quency and spontaneous Ca^{2+} release from the SR, leading to the detrimental phenotypes. Therefore, further studies on the effects of phosphorylation state in various signaling proteins are required.

S100A1. S100A1 belongs to the S100 protein family, which is the largest EF-hand Ca^{2+}-binding protein family, and is predominantly expressed in cardiomyocytes, where it is localized in the SR, sarcomere, and mitochondria (84). Myocardial expression levels of S100A1 are decreased in human and nonhuman failing hearts (99, 122). Overexpression of S100A1 transgenic mice showed an augmentation of basal cardiac function in vivo and enhancement of Ca^{2+} transient in isolated cardiomyocytes that remained to respond to β-adrenergic signal (81). S100A1 transgenic mice subjected to MI did not show HF with preserved global contractile performance and superior survival (83). Adenoviral gene delivery of S100A1 increased contractile function, systolic Ca^{2+} amplitude and SR Ca^{2+} uptake, reduced SR Ca^{2+} leak, and reversed reactivated fetal gene expression in failing cardiomyocytes (8, 80, 82), leading to normalized cardiac function and Ca^{2+} handling in postinfarcted failing rat heart (82, 93). Recently, Pleger et al. (94) have reported that delivery of AAV9-S100A1 to the LV noninfarcted myocardium in pig chronic MI model prevents and reverses cardiac dysfunction and LV remodeling along with normalized cardiomyocyte Ca^{2+} cycling and SR Ca^{2+} handling (94).

Interestingly, the studies of cardiac overexpression of S100A1 suggest that hypercontractile phenotype of transfected cardiomyocytes is independent from β-AR downstream signaling and related protein expression (9, 80, 81). This may be related to the therapeutic effectiveness of S100A1 on HF since chronic stimulation of β-AR signaling and its downstream effectors such as PKA eventually leads to cardiac hypertrophy and HF. The molecular targets of S100A1 are *1*) SR Ca^{2+}-handling proteins, *2*) cardiac titin, and *3*) mitochondrial proteins (104). S100A1 enhances diastolic Ca^{2+} uptake from SERCA2a, diminishes diastolic Ca^{2+} leakage, and increases systolic Ca^{2+} release through RyR through interaction with the RyR and the SERCA2/PLN complex. S100A1 regulates titin-actin interaction results in the reduction of titin-mediated vicious break and improvement of myofilament sliding. Additionally, S100A1 enhances ATP production through interaction with inner mitochondrial membrane and matrix molecules. Although our understanding of how S100A1 organizes cardiomyocyte Ca^{2+} dynamics, structure and metabolism is still incomplete and further studies are warranted, S100A1 may be a promising therapeutic target on HF.

Cytokine Therapy

Many of the growth factors, cytokines, and receptors have been reported to be potent as a therapeutic regimen for cardiac repair following MI. Most of the factors improve cardiac function and attenuate LV remodeling via angiogenesis, antiapoptotic effects, and stem cell homing. Among these factors, G-CSF and Epo are extensively studied, and several clinical trials are achieved.

Granulocyte colony-stimulating factor. G-CSF is a 25-kDa glycoprotein cytokine that stimulates the proliferation and survival of granulocytes lineage-derived cells. Orlic et al. (86) reported that G-CSF stimulates bone marrow cells to mobilize into the infarcted area and to differentiate into cardiomyocytes in mice. However, there is still a controversy about G-CSF-mediated cardiac regeneration by bone marrow stem cells (22, 78). The mechanisms other than cardiac regeneration have emerged from the findings that G-CSF receptor is expressed on cardiomyocytes and endothelial cells and that its downstream Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is essential for the effect of G-CSF in the heart (29). It has been reported that G-CSF reduces the number of apoptotic cells in the ischemic border zone and increases Akt activities in the myocardium in mice, rats, and swines in an acute phase of MI (52, 85, 117). G-CSF also increased the production of nitric oxide (NO) through Akt/endothelial NO synthase pathway and enhanced the proliferation and migration of endothelial cells (88, 124). Neurtophils and macrophages play important roles in accelerating the infarct healing process. G-CSF-mediated infiltration of inflammatory cells is the first step to regulate cardiac healing. Fujita et al. (30) have reported that G-CSF mobilizes monocytes/macrophage, which differentiate into myofibroblasts and enhance cardiac repair. G-CSF treatment improved early postinfarct ventricular expansion through enhancement of expression of transforming growth factor-β_1 and promotion of reparative collagen synthesis in the infarcted heart (92). Although there was a conflicting report showing that G-CSF reduces ratio of metalloproteinases to their tissue inhibitors and aggravates excessive fibrosis deposition in infarcted areas of the myocardium (15), the more sophisticated protocol of dose and duration of G-CSF treatment may ensure the beneficial effect on the infarct healing process. Recently, several reports suggest that G-CSF exerts pleiotropic effects on cardiac tissue such as improvement of impaired mitochondrial electron transport and oxygen consumption (46). Cardioprotective effects of G-CSF have been reported in chronic myocardial ischemia and HF through angiogenesis (42, 17), reduction of apoptosis (42), prevention of the formation of excessive granulation tissue (17), and reduced expression of the angiotensin II type-1 receptor and tumor necrosis factor-α (69). These results highlight that the effects of G-CSF treatment might also occur in the chronic phase through similar or distinct mechanism in acute phase. Figure 3 showed a diagram of cellular and molecular interaction in cardiac repair after G-CSF therapy after MI.

Many clinical studies using G-CSF for patients of acute MI have been reported. Table 1 shows a summary of clinical studies with G-CSF in infarcted patients. Since the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor Trial (FIRSTLINE-AMI) first reported that patients receiving G-CSF had increased mobilization of $CD34^+$ mononuclear cells, had improved ejection fraction, and had prevented LV remodeling without safety concerns (51), several randomized, controlled trials have been conducted; however, the results of these trials are discordant, and recent published reports of the meta-analysis of the randomized controlled trials are still inconclusive. Kang et al. (56) have reported that G-CSF treatment improves LV ejection fraction in acute MI from 14 randomized controlled trials (56). The result of the meta-analysis by Zohlnhöfer et al. (137) has shown that G-CSF treatment does not enhance the improvement of ejection fraction from 10 randomized controlled trials. Although clinical trials have shown equivocal results, stem cell

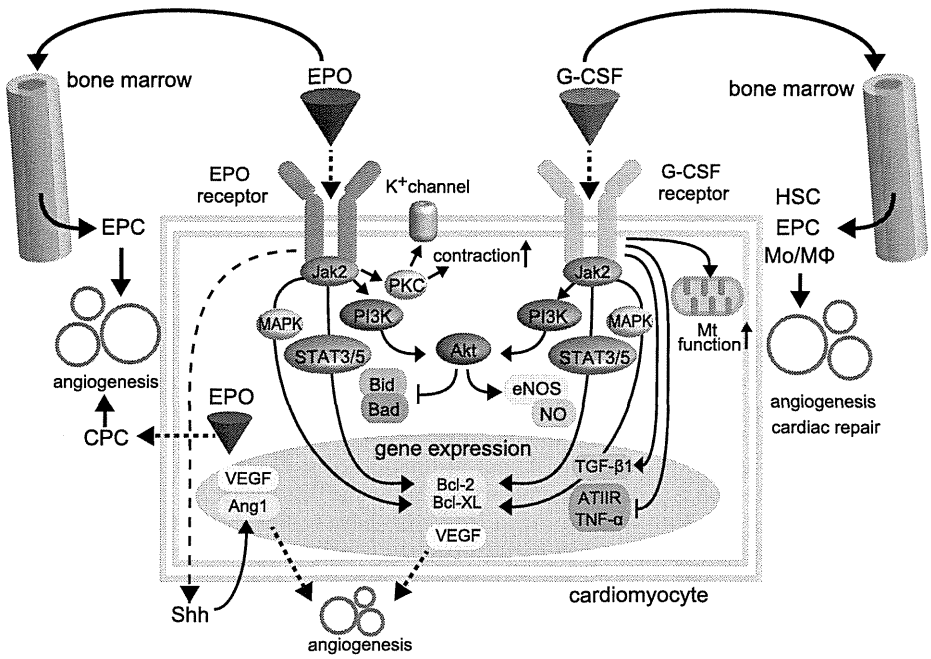

Fig. 3. Schematic view of granulocyte colony-stimulating factor (G-CSF) and erythropoietin (EPO) effects on infarcted hearts. G-CSF and EPO mobilize hematopoietic cells and endothelial progenitor cells (EPCs) from bone marrow to induce angiogenesis and cardiac repair. G-CSF and EPO activate Janus-activated tyrosine kinase 2 (JAK2) and its downstream antiapoptotic and angiogenic signaling through STAT, PI3 kinase (PI3K)/Akt, and MAPK signaling. G-CSF promotes early myocardial healing through enhancement of transforming growth factor-β_1 (TGF-β_1) and prevents later excessive fibrosis through downregulation of angiotensin II type-1 receptor (ATIIR) and TNF-α. G-CSF improves impaired mitochondria (Mt) function. EPO induce secretion of sonic hedge hoc (Shh) from cardiomyocyte and cardiomyocyte itself secrets EPO. These factors also contribute to vascular formation. Ang-1, angiopoietin1; CPC, cardiac progenitor cell; eNOS, endothelial nitric oxide (NO) synthase; HSC, hematopoietic stem cell; MΦ, macrophage; MAPK, mitogen-activated protein kinase; Mo, monocyte; PKC, protein kinase C; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

mobilization with G-CSF is still promising because of its feasibility and general applicability. In addition to an elucidation of the underlying mechanisms, additional careful and well-designed protocols in terms of dose of G-CSF, timing of the treatment, and profiles of patients are still necessary to examine because it has been reported that the degree of improvement in LV ejection fraction is inversely associated with the time to G-CSF administration in patients after reperfusion and LV ejection fraction at baseline (1, 120).

Erythropoietin. Erythropoietin (Epo) exerts its hematopoietic effects by stimulating the proliferation of early erythroid precursors and the differentiation of later precursors of the erythroid lineage (64). Independent of erythropoiesis, recently EPO was shown to protect the heart from ischemic injury (77, 118). EPO receptor was expressed in both ventricular myocytes and endothelial cells (21). The interaction between EPO and its receptors leads to dimerization of the receptors and autophosphorylation of JAK-2. JAK-2 then phosphorylates different signal transducers and activates several downstream signaling pathways, including STAT5, PI3K/Akt, and mitogen-activated protein kinase (MAPK). Erythropoietin prevented apoptosis of endothelial cells and cardiomyocytes by activating the protein kinase C and PI3K/Akt pathway (38). In addition, ATP-dependent and Ca^{2+}-activated potassium channels also mediate the protective effects of EPO in ischemic reperfusion injury (13). Recently, it has been reported that EPO induces cardiac mitochondrial biogenesis through EPO receptor-dependent activation of endothelial NO synthase, serving to protect cardiac oxidative phosphorylation during hypoxia (13). EPO promotes angiogenesis by several distinct mechanisms. Westenbrink et al. (129) have reported that EPO stimulates VEGF protein expression through STAT3 pathway predominantly in cardiomyocytes, and secreted VEGF induces the proliferation of endothelial cells and promotes the incorporation of endothelial progenitor cells (129). Ueda et al. (125) have reported that EPO induces angiogenesis by stimulating cardiomyocytes to secrete VEGF and angiopoietin 1 through sonic hedge hoc signaling independent of STAT3 pathway. Hochs et al. (48) have reported that STAT3 is crucial for expression of EPO in cardiomyocytes and cardiomyocytes-derived EPO activates blood vessel formation by cardiac progenitor cells through C-C motif ligand-2/C-C motif receptor-2 system (48). Besides protective and angiogenic effects of EPO, it has been recently reported that EPO exhibits direct positive inotropic and lusitropic effects in cardiomyocytes and ventricular muscle preparation through PI3K and PKCε isoform signaling to directly affect both Ca^{2+} release dynamics and myofilament function (45). Figure 3 shows a

Table 1. *Overview of randomized controlled trials with G-CSF in infarcted patients*

Trial	*N*	Age, yr	Dose, μg/kg (days)	Cardiac function measurement	Mean follow-up duration, mo	Outcomes
Ince et al. (51)	50	50	10 (6)	ECHO	4	LVEF↑, MACE→
Zohlnhöfer et al. (136)	114	59	10 (5)	MRI	4	LVEF→, MACE→
Valgimigli et al. (127)	20	60	5 (4)	SPECT	6	LVEF→, MACE→
Ripa et al. (101)	78	56	10 (6)	MRI	6	LVEF→, MACE→
Engelmann et al. (27)	44	58	10 (5)	MRI	3	LVEF→, MACE→
Takano et al. (119)	40	62	2.5 (5)	SPECT	6	LVEF↑, MACE→
Leone et al. (67)	41	55	10 (5)	ECHO	6	LVEF↑, MACE→

N represents the total number of patients. Age is shown as mean. G-CSF, granulocyte colony-stimulating factor; ECHO, echocardiography; LVEF, left ventricular ejection fraction; MACE, major adverse cardiac events; MRI, magnetic resonance imaging; SPECT, single-photon emission computed tomography.

diagram of cellular and molecular interaction in cardiac repair after EPO therapy after MI.

A single bolus of EPO in patients with acute MI showed that EPO was safe and well tolerated (70). EPO stimulated endothelial progenitor cell mobilization; however, LV ejection fraction was similar between the EPO-treated and control groups in this pilot study. Subsequently, A Prospective, Ransomized, Clinical Study to Examine the Effects of a Single Bolus Erythropoietin on Left Ventricular Function in Patients with an Acute Myocardial Infarction (HEBE-III) was initiated to assess the effects of EPO in a larger number of acute MI patients. The results showed that a single dose of EPO after successful percutaneous coronary intervention did not improve LV ejection fraction, but the treatment with EPO related less major adverse cardiovascular events and a favorable clinical safety profile (128). No improvement on cardiac function or reduction in infarct size was observed in A Randomized, Double-Blind, Placebo-Controlled Trial of Erythropoietin in Patients with ST-Segment Elevation Myocardial Infarction Undergoing Percutaneous Coronary Intervention (REVIVAL-3), which was another prospective, randomized, double-blind, placebo-controlled trial of EPO in patients with acute MI (87). Currently, the Reduction of Infarct Expansion and Ventricular Remodeling with Erythropoietin after Large Myocardial Infarction Trial (REVEAL) and Exogenous erythroPoietin in Acute Myocardial Infarction: New Outlook aNd Dose Association Study (EPAMINONDAS) trials are ongoing (2, 76). Table 2 shows a summary of clinical studies with EPO in infarcted patients. Despite the lack of beneficial effects of EPO on cardiac function in patients of MI, treatment with erythropoiesis-stimulating agents for chronic HF with anemia showed the improvement of LV ejection fraction along with better exercise tolerance and reduction of symptoms (63). Since it has been reported that there is an ideal dose of EPO for MI (134), the results of further clinical trials may provide us a new insight of the safety and efficacy of EPO in cardiovascular disease.

Summary

HF is a major clinical cause of morbidity and mortality, and the need for better treatments has been recognized for decades. Many new approaches to HF therapy including cells, drugs, and genes have been explored, and it is important to note that these therapies are complementary. Gene therapy means the delivery of genetic materials into cells to achieve therapeutic effects. AAV own attractive characters such as the defective replication, lack of human pathology, and prolonged transgene expression in clinical studies. Because β-adrenergic signaling and excitation-contraction coupling are crucial for cardiac contraction, gene therapy targeting for GRK2, AC6, SERCA2a, PLN, IN-1, IN-2, and S100A1 have shown promising preclinical data. Clinical trials of AC6 and SERCA2a have launched. A growing amount of evidence indicates that G-CSF and EPO promote cardiac repair after MI through mobilization of the stem or progenitor cells from bone marrow and also through direct pleiotrophic cardiovascular effects such as antiapoptosis, angiogenesis, regulation of fibrosis, and improved mitochondrial function. The preclinical studies have been actively investigated with promising data, which show that G-CSF and EPO improve cardiac function in various kinds of models of HF.

Future Directions

A recent advance in our understanding of the molecular mechanisms associated with HF clarifies several key molecules, which are promising targets for gene therapy. Gene therapy is considered as a viable adjunctive treatment to mechanical and pharmacological therapies for HF; however, further development of advanced vectors based on elaborated preclinical experiments by using large animals are still needed. The more favorable characters of next generation vectors include the regulated expression pattern by which we can manipulate the on/off system or environment-dependent expression such as tetracycline regulatory system and hypoxia-inducible factor-1α-dependent gene expression. Because of the complexity of the pathophysiology of HF, the more target molecules that are amenable to genetic manipulations have to be considered. These include key molecules that govern the homing and differentiation of stem cells, cell death/survival, and myocyte cell cycle. Further studies along with the development of more selective and noninvasive vector delivery systems will undoubtedly lead to safer and more effective gene therapy for HF.

Although most of the meta-analyses suggest that the administration of G-CSF or EPO does not improve the beneficial effects of standard HF therapy, safety of the usage of G-CSF and EPO has been demonstrated. In addition, some methodological issues, such as patient age, time of onset of symptoms to percutaneous coronary intervention, and time to administration of cytokines, need to be carefully addressed. To clarify the efficacy of G-CSF and EPO treatment, the establishment of better protocols in terms of the appropriate indications, doses, therapeutic window, as well as more mechanistic insight of molecular pathways under G-CSF and EPO are needed.

Table 2. *Overview of randomized controlled trials with EPO in infarcted patients*

Trial	*N*	Age	Dose	Cardiac function measurement	Mean follow-up duration, mo	Outcomes
Lipsic et al. (70)	50	50	300 μg (~60,000 IU) darbepoetin-α single bolus bIV	Radionuclide ventriculography	4	LVEF→, MACE→
Voors et al. (128)	529	61	60,000 IU epoietin-α single bolus IV	Radionuclide ventriculography	1.5	LVEF→, MACE→
Ott et al. (87)	138	61	33,300 IU epoietin-β single bolus IV	Angiography	6	LVEF→, MACE→
Melloni et al. (76)	210		15, 30, or 60 × 10^3 IU epoietin-α single bolus IV	MRI	3	Ongoing
Andreotti et al. (2)	102		100 or 200 IU·kg^{-1}·day^{-1} epoietin-α triple 30 min IV	MRI	12	Ongoing

N represents the total number of patients. Age is shown as mean. EPO, erythropoietin; IU, international units; IV, intravenous.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.N. and I.K. conception and design of research; T.N. drafted manuscript; I.K. edited and revised manuscript; I.K. approved final version of manuscript.

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ORIGINAL ARTICLE
Cardiovascular Intervention

Early Experiences of Transcatheter Aortic Valve Replacement in Japan

Koichi Maeda, MD; Toru Kuratani, PhD; Isamu Mizote, PhD; Kazuo Shimamura, PhD; Yasuharu Takeda, PhD; Kei Torikai, PhD; Satoshi Nakatani, PhD; Shinsuke Nanto, PhD; Yoshiki Sawa, PhD

Background: Transcatheter aortic valve replacement (TAVR) is a new alternative treatment with acceptable early results for patients with aortic valve stenosis considered to be inoperable. The first TAVR was performed in Japan in October 2009, and a total of 51 have been performed up to February 2012.

Methods and Results: Because it is not possible to disclose details for 36 patients at the time of writing due to ongoing clinical trials, the early and mid-term results of 15 patients are presented for the Edwards SAPIEN valves. Age was 83.4±6.1 years. Mean pressure gradient and aortic valve area were 60.3±21.1 mmHg and 0.64±0.19 cm^2, respectively. Left ventricular ejection fraction was 55.5±15.4%. The Logistic EuroSCORE, EuroSCORE II, and Society of Thoracic Surgeons score were 28.5±21.5%, 11.1±15.8%, and 10.0±7.4%, respectively. All of the procedures were successful and did not require conversion to surgery. Perioperative stroke did not occur, although pacemakers were implanted in 2 patients (13.3%). At discharge, mean pressure gradient and aortic valve area were improved to 10.8±4.4 mmHg and 1.77±0.36 cm^2, respectively. Except for 1 patient who died of cancer 7 months after operation, all patients were alive at the time of writing (11–848 days after procedure; mean follow-up period, 184 days).

Conclusions: Satisfactory early and mid-term results have been achieved with TAVR, indicating that this is a good alternative to treat aortic valve stenosis. (*Circ J* 2013; **77**: 359–362)

Key Words: Aortic valve stenosis; Edwards SAPIEN; Transcatheter aortic valve replacement

Aortic valve stenosis (AS) is an important cause of cardiac morbidity and mortality in elderly patients,[1] and prognosis when untreated is poor.[2] Until recently, aortic valve replacement (AVR) was the only treatment for AS, and the result was satisfactory.[3,4] There are a number of patients, however, who do not undergo surgery for various reasons.[5,6] Transcatheter AVR (TAVR) is a new alternative treatment for AS in patients considered to be inoperable or at high risk for conventional AVR, with acceptable early results reported.[7–9] We performed the first TAVR procedure in Japan in October 2009. Thereafter, PREVAIL JAPAN, a clinical trial of the Edwards SAPIEN (Edwards Lifesciences), and MDT-2111 JAPAN, a clinical trial of the Medtronic CoreValve prosthesis (Medtronic, Minneapolis, MN, USA), were started in April 2010 and October 2011, respectively. We have treated a total of 51 patients with TAVR between October 2009 and February 2012 at Osaka University Graduate School of Medicine.

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We are not allowed to disclose such details as baseline findings and clinical outcomes including safety and efficacy endpoints for patients participating in the PREVAIL JAPAN and MDT-2111 clinical trials before their data are submitted. Thus, we retrospectively evaluated 15 patients with the Edwards SAPIEN prosthesis in the present study.

Methods

Subjects

We enrolled patients with severe aortic stenosis and cardiac symptoms for whom conventional surgery to replace the aortic valve was associated with high risk. Severe aortic stenosis was defined as aortic valve area <0.8 cm^2 or effective orifice area index <0.5 cm^2/m^2, mean aortic valve gradient ≥40 mmHg, or peak aortic jet velocity ≥4.0 m/s. All the patients had New York Heart Association class II or greater symptoms.

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Table 1. Baseline Characteristics

	Total (n=15)	Trans-apical (n=7)	Trans-iliofemoral (n=8)
Age (years)	83.4±6.1 (70–91)	87.5±3.8 (80–92)	85.0±3.2 (81–94)
Female	7 (46.7)	2 (28.6)	5 (62.5)
Body surface area (m^2)	1.47±0.15 (1.16–1.73)	1.48±0.13 (1.29–1.68)	1.46±0.18 (1.16–1.73)
EuroSCORE (%)	28.5±21.5 (7.9–88.9)	20.2±10.0 (7.9–33.1)	35.6±26.7 (11.7–88.9)
EuroSCORE II (%)	12.3±16.7 (2.1–65.1)	5.6±3.3 (2.1–10.7)	15.9±20.8 (3.0–65.1)
STS score (%)	10.0±7.4 (4.3–19.2)	7.9±4.1 (4.6–15.0)	12.0±9.3 (4.3–31.1)
LVEF (%)	55.5±15.4 (27–74)	64.1±10.0 (46–73)	48.0±15.8 (27–70)
AVA (cm^2)	0.64±0.19 (0.35–0.93)	0.71±0.18 (0.35–0.93)	0.58±0.18 (0.36–0.86)
Pressure gradient (mmHg)	60.3±21.1 (32–103)	65.1±20.6 (44.4–103)	56.1±22.0 (37–102)
Diabetes	4 (26.7)	1 (14.3)	3 (37.5)
Peripheral artery disease	3 (20.0)	1 (14.3)	2 (25.0)
Previous stroke	3 (20.0)	0 (0)	3 (37.5)
Cerebrovascular disease	13 (86.7)	6 (85.7)	7 (87.5)
Respiratory dysfunction	8 (53.3)	3 (42.9)	5 (62.5)
Coronary artery disease	6 (40.0)	1 (14.3)	5 (62.5)
Previous cardiac surgery	2 (13.3)	0 (0)	2 (25.0)

Data given as mean±SD (range), or n (%). AVA, aortic valve area; LVEF, left ventricular ejection fraction; STS, Society of Thoracic Surgeons.

At least 1 surgeon and 1 cardiologist had to agree that the patient was not a suitable candidate for surgery. Pertinent exclusion criteria were bicuspid or non-calcified aortic valve; diameter of aortic annulus based on trans-esophageal echocardiography <18 mm or >25 mm; aortic dissection or ilio-femoral dimensions or disease precluding safe sheath insertion; left ventricular ejection fraction (LVEF) <20%; untreated coronary artery disease requiring revascularization; severe (>3+) mitral or aortic regurgitation or prosthetic valve; acute myocardial infarction within previous 1 month; upper gastrointestinal bleeding within previous 3 months; transient ischemic attack or stroke within the previous 6 months; any cardiac procedure other than balloon valvuloplasty within previous 1 month or within previous 6 months for drug-eluting stent; and life expectancy <12 months due to associated non-cardiac comorbid condition.

Between October 2009 and February 2012, 124 patients with severe AS, who were considered to be inoperable or at high risk for conventional aortic valve surgery, were referred to Osaka University Graduate School of Medicine. We discussed therapies for these patients at the heart multi-disciplinary meetings attending by cardiac surgeons, cardiologists, and anesthesiologists. Consequently, in these high-risk patients, we performed TAVR in 51 patients, surgical AVR in 12 patients, and prescribed medication in 59 patients. The others (2 patients) had not undergone TAVR at the time of writing. Of the 51 TAVR patients, 15 underwent TAVR with SAPIEN, for which we are able to disclose the details. The patients were 9 men and 6 women, with a mean age of 83.4±6.1 years (range, 70–91 years). The Logistic EuroSCORE, EuroSCORE II, and Society of Thoracic Surgeons score were 28.5±21.5% (range, 7.9–88.9%), 11.1±15.8% (range, 2.1–65.1%), and 10.0±7.4% (range, 4.3–31.14%), respectively. Some patients had various comorbidities not reflected by these risk scores, including shaggy or porcelain aorta (n=2), or aortic aneurysm (n=2). Preoperative transthoracic echocardiography (TTE) indicated a mean aortic valve area of 0.64±0.19 cm^2 (range, 0.35–0.93 cm^2) and mean pressure gradient of 60.3±21.1 mmHg (range, 32–103 mmHg). LVEF was 55.5±15.4% (range, 27–73%) and there were 3 patients with low LV function (LVEF, 27–38%). The diameter of the aortic annulus was 21.3±1.1 mm (range, 19.4–23.2 mm; Table 1). We evaluated clinical outcomes included 30-day mortality, hospital mortality, complications, and mid-term results. Statistical analysis was performed using JMP version 9 (SAS Institute). Data are given as mean± SD. $P<0.05$ was considered statistically significant.

TAVR Procedure

Patients with adequate iliofemoral access underwent TAVR using an Edwards SAPIEN via a trans-iliofemoral approach with standard surgical cutdown of the common iliac artery or common femoral artery, and Retroflex III delivery system, while others underwent TAVR using an Edwards SAPIEN via a trans-apical approach with a mini left anterior thoracotomy and Ascendra delivery system. In particular, in patients with aortic disease including severe tortuosity of abdominal aorta, shaggy aorta except for ascending aorta, and aneurysms, we usually choose a trans-apical approach. In consequence, trans-apical approach and trans-iliofemoral were performed in 7 and 8 patients (femoral, 2; iliac, 6), respectively. All procedures were performed by a team composed of cardiac surgeons, cardiologists, and anesthesiologists in hybrid operating room. Under general anesthesia, balloon aortic valvuloplasty, and rapid ventricular pacing were routinely used. We used a 3-D navigation system with *syngo* DynaCT (Artis Zee; Siemens AG, Forchheim, Germany) to ensure precise deployment of devices in 8 cases. Furthermore, we routinely perform TAVR with cardiopulmonary support (CPS) in patients with low LV function (LVEF <40%) to avoid immediate conversion to on-pump technique due to hemodynamic instability. In consequence, we used elective CPS in 3 patients with low LV function. Transesophageal echocardiography was utilized during the procedure in all cases, except for 1 patient who had an intractable esophagus ulcer, in which case intracardiac echocardiography was utilized.

Results

Edwards SAPIEN valves were successfully implanted in all 15 patients without conversion to surgery. A 23-mm Edwards

Table 2. Procedural Data

	Total (n=15)	Trans-apical (n=7)	Trans-iliofemoral (n=8)
Operative time (min)	114±28 (75–180)	119±28 (100–180)	109±28 (75–160)
Contrast agent (ml)	128±66 (70–300)	91±19 (70–125)	161±75 (110–300)
Procedural success	15 (100)	7 (100)	8 (100)
Transcatheter heart valve, Edwards SAPIEN prosthesis			
23mm	10 (66.7)	5 (71.4)	5 (62.5)
26mm	5 (33.3)	2 (28.6)	3 (37.5)
Cardiopulmonary support	3 (20)	0 (0)	3 (37.5), Elective 3
Use of DynaCT	8 (53.3)	6 (85.7)	2 (25.0)
Intraoperative complications	1 (6.7)	0 (0)	1 (12.5), Approach site dissection 1
Patients extubated in OR	12 (80.0)	4 (57.1)	8 (100)

Data given as mean±SD (range), or n (%). OR, operating room.

Table 3. Postoperative Transthoracic Echocardiography

	Total (n=15)	Trans-apical (n=7)	Trans-iliofemoral (n=8)
LVEF (%)	59.1±14.9 (32–76)*	64.9±12.4 (43–76)	54.1±15.8 (32–76)*
Mean AVA (cm^2)	1.77±0.36 (1.22–2.43)†	1.72±0.27 (1.43–2.10)†	1.81±0.44 (1.22–2.43)†
mean gradient (mmHg)	10.8±4.4 (4.7–17.4)†	10.2±5.5 (4.7–17.0)**	11.3±3.6 (7.2–17.4)**
Perivalvular leakage			
None	2 (13.3)	1 (14.3)	1 (12.5)
Trivial	1 (6.7)	0 (0)	1 (12.5)
Mild	12 (80.0)	6 (85.7)	6 (75.0)
Moderate	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)

Data given as mean±SD (range), or n (%). *P<0.05, **P<0.005, †P<0.0005 compared with preoperative data. AVA, aortic valve area; LVEF, left ventricular ejection fraction.

Table 4. Early Clinical Results

	Total (n=15)	Trans-apical (n=7)	Trans-iliofemoral (n=8)
30-day mortality	0 (0)	0 (0)	0 (0)
Hospital mortality	0 (0)	0 (0)	0 (0)
Postoperative complications			
Stroke	0 (0)	0 (0)	0 (0)
Bleeding	1 (6.7)	1 (14.3)	0 (0)
Tamponade	0 (0)	0 (0)	0 (0)
Tracheotomy	0 (0)	0 (0)	0 (0)
PMI	2 (13.3)	1 (14.3)	1 (12.5)
Wound infection	0 (0)	0 (0)	0 (0)
Postoperative hospital stay (days)	20.1±12.0 (9–50)	22.7±15.7 (10–50)	17.8±7.9 (9–29)
Home discharge	11 (73.3)	6 (85.7)	5 (62.5)

Data given as mean±SD (range), or n (%). PMI, pacemaker implantation.

SAPIEN prosthesis was used in 10 patients and the 26-mm prosthesis was used in 5 patients (Table 2). Pre-implantation balloon valvuloplasty was done in all cases. In terms of perivalvular aortic regurgitation, none, trivial, and mild were found in 2 patients (13.3%), 1 patient (6.7%), and 12 patients (80%), respectively. Regarding intraoperative complications, an endovascular repair was required for dissection at the iliac approach site in 1 case. The mean operation time was 114±28 min (range, 75–180 min) and 12 patients (80%) were able to be extubated in the operating room. One patient who had the transapical approach required reoperation for bleeding on postoperative day 1. Furthermore, 1 patient with a huge abdominal aortic aneurysm and another with a descending aortic aneurysm underwent endovascular repair on postoperative day 1 and 51, respectively. There was no occurrence of perioperative stroke, while pacemakers were implanted in 2 patients (13.3%) for sick sinus syndrome and systolic anterior motion. At discharge, TTE demonstrated that there was significant hemodynamic improvement with a mean aortic valve area of 1.77±0.36 cm^2 (range, 1.22–2.43 cm^2) and mean pressure

Table 5. Mid-Term Clinical Outcome

	Total (n=15)	Trans-apical (n=7)	Trans-iliofemoral (n=8)
All cause mortality, mean follow-up: 187 days (11–848)	1 (6.7)	0 (0)	1 (12.5), Cancer 1
Cardiovascular mortality	0 (0)	0 (0)	0 (0)
Re-hospitalization (caused by cardiovascular event)	1 (6.7)	0 (0)	1 (12.5), TEVAR for DTA aneurysm 1

Data given as n (%). DTA, descending thoracic aorta; TEVAR, thoracic endovascular aortic repair.

gradient of 10.8±4.4 mmHg (range, 4.7–17.4 mmHg). Also, LVEF improved significantly from 55.5±15.4% to 59.1±14.9% ($P<0.05$; Table 3). Duration of postoperative stay was 20.1±12.0 days, and 11 patients achieved home discharge (Table 4).

Except for 1 patient, all patients were alive with a mean follow-up period of 184 days (range, 11–848 days). One patient died of unknown cancer 7 months after operation. There was only 1 patient who had cardiovascular-related re-hospitalization (Table 5). This patient underwent elective thoracic endovascular repair for a descending aortic aneurysm, which was found preoperatively.

Discussion

TAVR has recently been shown to be feasible for patients with severe AS who are considered inoperable, with satisfactory early results reported.[7–9] We were the first to perform TAVR in Japan in October 2009. Presently, 2 clinical trials are underway and we have performed TAVR in 51 patients between October 2009 and February 2012. Early outcomes are satisfactory, although there have been some difficult cases, including poor calcification at the annulus, and low LV function. We have strategies for TAVR at Osaka University Graduate School of Medicine.

First, in patients with aortic disease, including severe tortuosity of abdominal aorta, shaggy aorta except for ascending aorta, and aneurysms, we usually choose a trans-apical approach. Discussion based on preoperative multislice computed tomography is mandatory to reduce the risk of serious complications such as aortic rupture or thromboembolisms. We note that such preoperative examinations were sufficiently thorough to prevent serious complications, and there were no strokes or hospital deaths in the present study.

Second, major complications may occur during the procedure and, in particular, patients with low LV function may require immediate conversion to an on-pump technique due to hemodynamic instability. Therefore, we routinely perform TAVR with CPS in patients with low LV function (LVEF <40%) and achieve acceptable outcomes. Because CPS time is usually short (approximately 10 min), we have seen no complications due to CPS.

Third, nearly all of the present patients were able to start early rehabilitation and the majority (n=12, 80%) were extubated in the operating room. We have tried to reduce postoperative pain especially in the case of trans-apical approach. In consequence, 9 continuous patients, including 4 patients who had the trans-apical approach, achieved extubation in the operating room. Because TAVR is less invasive than conventional AVR, patients can achieve early extubation and start early rehabilitation, thereby reducing the rate of perioperative complications. Consequently, we achieved short postoperative stay (20.1±12.0 days) and a relatively high rate of home discharge (73.3%) even in high-surgical-risk patients.

Conclusions

We have performed TAVR in 51 patients, from October 2009 to February 2012. In 15 cases that we have been allowed to report upon, satisfactory results have been achieved. TAVR may be an alternative to surgery for AS.

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STEM CELLS TRANSLATIONAL MEDICINE℠

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Bioengineered Myocardium Derived from Induced Pluripotent Stem Cells Improves Cardiac Function and Attenuates Cardiac Remodeling Following Chronic Myocardial Infarction in Rats

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Key Words. Induced pluripotent stem cells • Bioengineered myocardium • Implantation • Myocardial infarction

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ABSTRACT

Cell-based therapies are promising strategies for myocardial repair following myocardial infarction. Induced pluripotent stem (iPS) cells have the potential to generate many cardiomyocytes, and they hold significant promise for the application of regenerative medicine to heart failure. Here, we developed cardiac tissue sheets, termed bioengineered myocardium (BM), from mouse iPS cells and measured cardiac performance following BM implantation in a rat chronic myocardial infarction model. Immunostaining analyses revealed that the α-actinin$^+$ cell population was isolated with more than 99% purity under specific culture conditions. To evaluate the contribution of BM to the improvements in cardiac performance, we induced myocardial infarction in 30 F344/NJcl-*rnu/rnu* rats by left anterior descending coronary ligation. The rats were randomly divided into two groups, 2 weeks after ligation: a BM implantation group ($n = 15$) and a sham group ($n = 15$). Echocardiography and catheter examination showed that the BM implantation significantly improved cardiac function and attenuated cardiac remodeling compared with the sham group. Histological analyses demonstrated that the implanted BM survived at the epicardial implantation site 4 weeks after implantation. The implanted BM survived and attenuated left ventricular remodeling in the rat chronic myocardial infarction model. Thus, BM derived from iPS cells might be a promising new treatment for heart failure. STEM CELLS TRANSLATIONAL MEDICINE *2012;1:430–437*

INTRODUCTION

Even though remarkable progress has been made in the medical and surgical management of cardiac diseases, heart failure remains a major cause of death worldwide [1, 2]. Cell-based therapies using skeletal myoblasts, bone marrow mononuclear cells, mesenchymal stem cells, and cardiac stem cells were recently introduced for the clinical treatment of ischemic heart disease [3–6]. Bolli et al. [6] reported that intracoronary infusion of autologous cardiac stem cells is effective in improving left ventricular (LV) systolic function and reducing infarct size in patients with heart failure after myocardial infarction (MI). However, other studies [3–5] showed that their effectiveness is limited and that long-term outcomes are unfavorable.

Growth factors secreted from the transplanted cells are thought to improve the damaged myocardium to a certain extent; this occurs via a paracrine effect [7, 8]. Myoblasts or bone marrow mononuclear cells may improve cardiac performance via a paracrine effect, without directly contributing to the improvement of systolic performance by enhancing the contractile ability of the heart [9, 10]. Several researchers have developed certain stem or progenitor cells that can differentiate into cardiomyocytes or generate cardiomyocytes via cell fusion [11, 12], in order to obtain cells that can compensate for lost cardiomyocytes and greatly promote myocardial regeneration in a severely damaged myocardium following transplantation [13, 14]. However, the differentiation rate of these stem or progenitor cells into cardiomyocytes, or their fusion rate, is relatively low. Therefore, improvement of the impaired cardiac performance by these cells is probably mainly due to paracrine effects.

We think that two important issues need to be addressed in order to improve the effectiveness of cell-based therapies for heart failure. First, autologous stem cells, which differentiate into definitive cardiomyocytes at high rates, need to be used to greatly enhance the contractile ability of the myocardium in situations where

large numbers of cardiomyocytes are damaged. Second, these cells need to be delivered to the damaged myocardium at the maximum dose by using sophisticated cell delivery systems. For example, the development of systems that will enable newly generated cardiomyocytes to integrate with each other ex vivo to form cardiac tissue, and enable this cardiac tissue to synchronously contract with the host myocardium following implantation, should be investigated. Such a strategy may provide the best method for regenerating a severely damaged myocardium.

An intelligent cell delivery system, termed cell sheet technology, was recently developed [15–17]. Conventional methods of cell delivery present some disadvantages, such as needle injection, loss of transplanted cells by leakage, poor survival of grafted cells, myocardial damage resulting from injury by the needle and subsequent acute inflammation [18, 19], and the potential to cause a lethal arrhythmia [20]. However, a cell sheet can deliver substantially higher cell numbers to the damaged myocardium than can needle injection, and it can induce significant myocardial regeneration [21]. Pluripotent stem cells have been generated from mouse and human somatic cells by using specific transcription factors (OCT4, SOX2, c-MYC, and KLF4 or OCT4, SOX2, NANOG, and LIN28) [22–24]; cell-based therapy using such induced pluripotent stem (iPS) cells is expected to find widespread clinical applications. There have been several reports on the differentiation of iPS cells into cardiomyocytes [25–27], but it is unknown whether cardiac tissue sheets derived from iPS cells can improve cardiac function in a chronic MI model. In this study, we developed cardiac tissue sheets termed bioengineered myocardium (BM) from mouse iPS cells, and investigated the efficacy of BM derived from iPS cells in a chronic MI model.

Materials and Methods

Cardiac Differentiation of iPS Cells and Purification of iPS Cell-Derived Cardiomyocytes

We cultured and maintained mouse iPS cells (256H18) [28] and embryonic stem (ES) cells (B6G2) on feeder layers of mitomycin-C-treated STO cells (supplemental online data) [22]. For differentiation, 500 iPS cells were resuspended in 30-μl aliquots of differentiation medium (DM; growth medium without leukemia inhibitory factor) and cultured in 96-well HydroCell plates (CellSeed, Tokyo, http://www.cellseed.com) for 2 days. On day 2, an additional 30 μl of DM containing 4 μM 6-bromoindirubin-3′-oxime (BIO; a glycogen synthase kinase-3β inhibitor, to activate the Wnt-signaling pathway) (Calbiochem, San Diego, http://www.emdbiosciences.com) [29] was added to each well. On day 5, the individual embryoid bodies (EBs) obtained were transferred to gelatin-coated 60-mm dishes (200 EBs per dish). The medium was replenished with fresh DM every day. On day 11, the medium was changed to no-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) (F. Hattori and K. Fukuda, WO2007/088874; PCT/JP2007/051563, 2007), and the cells were maintained for a further 2–3 days. The medium was then replaced with DM, and on day 15, the cells were used for reverse transcription-polymerase chain reaction (RT-PCR), quantitative RT-PCR, or immunocytochemistry (supplemental online data).

Development of the iPS Cell-Derived Contractile BM

To develop the contracting BM from iPS cells, individual EBs were seeded onto 24-well UpCell plates (CellSeed) (40 EBs per well) on day 5. Each UpCell plate contains cell culture surfaces to which a temperature-responsive polymer, poly(*N*-isopropylacrylamide), is grafted, and from which cells can be detached as a cell sheet simply by reducing the temperature without any enzymatic treatments [15–17].

Next, the medium was changed to DM and no-glucose DMEM as outlined above, until day 15. The iPS cell-derived cardiomyocytes were then detached at room temperature as a cell sheet with a diameter of approximately 10 mm (supplemental online Fig. 1). This cardiac tissue sheet, termed BM, exhibited spontaneous contraction (supplemental online Movie 1) and was used for implantation.

Animal Experiments

We used F344/NJcl-*rnu/rnu* female rats (CLEA Japan, Tokyo, http://www.clea-japan.com) in which MI was induced by left anterior descending (LAD) coronary ligation [21, 30, 31]. Two weeks later, the animals were randomly divided into two groups: the BM implantation group ($n = 15$) and the sham group ($n = 15$) in which we carried out MI surgery, and opened and closed the rat chests 2 weeks later. BM was implanted directly over the scar area without sutures. After detachment from the temperature-responsive dish, the BM was picked up and spread over the surface of the heart, following which it was allowed to attach to the host myocardium for 10–15 minutes. The animal experiments were performed in compliance with 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 Resource and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The ethics committee of Osaka University approved all experiments.

Assessment of Cardiac Performance

Echocardiography was performed before implantation and 1, 2, and 4 weeks after implantation. We measured the anterior wall dimension, posterior wall dimension, left ventricular diastolic dimension (LVDd), and left ventricular systolic dimension (LVDs), and then we calculated the LV ejection fraction (EF) and fractional shortening (FS). EF and FS were calculated using the following formulas:

$$EF\ (\%) = [(LVDd^3 - LVDs^3)/LVDd^3] \times 100$$

$$FS\ (\%) = [(LVDd - LVDs)/LVDd] \times 100$$

A catheter examination was performed 4 weeks after implantation; sternotomy was performed using a thin catheter tip, and hemodynamics parameters such as dP/dt (an index that is used clinically to characterize the contractile ability of the heart) maximum (max), dP/dt minimum (min), the time constant of isovolumic relaxation (τ), end-systolic elastance (Ees), and end-diastolic elastance (Eed) were determined.

Preparation of LV Myocardium Specimens for Histological Analysis and RNA Isolation

After performing echocardiography and catheter examination, the hearts ($n = 15$ per group) were removed 4 weeks following

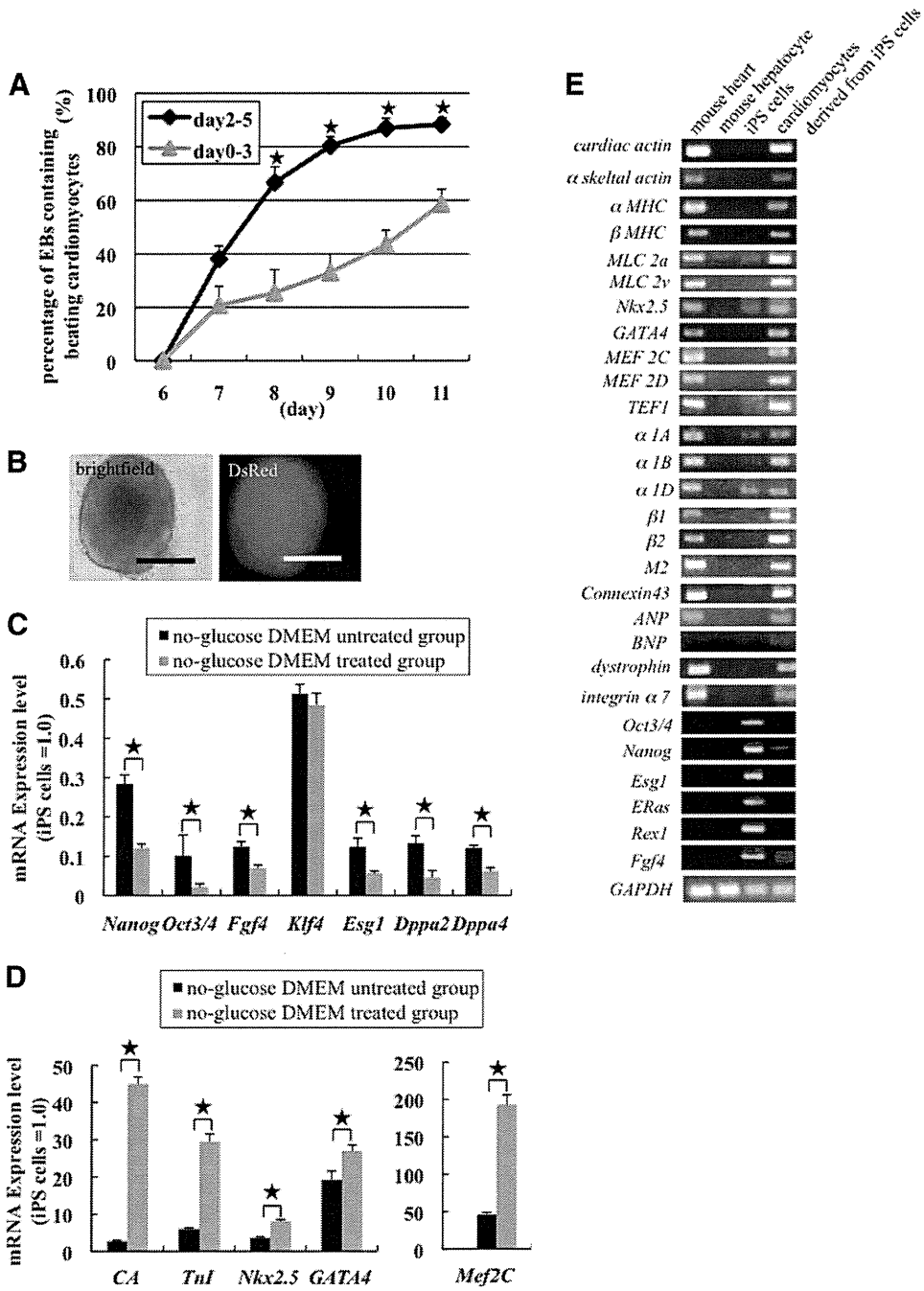

Figure 1. Efficiency of differentiation of mouse iPS cells into cardiomyocytes. **(A):** Number of EBs with beating foci. ⋆, $p < .05$. **(B):** Image of an EB derived from iPS cells obtained on day 5. DsRed was expressed in the EB because the mouse iPS cell line 256H18 was used. Scale bars = 300 μm. **(C–E):** Gene expression in iPS cell-derived cardiomyocytes. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses of the expression levels of undifferentiated cell marker genes (*Nanog*, *Oct3/4*, *Fgf4*, *Esg1*, *Dppa2*, and *Dppa4*) **(C)** and cardiac marker genes (*cardiac actin* [*CA*], *Troponin I* [*TnI*], *Nkx2.5*, *GATA4*, and *Mef2C*) **(D)** in the no-glucose DMEM-treated group and the no-glucose DMEM-untreated group ($n = 5$ per group). ⋆, $p < .05$. **(E):** RT-PCR analyses of cardiac marker genes, such as those for structural proteins (*cardiac actin* to *MLC2v*), transcription factors (*Nkx2.5* to *TEF1*), adrenergic receptors (α*1A* to β*2*), muscarinic receptors (*M2*), other proteins (*connexin 43* to *integrin* α*7*), and markers for undifferentiated cells (*Oct3/4* to *Fgf4*) in native cardiomyocytes, native hepatocytes, iPS cells, and iPS cell-derived cardiomyocytes. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DsRed, *Discosoma* red fluorescent protein; iPS, induced pluripotent stem.

implantation and sectioned into two pieces each. Each heart was cut into cross-sections for nine specimens in each group, with one section snap-frozen in liquid nitrogen and two others embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, http://www.sakura-finetek.com) for immunostaining and Masson's trichrome staining. Fibrosis areas were calculated using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com). The remaining six specimens in each group were transverse-sectioned; the apex-side specimens were

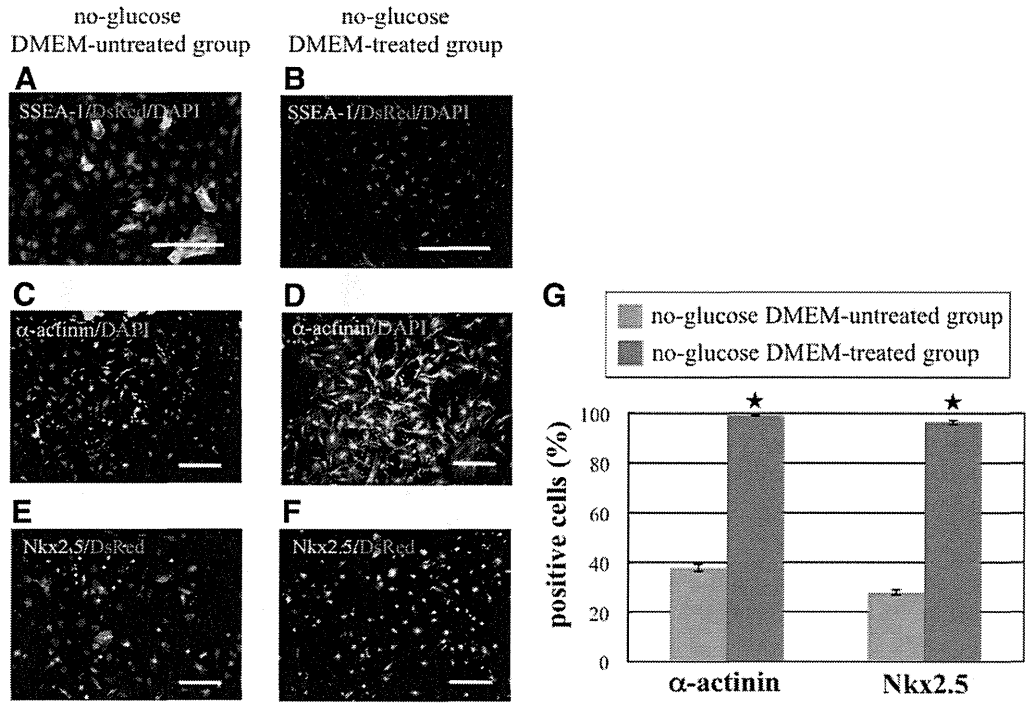

Figure 2. Immunostaining of induced pluripotent stem (iPS) cell-derived cardiomyocytes. **(A–F):** Microscopic images showing differentiated cells on day 15 in the no-glucose DMEM-untreated group **(A, C, E)** and in the no-glucose DMEM-treated group **(B, D, F)**. **(A, B):** Immunostaining of SSEA-1 (green). Nuclei were stained with DAPI (blue); DsRed (red) was expressed in the cytosol of iPS cell-derived cells. Scale bars = 100 μm. **(C, D):** Immunostaining of α-actinin (green). Nuclei were stained with DAPI. Scale bars = 300 μm. **(E, F):** Immunostaining of Nkx2.5 (green) and DsRed (red) expression in the cytosol of iPS cell-derived cells. Scale bars = 300 μm. **(G):** Quantification of α-actinin- and Nkx2.5-positive cells by imaging analysis. In the no-glucose DMEM-untreated group (gray columns), the percentages of α-actinin- and Nkx2.5-positive cells were 37.87% and 27.92%, respectively. In contrast, the percentages of α-actinin- and Nkx2.5-positive cells in the no-glucose DMEM-treated group (red column) were 99.18% and 96.45%, respectively. ⋆, $p < .05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DsRed, *Discosoma* red fluorescent protein; SSEA, stage-specific embryonic antigen.

dissected to remove the right ventricular free wall and divided into two pieces from either the infarction or the remote sites. Each specimen was stored in RNAlater solution (Qiagen, Hilden, Germany, http://www1.qiagen.com) for RNA isolation and in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) for the enzyme-linked immunosorbent assay (ELISA) (supplemental online data).

Statistical Analysis

All values are expressed as mean (SD). Statistical comparison of the data was performed using unpaired two-sided *t* tests. A *p* value of <.05 was considered statistically significant.

RESULTS

Expression of Several Cardiac Markers by iPS Cell-Derived Cardiomyocytes and Procurement of α-Actinin-Positive Cardiomyocytes with More than 99% Purity

To obtain high-purity cardiomyocytes from iPS cells (256H18 clone), we first generated EBs by treatment with BIO and determined the best culture conditions to induce the differentiation of iPS cells into cardiomyocytes. When EBs were cultured with BIO from day 2 until day 5, spontaneous contraction occurred within the small foci in approximately 88% of the EBs on day 11. In contrast, when EBs were cultured with BIO from day 0 until day 3, the number of beating foci significantly decreased from day 8 to day 11 when compared with the cells cultured with BIO from day 2 to day 5 (Fig. 1A). The EBs formed from the 256H18 iPS cell line expressed *Discosoma* red fluorescent protein (DsRed) in the cytosol (Fig. 1B). When EBs derived from ES cells were treated with BIO on days 2–5, they exhibited spontaneous contraction equivalent to that of the iPS cells treated with BIO for the same period (supplemental online Movie 2).

In order to avoid teratoma formation after implantation, we changed the DM to no-glucose DMEM for days 11–12 or 13, and then reverted to DM the next day. This method enabled us to easily select differentiated cardiomyocytes from undifferentiated cells based on their different energy consumption requirements.

We performed RT-PCR, quantitative RT-PCR, and immunostaining with differentiated cells from day 15 to investigate the purification efficiency. Quantitative RT-PCR analysis revealed that the expression of undifferentiated cell marker genes (*Nanog*, *Oct3/4*, *Fgf4*, *Esg1*, *Dppa2*, and *Dppa4*) was significantly lower in the no-glucose DMEM-treated group than in the no-glucose DMEM-untreated group. These data indicated that iPS cells cultured in no-glucose DMEM had lower potential to form tumors following implantation (Fig. 1C). The expression of cardiac marker genes (*cardiac actin*, *TnI*, *Nkx2.5*, *Gata4*, and *Mef2C*) was significantly higher in the no-glucose DMEM-treated group than in the no-glucose DMEM-untreated group (Fig. 1D). Therefore, we surmised that no-glucose DMEM treatment significantly increases the purity of differentiated iPS cell-derived cardiomyocytes by destroying noncardiomyocytes. RT-PCR analysis further revealed that iPS cell-derived cardiomyocytes treated with BIO and no-glucose DMEM expressed several cardiac markers,

such as structural proteins, transcription factors, adrenergic and muscarinic receptors, and connexin 43, to nearly the same extent as the native cardiomyocytes did (Fig. 1E). Conversely, markers (*Oct3/4*, *Nanog*, *Esg1*, *Eras*, *Rex1*, and *Fgf4*) for undifferentiated cells were downregulated in iPS cell-derived cardiomyocytes compared with iPS cells (Fig. 1E). Immunostaining of stage-specific embryonic antigen-1 (SSEA-1) (a marker of undifferentiated cells) revealed that a few SSEA-1-positive cells remained alive in the no-glucose DMEM-untreated group even at day 15, but that SSEA-1-positive cells were observed in the no-glucose DMEM-treated group (Fig. 2A, 2B). In contrast, immunostaining of α-actinin and Nkx2.5 (cardiac markers) revealed that the percentages of α-actinin and Nkx2.5-positive cells in the no-glucose DMEM-untreated group were 37.87% and 27.92%, respectively, but those in the no-glucose DMEM-treated group were 99.18% and 96.45%, respectively (Fig. 2C–2G). Thus, we successfully obtained high-purity cardiomyocytes from iPS cells by using the combined BIO and no-glucose DMEM culture methods.

Improvement in Cardiac Performance and Attenuation of Remodeling by the BM Derived from iPS Cells

We next investigated the therapeutic effect of the BM derived from iPS cells in vivo by inducing MI in F344/NJcl-*rnu/rnu* rats (nude rats) and then implanting the BM derived from iPS cells onto the infarcted area 2 weeks after the infarction. Echocardiographic analysis revealed that FS improved and LVDd decreased in the group implanted with the BM derived from iPS cells (i.e., the BM group) 4 weeks following implantation (Fig. 3A, 3B; supplemental online Table 1). Thus, the BM improved systolic performance and attenuated LV dilatation. The systolic and diastolic performances also improved after implantation, as revealed by pressure-volume analysis (Fig. 3C). The hemodynamics indices were then determined by performing a catheter examination. Significantly higher Ees and lower Eed, higher dP/dt max and lower dP/dt min, and lower τ were observed in the BM group compared with the sham group 4 weeks after implantation (Fig. 4A, 4B). Thus, these parameters indicate that the BM derived from iPS cells improved systolic and diastolic performances.

Survival and Attenuation of Fibrosis by the BM Derived from iPS Cells at the Epicardial Implantation Site 4 Weeks After Implantation

We obtained LV myocardial specimens 4 weeks after implantation to confirm the survival of the implanted BM derived from iPS cells. Masson's trichrome staining revealed less fibrosis and increased thickness of the anterior wall at the site where the BM was implanted (Fig. 5A, 5B, 5E, 5F). Fluorescence microscopy revealed that the implanted BM, which expressed DsRed in the cytosol, survived at the epicardial implantation site (Fig. 5C, 5D, 5G, 5H). Immunohistochemistry revealed that DsRed-positive surviving cells expressed α-actinin in the sarcomere (Fig. 5I–5N). In addition, the percentage of fibrosis in the BM group was significantly lower than that in the sham group (supplemental online Fig. 2). Furthermore, ELISAs performed for hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) revealed that the expression levels of these angiogenic proteins were higher in the BM group than in the sham group (supplemental online Fig. 3). These data indicated that the BM derived from iPS cells also induced a paracrine or autocrine effect by secreting angiogenic factors and inducing angiogenesis in the impaired myocardium. Thus, these data indicate that the BM derived from iPS cells may improve cardiac performance in severely damaged myocardium by directly affecting contractile function and inducing a paracrine effect on angiogenic proteins.

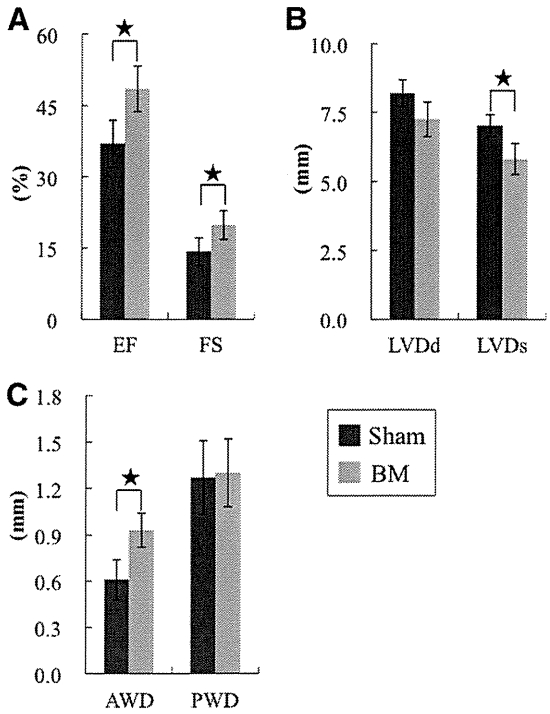

Figure 3. Effects of the induced pluripotent stem cell-derived BM on postinfarct ventricular function at 4 weeks by echocardiography. Shown is echocardiographic assessment of EF **(A)** (left) and FS **(A)** (right), LVDd **(B)** (left), LVDs **(B)** (right), AWD **(C)** (left), and PWD **(C)** (right) in the BM group ($n = 15$) and the sham group ($n = 15$). ★, $p <$ .05. The systolic performance was significantly improved and the left ventricular dilatation was attenuated in the BM group compared with the sham group. Abbreviations: AWD, anterior wall dimension; BM, bioengineered myocardium; EF, ejection fraction; FS, fractional shortening; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; PWD, posterior wall dimension.

Discussion

We previously demonstrated that neonatal cardiomyocyte sheets can survive in the hearts of rats with chronic MI and that cardiac performance was improved by the successful electrical and histological integration of these sheets into the host myocardium [30]. However, this strategy cannot be used in clinical settings because the large numbers of neonatal cardiomyocytes required cannot be obtained under ethical guidelines. Therefore, we attempted to use autologous iPS cells, due to their high cardiomyocyte differentiation rates. Such cells may completely regenerate a damaged myocardium and contribute to the armamentarium of heart failure management in clinical settings.

Our data indicate that the iPS cell-derived cardiomyocytes, differentiated by combined treatment with BIO and no-glucose DMEM, expressed cardiac markers and were highly similar to native mouse cardiomyocytes. As seen in supplemental online Figure 4, the organization of α-actinin in iPS cell-derived cardiomyocytes was random and similar to that of mouse neonatal cardiomyocytes. We postulate that the iPS cell-derived cardiomyocytes generated using our protocol included embryonic- and adult-phenotype ventricular and atrial myocytes.

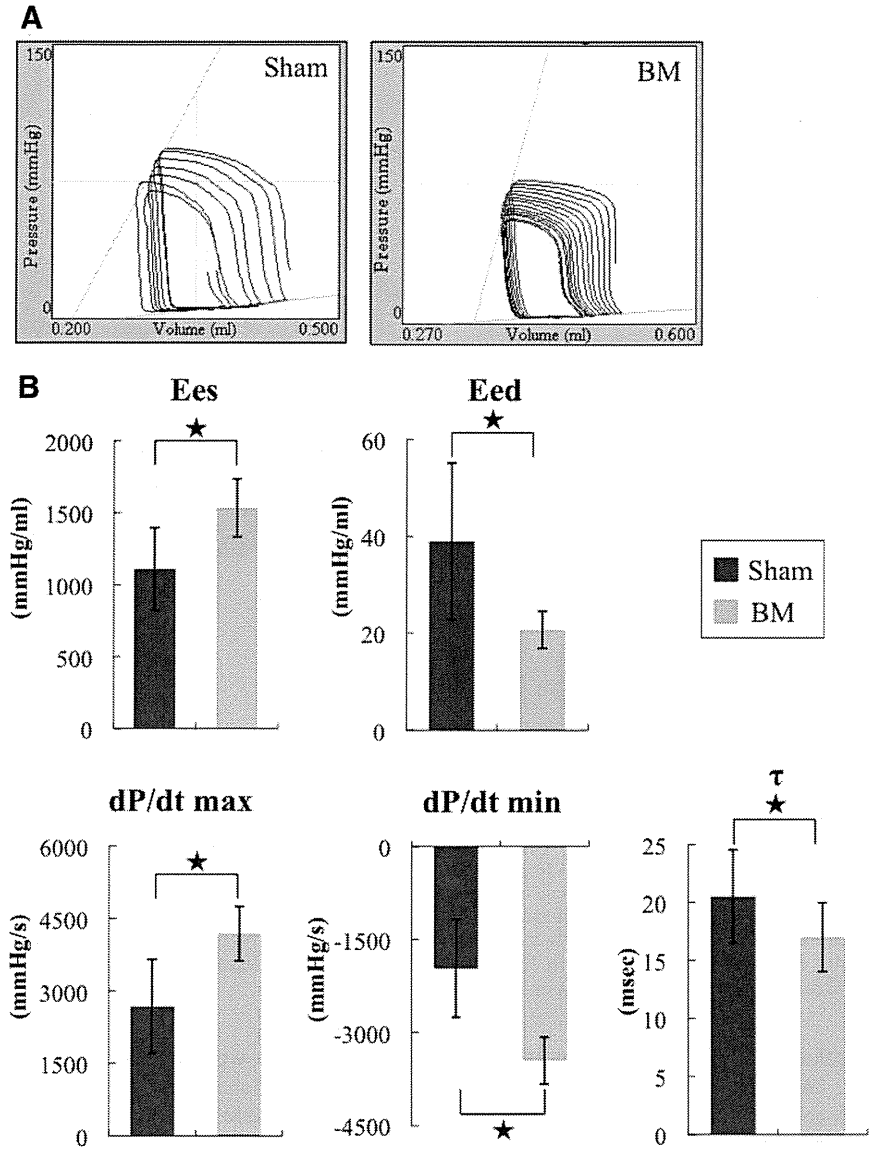

Figure 4. Effects of the induced pluripotent stem cell-derived BM on postinfarct ventricular function at 4 weeks by catheter examination. **(A):** Representative pressure-volume loops of the sham and BM groups. The systolic and diastolic performances of the BM group were significantly better than those of the sham group. **(B):** Catheter examination of Ees, Eed, dP/dt max, lower dP/dt min, and lower τ in the BM group ($n = 15$) and the sham group ($n = 15$). ⋆, $p < .05$. Abbreviations: BM, bioengineered myocardium; Eed, end-diastolic elastance; Ees, end-systolic elastance; max, maximum; min, minimum.

The most significant finding of the present study is the ability of the iPS cell-derived BM to improve cardiac performance, attenuate cardiac remodeling, recover wall thickness, and survive at the epicardial implantation site after implantation. These findings led us to explore the mechanism by which this improvement occurs. We believe that this improvement is achieved by two effects: (a) a direct effect of the BM synchronously contracting with the host myocardium, and (b) a paracrine effect of growth factors secreted at the site of implantation. We have previously demonstrated that (noncontracting) fibroblast sheets failed to improve systolic performance compared with neonatal cardiomyocyte sheets, and we did not observe recovery of wall thickness in the fibroblast sheet-implantation group [30]. In this study, the implanted contracting BM improved cardiac performance, recovered wall thickness, and survived at the epicardial implantation site for up to 4 weeks after implantation. This suggests that the BM, similar to neonatal cardiomyocyte sheets [30], synchronously contracts with the host myocardium and directly improves systolic performance. However, as seen in Figure 5M and 5N, DsRed-negative cells were observed in the BM on magnification images. We speculate the following explanation. In the previous study [30], we showed that host cells such as fibroblasts, endothelial cells, and some stem cells migrate into the transplanted sheet in response to HGF and VEGF. Therefore, host cells may similarly migrate into the BM. Moreover, we did not dissociate cardiomyocytes before implantation of the BM, because this destroys the extracellular matrix and gap junctions. As seen in Figure 2D, almost all of the surviving cells in no-glucose DMEM were α-actinin$^+$ cells. However, once the cardiomyocytes are dissociated, they do not reconstruct a sheet even if high-purity cardiomyocytes are seeded at a high density onto UpCell. Then, in this study, to obtain the BM as a sheet, we did

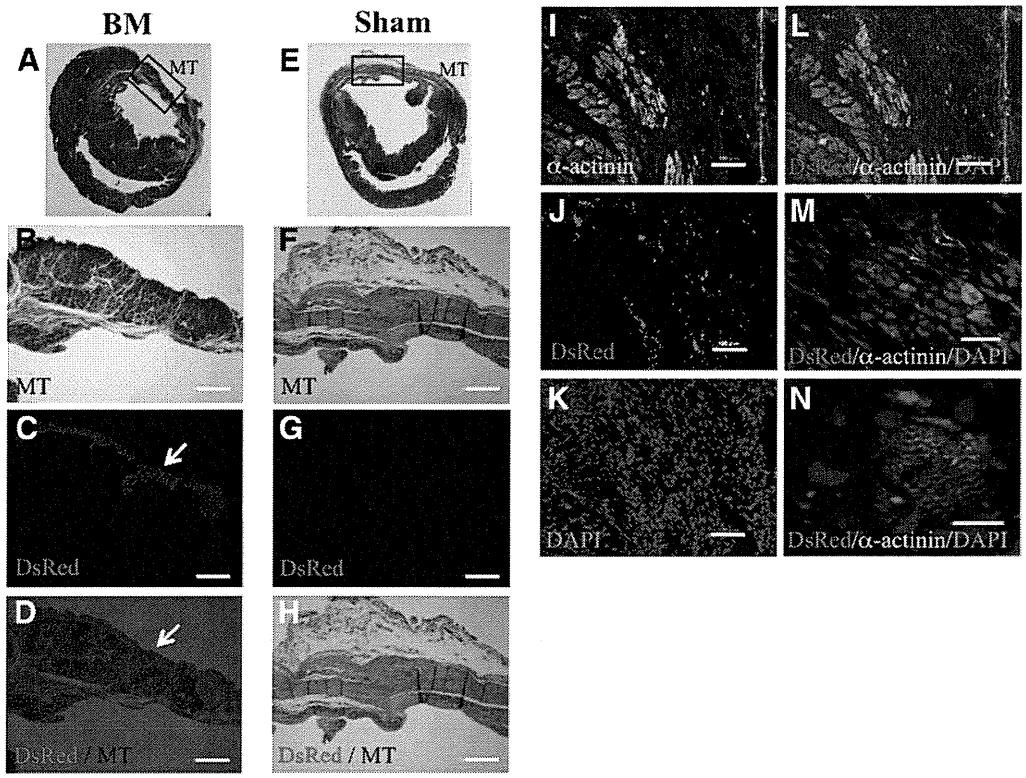

Figure 5. Histological examination of short-axis specimens at 4 weeks. **(A–H):** Microscopic images showing the whole heart and the anterior wall region in the BM group **(A–D)** and the sham group **(E–H)**. MT staining **(A, B, E, F)**. DsRed **(C, G)** was expressed in the cytosol of induced pluripotent stem (iPS) cell-derived cells in the BM group. **(D):** Merged image of **(B)** and **(C)**. **(H):** Merged image of **(F)** and **(G)**. The iPS cell-derived BM survived at the epicardial implantation site (arrows in **[C, D]**), attenuated fibrosis, and increased the thickness of the anterior wall. Scale bars = 500 μm. **(I–N):** Fluorescent microscopic images showing a part of the anterior wall region in the BM group. **(I):** Immunofluorescent histochemistry of α-actinin. DsRed was expressed in the cytosol of iPS-derived cells **(J)**; the nuclei were stained with DAPI **(K)**. **(L):** Merged image of **(I–K)**. Scale bars = 100 μm. **(M, N):** High-magnification images showing the colocalization of surviving DsRed- and α-actinin-positive cells in the anterior wall. Scale bars = 40 **(K)** and 10 μm **(L)**. Abbreviations: BM, bioengineered myocardium; DAPI, 4′,6-diamidino-2-phenylindole; DsRed, *Discosoma* red fluorescent protein; MT, Masson's trichrome.

not use a dissociation solution such as StemPro Accutase (Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com) or trypsin. Therefore, α-actinin$^+$ cells were not arranged uniformly in the BM sheet, and its thickness varied throughout. For example, the section of EBs were thicker than the region of outgrowth of cells from EBs, and there were some small holes in the BM, likely representing areas of dead cells resulting from the use of no-glucose DMEM. Host cells are more likely to migrate into such areas. ELISA results revealed that elevated levels of HGF and VEGF probably contributed to the improvement of systolic performance by inducing angiogenesis and the migration of some stem cells, as observed with the implantation of skeletal myoblast sheets. Alternatively, the nondamaged host myocardium probably continues to survive after LAD coronary ligation because of the multiple autocrine or paracrine effects induced by the implanted BM, thereby improving the systolic and diastolic performances.

iPS cells can cause tumors such as teratomas; therefore, there is some apprehension regarding their use in clinical settings. We did not observe SSEA-1-positive cells by immunostaining analysis in the no-glucose DMEM-treated group. However, it is also important to acknowledge that it takes only one cell to form a tumor, and it is virtually impossible to guarantee that 100% of the cells are differentiated and safe. Therefore, to investigate the effect of no-glucose DMEM treatment on cardiomyocyte purification, we examined mice for teratomas after implantation of the BM that had been maintained for various times in no-glucose DMEM. The teratoma formation (TF) rate decreased as the period of no-glucose DMEM treatment was extended, and there was no TF in the 3-day no-glucose DMEM treatment group (supplemental online Fig. 5). Therefore, any undifferentiated cells cannot survive in no-glucose DMEM for more than 3 days.

Conclusion

We propose that the BM derived from iPS cells can improve cardiac performance and attenuate remodeling in a rat infarction model. Additional studies are required to confirm the mechanical and electrical functions in vivo; nevertheless, the BM derived from iPS cells may be a promising part of the armamentarium for the treatment of heart failure.

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Author Contributions

K.M.: conception and design, data collection, data interpretation, manuscript writing; H.U.: conception and design, data collection, data interpretation, manuscript writing assistance; A.S.: conception and design, data interpretation, manuscript revision, final approval; S.M.: conception and design, data interpretation, manuscript writing and revision, final approval; T. Sakaguchi: data interpretation; T.H.: data collection, data interpretation; T. Shimizu and T.O.: methodological assistance; S.Y.: conception and design, methodological help; Y.S.: conception and design, administrative and financial support, data interpretation, final approval.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Feasibility, Safety, and Therapeutic Efficacy of Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Sheets in a Porcine Ischemic Cardiomyopathy Model

Masashi Kawamura, Shigeru Miyagawa, Kenji Miki, Atsuhiro Saito, Satsuki Fukushima, Takahiro Higuchi, Takuji Kawamura, Toru Kuratani, Takashi Daimon, Tatsuya Shimizu, Teruo Okano and Yoshiki Sawa

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