica, Heidelberg, Germany). Thickness of the constructs was also measured using micrograph images imported into NIH image software.

### *Data analysis*

Data are expressed as mean  $\pm$  SD. One-way ANOVA was used for multiple group comparison. If the *F* distribution was significant, a Bonferroni's test was used to specify differences between groups. A *p* value of  $< 0.05$  was considered significant.

## RESULTS

### *Neovascularization occurs within the myocardial tissue grafts over short time periods*

To examine survival and neovascularization in early stages, graft sites were opened 1, 2, 3, and 7 days after transplantation. Macroscopic spontaneous beating could

be detected in the case of the day 3 and 7 grafts. Azan staining of the day 1 graft revealed edematous tissue with thicker morphology and red blood cell islands invading randomly from the host tissue (Fig. 1A). At 3 days, the tissues became thinner with reconstructed microvascular networks within the grafts (Fig. 1B and D). After 7 days, a well-organized vascular network was observed (Fig. 1C, E, and F), with some microvessels demonstrating increased diameter throughout the transplanted grafts. These results demonstrated that sufficient neovascularization, allowing for blood supply to the entire area of the transplanted grafts, developed rapidly, within a few days.

#### *Physiological analyses of myocardial tissue grafts after long-term implantation*

Triple-layer constructs were analyzed for size, contractile function, and electrical properties at 1, 4, and 24 weeks after implantation. Functional beating of all grafts was preserved for the respective durations of implantation. The graft beating at 24 weeks is shown in the online supplemental video. Host rat body weight was  $83 \pm 18$  g at the time of transplantation and increased time dependently to  $110 \pm 21$  g,  $180 \pm 29$  g, and  $247 \pm 13$  g, respectively (Fig. 2A). Graft area also significantly increased in accordance with host growth, from  $0.63 \pm 0.15$  cm<sup>2</sup> to  $1.55 \pm 0.49$  cm<sup>2</sup> and  $1.89 \pm 0.22$  cm<sup>2</sup>, respectively (Fig. 2B), with graft development predominantly in the cephalocaudal direction. While original cell sheets were square-shaped, the ratio of length to width was  $1.32 \pm 0.16$  at 24 weeks. On the other hand, the contractile area ratio of the grafts, which indicates the area range between contracted and relaxed states, did not change significantly over time, with values of  $7.7 \pm 2.9\%$ ,  $8.2 \pm 2.4\%$ , and  $7.9 \pm 4.4\%$ , respectively (Fig. 2C).

We next examined the conduction velocity and contractile force under 150 bpm electrical stimulation (Fig. 3). Conduction velocity increased significantly in a time-dependent manner, from  $5.9 \pm 1.2$  cm/s to  $13.8 \pm 5.4$  cm/sec and  $18.2 \pm 3.0$  cm/s, respectively (Fig. 2D). Contractile force of 1-week grafts was too small to be detected, but increased at 4 and 24 weeks after transplantation to  $1.2 \pm 0.5$  mN and  $1.8 \pm 0.4$  mN, respectively (Fig. 2E). These data indicate that functional growth of transplanted myocardial tissue grafts occurred in proportion with host rat growth. Furthermore, 1-year survival of two myocardial tissue grafts was also confirmed, with the preservation of spontaneous and simultaneous pulsation.

#### *Histological analyses of the myocardial tissue grafts after long-term implantation*

Azan staining at 1, 4, and 24 weeks after transplantation revealed that grafts consisted of stratified myocardial tissue containing multiple microvessels (Fig. 4A–C). The thickness of the grafts also increased from  $86 \pm 5$  to

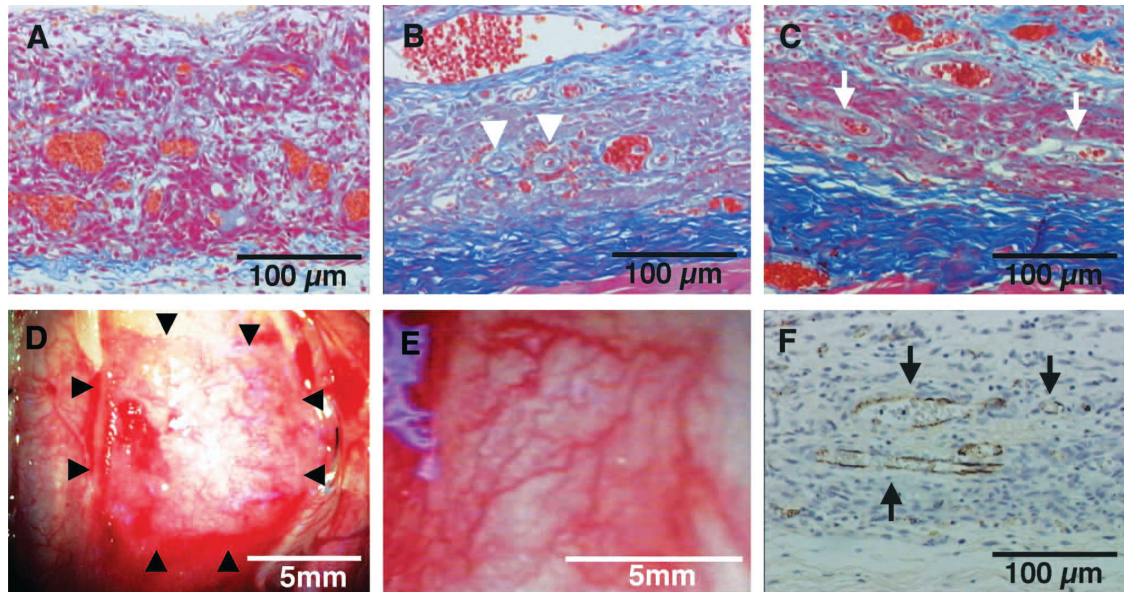
$94 \pm 11$  and  $104 \pm 12$   $\mu$ m, respectively (Fig. 2F). Immunofluorescent staining for sarcomeric actinin demonstrated well-differentiated sarcomeres, characteristic of elongated cardiomyocytes at all stages (Fig. 4D–F). Anti-connexin 43 antibody staining also demonstrated diffuse formation of gap junctions early (1 week) after transplantations and stable expression of them at long-term periods (Fig. 4G–I).

## DISCUSSION

In the present study, we demonstrate the successful reconstruction of 3-D, electrically coupled myocardial tissue using cell sheets. Histological analyses revealed the early formation of neovascular networks and well-differentiated cardiomyocytes with diffuse gap junctions present throughout the transplanted grafts. The implanted constructs also demonstrated growth and morphological and functional development, in accordance with host rat growth. Finally, the survival of beating myocardial tissue at the subcutaneous implantation sites was confirmed up to 1 year later, demonstrating the long-term survival of our bioengineered myocardial tissues *in vivo*.

Muller-Ehmsen *et al.* previously reported that by injection of an isolated cell suspension, neonatal cardiomyocytes were still present in infarcts 6 months after transplantation and contributed to improved cardiac function. However, they also reported cell loss due to various factors, such as physical strain during and after injection of the cell suspension, hypoxia, or cell wash-out through vascular or lymphatic networks.<sup>22</sup> It has also been reported that a significant amount of cell death also occurs shortly after cell injection due to acute ischemia.<sup>23</sup> In contrast, the transplantation of 3-D tissues fabricated by the layering of cardiomyocyte sheets harvested from temperature-responsive dishes allows for direct adhesion of the layered cell sheets not only to each other, but also to host tissues due to the presence of ECM on the basal sheet surface. This rapid adhesion to host tissues thus inhibits cell dislocation typically seen with injections of cell suspensions. Furthermore, we have reported in our previous study that cell sheets allow for more effective transplantation as large-area constructs without cell loss, in comparison to isolated cell injections, which often result in central tissue necrosis.<sup>20</sup> In this study, neither inflammatory changes nor tissue reduction were observed at any periods. These advantages in the transplantation of 3-D layered cell sheets, over direct injections of cell suspensions, may allow for improved efficacy in repairing damaged hearts.

Recently, several studies have reported the use of biodegradable scaffolds for myocardial tissue engineering. Although promising, complications such as insufficient cell migration into scaffolds and inflammatory re-



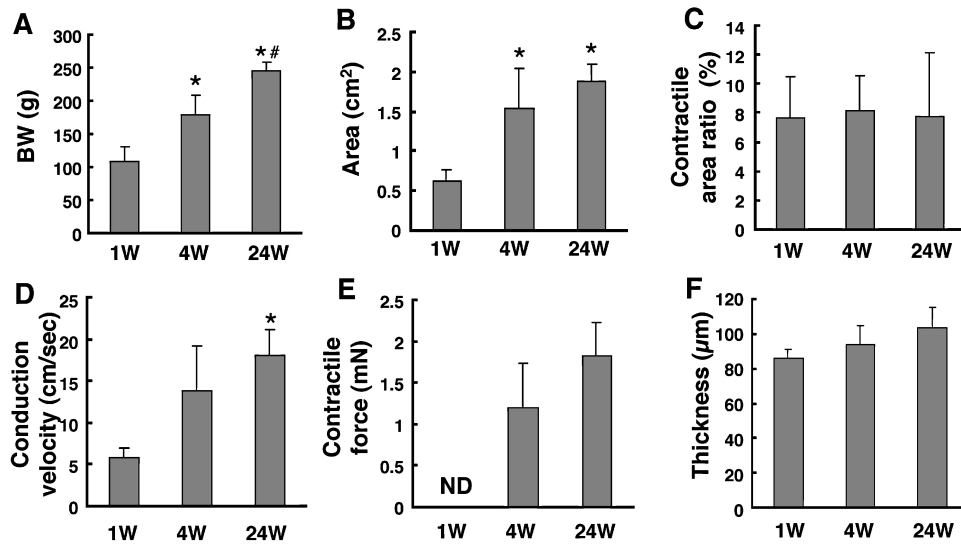
**FIG. 1.** Cross-sectional and macroscopic views of transplanted triple-layer grafts at early time periods. (A–C) Azan staining of the grafts. (A) Edematous and relatively thick tissue is observed in the day 1 construct, with numerous red blood cell islands arising randomly from the surrounding tissue. (B) At 3 days, the tissue became thinner, and microvascular structures can be observed within the construct (white arrowheads). (C) At 7 days, several blood vessel types are observed (white arrows). (D) Macroscopic view of a day 3 graft (black arrowheads) reveals diffuse microvascular formation within the construct. (E) A well-organized square-patterned vascular network is observed at 7 days. (F) Endothelial cell-specific factor VIII-related antigen staining of a day 7 graft reveals microvascular network formation that runs throughout the construct (black arrows).

actions due to scaffold biodegradation still occur.<sup>9,10</sup> In native myocardium, cells are considerably dense and intimately connected electrically via gap junctions. This morphology is in contrast to tissues such as cartilage, bone, and bladder (considered cell-sparse with large amounts of ECM) that have been successfully re-created using biodegradable scaffolds. In order to engineer myocardial tissue that resembles native tissue both in structure and function, it is critical to limit ECM formation, which often occurs upon scaffold biodegradation. Therefore, cell sheet engineering, which avoids the use of biodegradable scaffolds, seems favorable for myocardial tissue-engineering applications. Cardiomyocyte sheets contain electrically coupled cells and when layered, form both electrical and morphological connections immediately, resulting in cell-dense functional myocardial tissue.<sup>20</sup> In the present study, we also demonstrated that electrically communicative functional myocardial tissue, resembling native myocardium, may have increased potential for cardiac tissue repair due to long-term survival and growth *in vivo*.

Myocardial tissue grafts showed increased size, thickness, conduction velocity, and contractile force as host rats grew. Because cardiomyocytes rarely divide after birth, it is believed increases in both graft volume and contractile force are due to cardiomyocyte elongation and hypertrophy.<sup>24</sup> It is thought that the secretion of hor-

mones and growth factors from host rats, as well as mechanical stimuli, may cause these graft developments.<sup>20</sup> In the present study, transplanted grafts grew predominantly in cephalocaudal direction with elongated cardiomyocytes observed in sagittal cross sections. These phenomena seem likely due to the observation that growth of host rats occurred predominantly in the same direction. It is possible that bending and stretching exercise of the host animal leads to oriented reconstruction of the grafts. Previous studies have in fact revealed that *in vitro* stretching accelerates cardiomyocyte hypertrophy and introduces cell orientation.<sup>25</sup> Therefore, the application of mechanical loads to constructs should also be useful in myocardial tissue engineering.

In our results, conduction velocities of the fabricated grafts at 24 weeks ( $\sim 20$  cm/s) were larger in comparison with previously published values for bioengineered myocardial tissues ( $\sim 10$  cm/s). They are comparable to native neonatal rat ventricles and relatively small in comparison to native adult rat ventricles ( $\sim 30$  cm/s).<sup>26</sup> However, transplantation period-dependent acceleration of conduction velocities was consistent with native heart tissue growth, which demonstrates increasing velocity. The fact that graft growth was correlated with host development indicates a high potential for layered cardiomyocyte sheet transplantation for heart repair in younger patients with congenital defects.

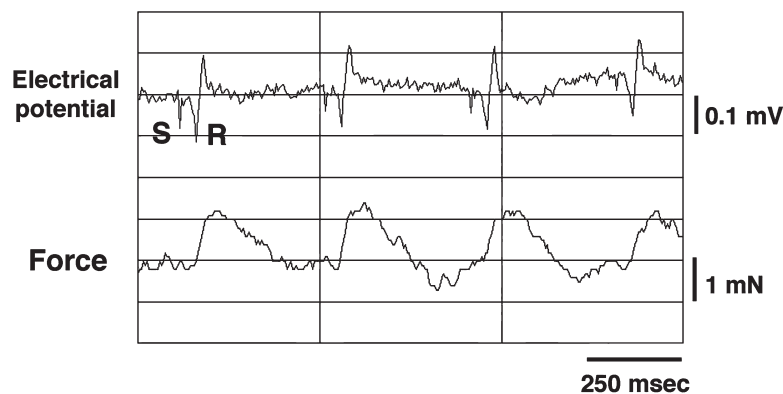


**FIG. 2.** Long-term comparison of myocardial tissue grafts. (A) Body weight of host nude rats significantly increased time dependently ( $n = 3$ ). (B) Graft area measured from still images captured in relaxation state also significantly increased in accordance with increasing host body weight ( $n = 3$ ). (C) Contractile area ratio, which was calculated from the area values in contractile state and relaxation state, did not change ( $n = 3$ ). (D) Conduction velocity calculated by measuring conduction time under 150 bpm electrical stimulation increased significantly in a time-dependent manner ( $n = 3$ ). (E) Contractile force measured by impaling a strain gauge was not detectable at 1 week (ND). At 4 weeks and 24 weeks, contractile force increased, with time ( $n = 3$ ). (F) Graft thickness measured with Azan staining also had a tendency to increase in accordance with host growth ( $n = 3$ ). Values represent mean  $\pm$  SD. One-way ANOVA followed Bonferroni's test were performed. \* $p < 0.05$  vs. 1 week; # $p < 0.05$  vs. 4 week.

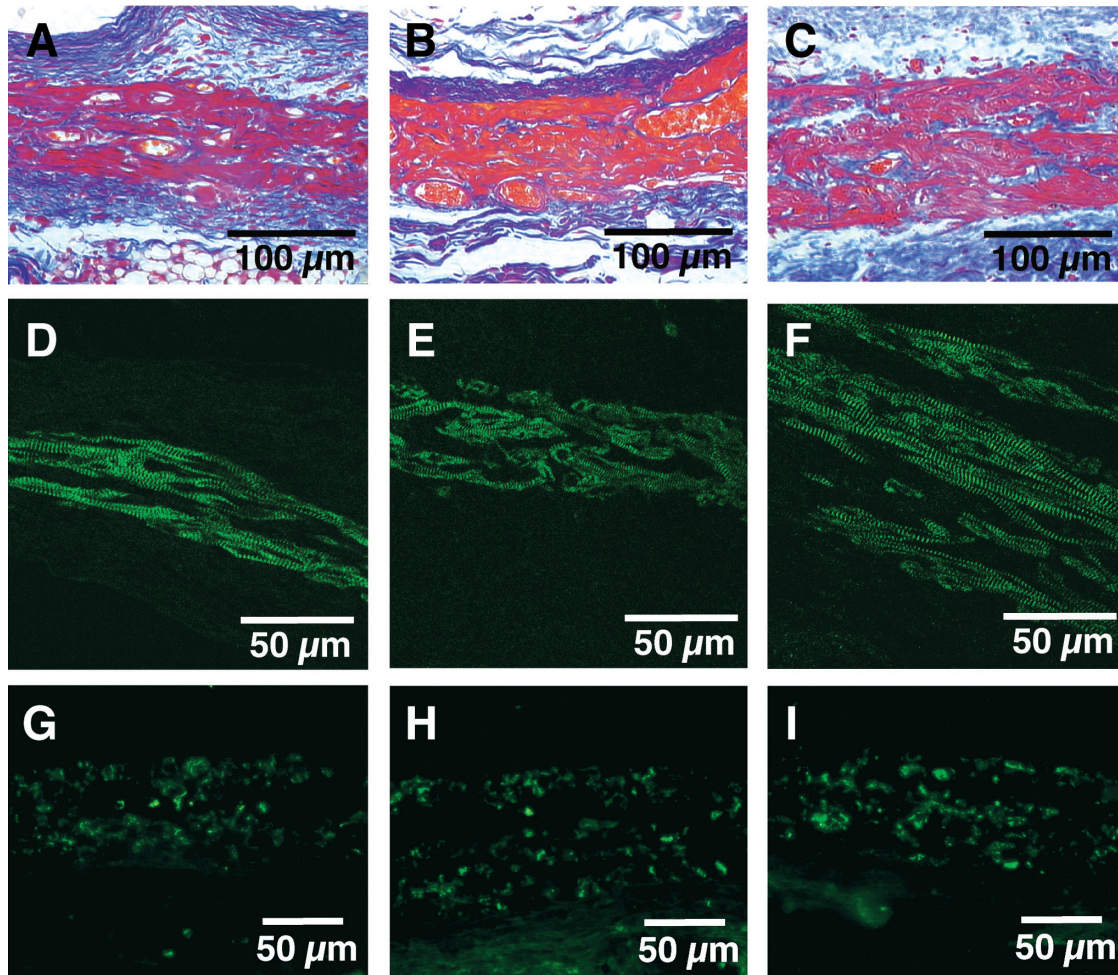
Regarding contractile forces, mean values for transplanted cell sheet constructs at 24 weeks ( $\sim 2$  mN) were larger in comparison to the values for *in vitro* myocardial tissues bioengineered by either our technology (data not shown) or collagen-based technique ( $< 1$  mN).<sup>7</sup> This indicates that *in vivo* condition should promote cardiomyocyte hypertrophy. On the other hand, the contractile forces of *in vivo* layered cell sheets were smaller than that of neonatal rat whole hearts ( $\sim 4$  mN).<sup>27</sup> Therefore, in the clinical setting, this contractile force seems to be insufficient for replacing heart function. Regarding

the effects of cardiac patch transplantation onto heart surface, cytokine secretions from the patches may promote neovascularization and inhibit heart remodeling.<sup>9</sup> Therefore, several-layer cell sheets may well rescue the patients suffering severe heart failure via these cytokine secretions rather than physical supports. For replacing heart wall as a future advanced therapy, it is critical to overcome size limitation of engineered tissues and to realize more functional multi-layer constructs.

Insufficient oxygen perfusion into 3-D engineered constructs still remains a common problem in tissue engi-



**FIG. 3.** Functional analyses of transplanted myocardial tissue grafts. Representative tracings of simultaneous detection of graft electrical potential (top) and contractile force (bottom). To analyze the conduction velocity of transplanted myocardial tissues, a recording microelectrode was positioned over the edge of the grafts and a stimulation bipolar electrode was set at the opposite edge. Under 150 bpm pacing, electrical potentials were recorded. The stimulus (S) and response (R) time intervals (conduction times) were measured. Conduction velocity was calculated as electrode distance (cm)/conduction time (sec). At the same time, graft contractile force was measured by impaling a strain gauge into the grafts.



**FIG. 4.** Histological analysis of myocardial tissue grafts after long-term implantation. Top panels (A, 1 week; B, 4 weeks; C, 24 weeks) show Azan staining. Middle panels (D, 1 week; E, 4 weeks; F, 24 weeks) demonstrate immunofluorescent staining of sarcomeric actinin. Bottom panels (G, 1 week; H, 4 weeks; I, 24 weeks) show immunofluorescent staining with anti-connexin 43 antibody. Azan staining shows striated myocardial tissue grafts and multiple vessels including orange-stained red blood cells. The thickness of the grafts increased time dependently (A–C). Actinin staining of sagittal cross sections demonstrates elongated cardiomyocytes and well-differentiated sarcomeres at all stages (D–F). Early diffuse depositions of connexin 43 are shown at 1 week and preserved over time (G–I).

neering. To fabricate thicker, functional myocardial tissue, new technologies to control blood vessel growth are needed. The present study revealed rapid vascularization within triple-layer grafts, resulting in the lack of necrotic tissue. The edematous stage, 1 day after the transplantation following sufficient neovascularization, may be induced by several angiogenic factors including vascular endothelial growth factor secreted by the transplanted cardiomyocytes.<sup>28</sup> This rapid vascular network formation should contribute to graft survival and longstanding pulsation. However, it is expected that primary local hypoxia may limit the number of cell sheets that can be layered to construct viable tissues. To overcome this limitation, growth factor administration, gene transfer, or co-culture

with vascular progenitor cells may accelerate vascular network growth and formation and may contribute to the fabrication of thicker tissues. Further research is needed to elucidate these critical points.

In this study, we transplanted the constructs into the relatively flat parts of dorsal subcutaneous tissues for measuring area, conduction velocity, and contraction force conveniently. In the clinical setting, direct transplantation of bioengineered grafts onto myocardial surface is a general approach. There may be significant differences about construct properties between their two transplantation sites, because heart beating may affect the graft remodeling and blood supply process may be different between them. Further studies will be needed to

clarify these points. On the other hand, ectopic transplantation of the constructs fabricated in subcutaneous tissue may be one of the future alternative approaches. Some *in vivo* conditionings are easily applied in subcutaneous tissues and may promote graft functions. We have already attempted to transplant triple-layer grafts repeatedly in subcutaneous tissue to clear size limitation due to primary insufficient neovascularization. These data will appear in the near future.

In conclusion, the survival and growth of functional myocardial tissue grafts fabricated by the layering of cell sheets were confirmed for up to one year. These results demonstrate that myocardial tissue fabrication based on cell sheet engineering may prove useful for permanent myocardial tissue repair.

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