Conclusions

This study clearly revealed that adipocyte-produced APN and HGF exert significant immunosuppressive effects, not only on Th1 cells, but also on Th17 cells in a typical model of autoimmune disorders. In addition, this tissue-engineered iACS improved the cardiac performance of autoimmune myocarditis via the suppression of autoimmune cellular activity, induction of immune-tolerance, and reversal of LV remodeling. This strategy of using a tissue-engineered drug-delivery system might be applicable to clinical treatments for fulminant myocarditis.

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Disclosures

There were no competing interests.

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Supplementary Files

Supplementary File 1

Supplementary Methods

Supplementary File 2

Table S1. Hemodynamic indices 5 weeks after the operation

Table S2. PCR primers used in real-time RT-PCR

Figure S1. T-cell proliferation assay.

Figure S2. Capillary formation on postoperative day 35 in each group.

Figure S3. Quantitative reverse transcription polymerase chain reaction (RT-PCR) results for profibrotic markers: TGFβ, TIMP1, TIMP2, TIMP3, MMP2, and MMP9, respectively (n=12 each).

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Safety and Efficacy of Autologous Skeletal Myoblast Sheets (TCD-51073) for the Treatment of Severe Chronic Heart Failure Due to Ischemic Heart Disease

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Background: Poor survival outcomes for patients with severe heart failure (HF) and the donor shortage for heart transplantation warrant the development of myocardial regenerative therapy. We performed a multicenter, phase II study to evaluate the safety and efficacy of autologous skeletal myoblast sheets (TCD-51073).

Methods and Results: In 3 study sites, we enrolled 7 patients with severe chronic HF due to ischemic heart disease despite maximal therapy, all of whom underwent transplantation of TCD-51073. No serious arrhythmia was reported, and no changes were noted in the frequency of ventricular extrasystole frequency. The primary efficacy endpoint of the change in left ventricular ejection fraction (LVEF) on gated blood-pool scintigraphy at 26 weeks after transplantation showed that 5 subjects were responders (classified as "improved" or "unchanged"). In addition, LVEF on echocardiography improved over time, with a change in LVEF of 7.1±2.8% at 26 weeks posttransplantation. Among the 7 subjects, 6 showed improvement in New York Heart Association functional class by at least 1 class. The 6-min walk distance was 410.1±136.1 m before transplantation and 455.4±103.7 m at 26 weeks after transplantation.

Conclusions: This study demonstrated the feasibility and safety of the transplantation of TCD-51073 in the patients with severe chronic HF due to ischemic heart disease, suggesting that TCD-51073 might maintain or improve cardiac function, symptoms, and physical function. (*Circ J* 2015; **79:** 991–999)

Key Words: Heart failure; Multicenter study; Myoblast sheets; Regenerative therapy

Over the past 20 years or so, the treatment of chronic heart failure (HF) has been progressed by use of drug therapies such as β-blockers, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), and aldosterone antagonists, or device therapy such as cardiac resynchronization therapy. Hence, clinical outcomes of patients with chronic HF are now significantly better, but patients with severe chronic HF who do not respond well to standard drug therapies still have poor outcomes.[1] In addition, although patients with end-stage severe HF benefit from heart transplantation or left ventricular assist system (LVAS) implantation,[2] these procedures are indicated for only a limited number of such patients worldwide where the number of heart transplant donors is limited. Thus, the therapeutic strategies available for patients with severe HF are still limited and new treatments need to be developed.

Since 15 years ago, treatment of heart disease with the use of patients' own somatic cells has been reported.[3] Among these treatments, using autologous skeletal myoblasts has been investigated in clinical trials, mainly in Western countries, in which cell transplantation was performed using myocardial injection during a surgical procedure through a thoracotomy, such as coronary artery bypass grafting (CABG) or LVAS implantation,[4] or using myocardial injection of cells through a cardiac catheter.[5] However, results from a European phase II clinical study (MAGIC trial) demonstrated that transplantation

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of autologous skeletal myoblasts was less effective than CABG as a control procedure.[6]

We developed an autologous skeletal myoblast sheet by using the cell sheet engineering approach developed by Okano et al of Tokyo Women's Medical University, Japan.[10] Following a number of nonclinical studies,[7–11] we conducted the first-in-human study at Osaka University in subjects with dilated cardiomyopathy who had received an LVAS, and we confirmed the feasibility and safety of the cell sheet, with successful weaning from the LVAS in 2 subjects.[12] We also conducted a phase I study in subjects with severe ischemic cardiomyopathy and dilated cardiomyopathy (UMIN ID; 000003273), and confirmed the safety and feasibility of the sheet in these cohorts. The results supported investigation of the practical use of autologous skeletal myoblast sheets on a commercial basis, leading to the development of TCD-51073 (Terumo Corporation, Tokyo, Japan).

We, therefore, conducted an exploratory, prospective, multicenter, uncontrolled, open-label phase II study of TCD-51073 in subjects with severe chronic HF due to ischemic heart disease, which we designed (1) to validate the method of efficacy evaluation, (2) to collect safety information, and (3) to confirm that transplantation or other procedures at multiple medical institutions was successfully conducted.

Methods

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki, Japanese Pharmaceutical Affairs Law, and Good Clinical Practice. The study was approved by the institutional review board at each study site, and all of the subjects provided prior informed consent to participate in the study.

Patients and Procedures

Patients This study included patients with ischemic heart disease who had impaired left ventricular systolic function and remained in HF status despite maximal oral therapy, including digitalis, diuretics, ACEIs, ARBs, β-blockers, aldosterone antagonists, and oral inotropic agents, and thus were at risk of worsening HF despite standard-of-care therapy. The inclusion criteria were: (1) patients who had chronic ischemic heart disease; (2) patients in New York Heart Association (NYHA) class III or IV HF; (3) patients who remained in HF status despite maximal oral therapy, including digitalis, diuretics, ACEIs, ARBs, β-blockers, aldosterone antagonists, and oral inotropic agents; (4) patients aged 20 years or older at the time of consent; (5) patients who were at risk of worsening HF despite standard-of-care therapy (eg, CABG, mitral valvuloplasty, LV restoration, cardiac resynchronization therapy, and percutaneous coronary intervention) conducted at least 3 months earlier; and (6) patients who had a LV ejection fraction (LVEF) ≤35% on resting echocardiography. The exclusion criteria were: (1) patients with evidence of skeletal muscle disease; (2) patients undergoing thyroid hormone treatment; (3) patients with infectious diseases (eg, infections caused by the human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and human T cell leukemia virus type 1); (4) patients who remained in shock because of worsening HF; (5) patients with irreversible non-cardiac organ failure; (6) patients with any malignancy; (7) patients who were pregnant or possibly pregnant; (8) patients with a history of alcoholism or drug addiction within 6 months before the day of consent; and (9) patients with severe pulmonary hypertension.

Procedure At each study site, skeletal muscle (required amount, 2–5 g) was harvested from the vastus medialis muscle of the subject by aseptic technique, under general anesthesia and with endotracheal intubation. In the Cell Processing Center of Terumo Corporation, skeletal myoblasts were isolated from the harvested skeletal muscle by enzymatic digestion in TrypLE Select™ (Thermo Fisher Scientific Inc, MA, USA) containing collagenase, expanded in culture in MCDB103 medium with fetal bovine serum for approximately 4 weeks, and cryopreserved. At least 7 weeks after the harvest of the skeletal muscle, the skeletal myoblasts were seeded at 6.0×10^7 cells per dish and incubated on temperature-responsive culture dishes (10 cm in diameter) to form a skeletal myoblast sheet (TCD-51073), as described previously.[7] Transplantation of TCD-51073 was performed through a left thoracotomy under general anesthesia, and 5 sheets of TCD-51073 (representing 3×10^8 cells) were transplanted onto a large area extending from the anterior wall to the lateral wall of the left ventricle. No other concomitant cardiac surgery, such as CABG or mitral valve repair, were performed.

Evaluation Methods

Study Protocol Evaluation for the study was performed up to 26 weeks after transplantation. A 2-year follow-up study is ongoing, which was begun after the evaluation period. Observations included physical examination (subjective and objective), NYHA classification, specific activity scale (SAS) score, and concomitant medications. Examinations included vital signs, body weight, laboratory tests, chest radiography, cardiac computed tomography (CT), echocardiography, resting standard 12-lead ECG, 24-h Holter ECG monitoring, 6-min walk test, cardiopulmonary exercise test (CPX), and gated equilibrium blood-pool scintigraphy. Myocardial perfusion single-photon emission CT and coronary angiography were performed for eligibility screening.

Primary Endpoint The primary endpoint was the change in LVEF on gated equilibrium blood-pool scintigraphy from pretransplantation to 26 weeks posttransplantation. This endpoint was chosen based on the advice provided by the Pharmaceuticals and Medical Devices Agency in Japan.

Secondary Endpoints The prespecified secondary endpoints included success or failure of the transplantation, LVEF, LV end-diastolic volume index, LV end-systolic volume index, NYHA classification, SAS, 6-min-walk distance (6MWD), peak oxygen uptake (peak $\dot{V}O_2$), anaerobic threshold (AT), B-type natriuretic peptide (BNP), and volumetric analysis of echocardiography performed on the long axes of apical 2- and 4-chamber views using the modified Simpson method.

Safety Endpoints Safety endpoints included arrhythmia, adverse events (AEs) and adverse drug reactions (ADRs) other than arrhythmia, serious arrhythmia (requiring inpatient hospitalization or prolonging the existing hospitalization for treatment or monitoring) and serious AEs (SAEs), AEs associated with harvest of skeletal muscle tissues, changes in vital signs over time, and changes in routine hematology and clinical chemistry parameters over time.

Study Administrative Structure The Case Adjudication Committee, which consisted of 2 independent cardiologists and 1 independent cardiac surgeon, was established to provide advice on the determination of patient eligibility and whether the study should be continued, as well as to evaluate efficacy. In addition, the Data and Safety Monitoring Committee, which consisted of 1 cardiac surgeon and 1 medical statistician, was established to review safety data from this study at appropriate intervals or at necessary times to provide recommendations

for continuation, modification, or termination of the study to the sponsor, as necessary. The patients were registered at the patient registration center (EPS Corporation, Tokyo, Japan), and cardiac CT scans were analyzed at the core laboratory (TITAN Inc, Tokyo, Japan).

Statistical Analysis

Primary Endpoint The patients were classified according to the change in LVEF from pretransplantation to 26 weeks posttransplantation as "improved" (ΔLVEF $\geq$5%), "unchanged" (5%>ΔLVEF>–3%), or "worsened" (–3% $\geq\Delta$LVEF). A responder was defined as a subject whose LVEF was classified as improved or unchanged, and the number of responders was determined.

Secondary Endpoints For continuous variables, summary statistical indexes (mean, standard deviation [SD and 2-sided 95% confidence interval [CI] for the mean, maximum, median, minimum, and number of subjects) were calculated. For discrete variables, frequency distribution tables were constructed.

Safety Endpoints For arrhythmia, and AEs and ADRs other than arrhythmia, the numbers of events and the subjects with events were determined. If an event was assessed as an ADR, the numbers of such events and the subjects with such events were determined. For significant AEs and SAEs, as well as AEs associated with harvest of skeletal muscle tissues, the numbers of events and the subjects with events were determined. For vital signs, routine hematology, and clinical chemistry parameters, summary statistical indexes (mean, SD, and 2-sided 95% CI for the mean, maximum, median, minimum, and number of subjects) were calculated and the time course of the changes was determined.

Results

Classification and Disposition of Subjects

From 3 Japanese study sites, we enrolled 7 patients with severe chronic HF due to ischemic heart disease who continued to be in NYHA class III or higher and have an LVEF $\leq$35% despite maximal therapy between May 2012 and October 2013 (Osaka University Hospital, 4 patients; Tokyo Women's Medical University Hospital, 2 patients; University of Tokyo Hospital, 1 patient). Of the 8 initial subjects who provided informed consent and reviewed by the Case Adjudication Committee, 1 was considered ineligible. All 7 eligible subjects received transplantation of TCD-51073 and completed the study without discontinuations.

Baseline Characteristics of the Patients

The baseline characteristics of the enrolled subjects are shown in **Table 1**. The mean age at eligibility screening was 56.3± 13.2 years (range, 35–71 years), and all of them were male. All the subjects had undergone percutaneous coronary intervention or CABG for ischemic heart disease but presented with symptoms of severe HF, were in NYHA class III, and had a LVEF of 29±3.7% (<30% in 3 subjects) on echocardiography. All the subjects were identified as having no indication for further revascularization. In addition, 2 subjects had an implantable cardioverter defibrillator or cardiac resynchronization therapy defibrillator, and 1 subject had undergone mitral valvuloplasty. Mitral regurgitation was reported in 6 subjects, as assessed pretransplantation by echocardiography. One subject who had a history of NYHA class IV symptoms was listed as a status 2 cardiac transplantation candidate by the Japan Organ Transplant Network.

Table 1. Characteristics of Study Patients Undergoing Cell Sheet Transplantation

Demographics	
No. of patients	7
Age, years (mean±SD)	56.3±13.2
>65 years	2 (28.6%)
Male sex	7 (100%)
Body weight (kg) [mean±SD]	70.3±9.5
Height (cm) [mean±SD]	168.9±6.0
Body surface area (m^2) [mean±SD]	1.8±0.1
Cardiac function	
NYHA functional class III	7 (100%)
LVEF (echocardiography) [mean±SD]	29.3±3.7
Risk factor	
Hypertension	3 (42.9%)
Hyperlipidemia	6 (85.7%)
Diabetes mellitus	3 (42.9%)
Oral medication	3 (42.9%)
Insulin	1 (14.3%)
Smoking	7 (100%)
Current or previous (within the past year)	0 (0.0%)
Cardiac history	
PCI or CABG	7 (100%)
PCI	6 (85.7%)
CABG	6 (85.7%)
Pacemaker implant	0 (0.0%)
ICD implant	1 (14.3%)
CRT implant	0 (0.0%)
CRT-D implant	1 (14.3%)
Valve surgery	1 (14.3%)
Left ventricular reconstruction	0 (0.0%)
IABP	1 (14.3%)
LVAD	0 (0.0%)
Myocardial infarction	6 (85.7%)
Nonsustained VT	2 (28.6%)
Atrial fibrillation or flutter	3 (42.9%)
Mitral regurgitation	6 (85.7%)
Medication	
ACEI or ARB	6 (85.7%)
ACEI	5 (71.4%)
ARB	1 (14.3%)
β-blocker	7 (100%)
Aldosterone receptor antagonist	5 (71.4%)
Diuretics	6 (85.7%)
Inotropic	2 (28.6%)
Antiplatelet	6 (85.7%)
Warfarin	5 (71.4%)
Amiodarone	4 (57.1%)
Hypoglycemic	3 (42.9%)
Statins	6 (85.7%)

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass grafting; CRT, cardiac resynchronization therapy; CRT-D, cardiac resynchronization-defibrillator therapy; IABP, intra-aortic balloon pump; ICD, implantable cardioverter defibrillator; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; SD, standard deviation; VF, ventricular fibrillation; VT, ventricular tachycardia.

Table 2. Summary of Adverse Events Among Patients Undergoing Cell Sheet Transplantation

Adverse event (MedDRA SOC)	Not related*						Cannot be ruled out	Total
	Underlying disease	Surgery	Concomitant medication	Patient's condition	Concurrent conditions	Other		
Congenital blood and lymphatic system disorders				1	1			2
Cardiac disorders	4	3					1	8
Gastrointestinal disorders		1	1			2		3
General disorders and administration site conditions	1	1		1				3
Hepatobiliary disorders		1		1		1		3
Infections and infestations		5		1				6
Injury, poisoning, and procedural complications		9				1		10
Investigations	1	3	3			4		11
Metabolism and nutrition disorders		2	4					6
Musculoskeletal and connective tissue disorders			1			2		3
Neoplasms benign, malignant, and unspecified (including cysts and polyps)					1			1
Nervous system disorders			1					1
Psychiatric disorders		1						1
Renal and urinary disorders		4				1		5
Reproductive system and breast disorders			1					1
Respiratory, thoracic, and mediastinal disorders		4						4
Skin and subcutaneous tissue disorders		4						4
Vascular disorders		2						2

*If there are 2 or more reasons, each is counted.

Table 3. Summary of Arrhythmia Among Patients Undergoing Cell Sheet Transplantation

Patient ID	Adverse event (MedDRA LLT)	Post Tx (days)	Relationship to study drug	Reason for "not related"
T01–01	Nonsustained VT	6	Not related	Underlying disease
T03–01	Atrial fibrillation	2	Not related	Underlying disease
T01–04	Nonsustained VT	4	Not related	Underlying disease, Possibility of concomitant medications
T02–02	Atrial flutter	4	Not related	Surgery
	Atrial fibrillation	5	Not related	Surgery
T01–05	Ventricular extrasystoles	1	Not related	Underlying disease, Possibility of concomitant medications, Surgery

Abbreviation as in Table 1.

Surgical Procedure

Harvest of the skeletal muscle and cell culture were successfully performed in all the enrolled subjects, who were transplanted with 5 sheets of TCD-51073. The proportion of skeletal myoblasts (CD56-positive cells) in the transplanted TCD-51073 was ≥60%, with a cell viability ≥75%, indicating evidence of fusion ability in all subjects. This product also complied with sterility, bacterial endotoxins, and mycoplasma tests.

Safety Evaluation

The AEs reported in this study are listed in **Table 2**. AEs associated with the harvest of the skeletal muscle were observed in 2 of the 7 subjects and included 2 events of wound complication in 2 subjects and 1 event of postprocedural swelling in 1 subject. These AEs all resolved without sequelae within 1–14 days. After TCD-51073 transplantation, 74 AEs were observed in the total group. Among these AEs, the most commonly observed were wound complications in 4 subjects, hypokalemia in 3, and postoperative fever in 3. No subject except those with SAEs, as described next, required specific treatment.

Serious arrhythmia, which required inpatient hospitalization or prolonged existing hospitalization for treatment or monitoring, was defined as a significant AE in this study, but no cases occurred. In contrast, 6 arrhythmia events occurred in 5 subjects, including 2 events of ventricular arrhythmia in 2 subjects, 1 event of ventricular extrasystole in 1 subject, 2 events of atrial fibrillation in 2 subjects, and 1 event of atrial flutter in 1 subject (**Table 3**). Among the 2 subjects with

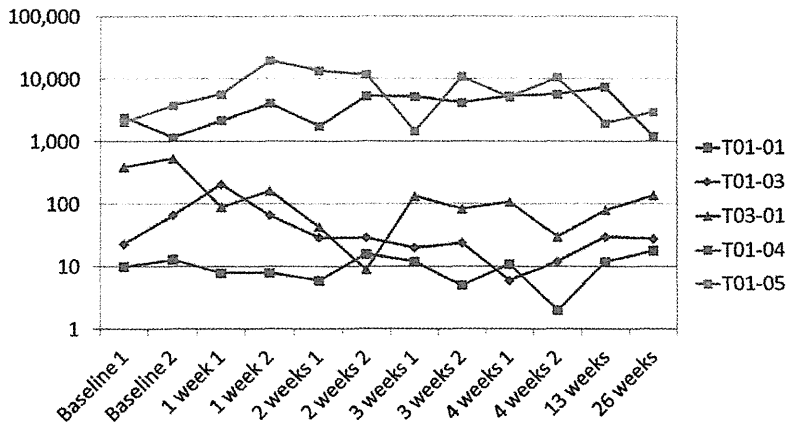


Figure 1. Serial change in the number of premature ventricular contractions on 24-h Holter ECG monitoring in 5 subjects with data available from their respective case report forms performed before cell sheet transplantation; twice weekly at 1, 2, 3, and 4 weeks after transplantation, and once weekly at 13 and 26 weeks after transplantation.

nonsustained ventricular arrhythmia, in subject T01-01 with existing nonsustained ventricular tachycardia, the nonsustained ventricular arrhythmia resolved with drug therapy on the day of onset. In subject T01-04, with existing multiple nonsustained ventricular tachycardias, the nonsustained ventricular arrhythmia did not resolve, but was mild and required no additional drug therapy or treatment. These arrhythmic events were all reported within 1 week after transplantation and were considered attributable to the transplantation procedure or underlying disease.

In addition, the subjects were hospitalized for 4 weeks after transplantation to further investigate whether arrhythmogenic effects were present and 24-h Holter ECG monitoring was performed before transplantation; twice weekly at 1, 2, 3, and 4 weeks after transplantation; and once weekly at 13 and 26 weeks after transplantation. The ECG changes over time in each subject who received TCD-51073 transplantation showed no specific trend, with no significant change in the frequency of ventricular extrasystole (**Figure 1**).

In this study, 3 SAEs occurred in 3 subjects, comprising colon cancer (subject T02-01, 182 days posttransplantation), prolonged HF (subject T01-04, 31 days posttransplantation), and aggravated HF (subject T03-01, 49 days posttransplantation). The colon cancer was diagnosed as a primary cancer based on pathological findings and was considered unrelated to TCD-51073. This subject underwent right hemicolectomy at 273 days posttransplantation and subsequently recovered. The prolonged HF was attributed to surgery, inappropriate drug administration, and the subject's lifestyle, and was considered unrelated to the transplanted TCD-51073. This subject was discharged from hospital at 141 days posttransplantation, but was re-hospitalized at 159 days posttransplantation. The patient was subsequently discharged from hospital at 172 days posttransplantation, and recovered. The relationship between the event of aggravated HF and the transplanted TCD-51073 was considered unknown. This subject was a Status 2 patient registered for heart transplantation and repeatedly hospitalized and discharged 4 times until 26 weeks posttransplantation, with drugs added and/or modified because of the aggravated HF during 3 of the hospitalizations. For all episodes of hospitalization, the subject recovered with intravenous infusion of inotropic agents and diuretics or modification of diuretics for fluid control and was discharged from hospital.

In the vital signs, physical examination results, and clinical laboratory test results, no serious abnormal changes occurred

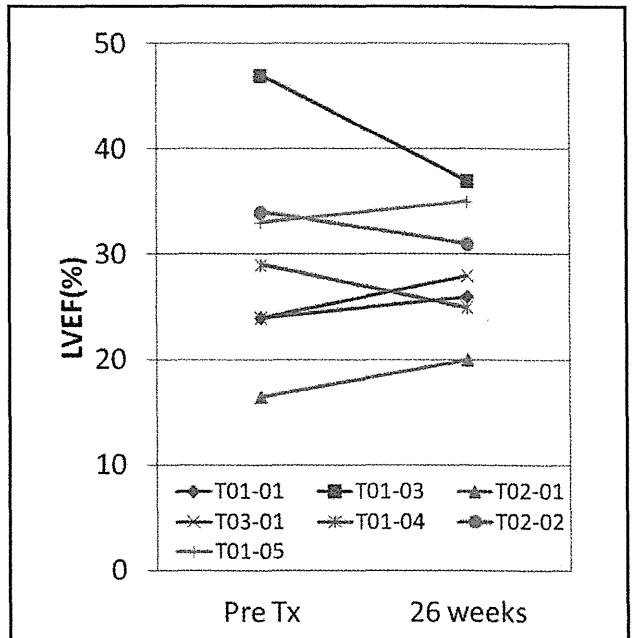


Figure 2. Change in left ventricular ejection fraction (LVEF) from pretransplantation to 26 weeks post cell sheet transplantation using gated equilibrium blood-pool scintigraphy.

in any of the subjects, although effects of the thoracotomy, and concomitant medications or changes associated with observed AEs were noted. For body weight, no serious abnormal changes occurred. No clinically significant abnormal changes or abnormal changes qualifying as SAEs were noted.

Primary Efficacy Endpoint Figure 2 shows the change in LVEF over time according to gated equilibrium blood-pool scintigraphy. The LVEF was found to be "unchanged" in 5 of the 7 subjects. A responder was defined as a subject in whom the LVEF was classified as "improved" or "unchanged" in the present study. Accordingly, 5 subjects were considered to be responders. Among the 2 subject with worsened LVEF, 1 subject (subject T01-04) received continuous administration of drugs that affected LVEF values, including intravenous inotropic agents (eg, dobutamine) and diuretics, for the management of AEs that occurred after transplantation (prolonged

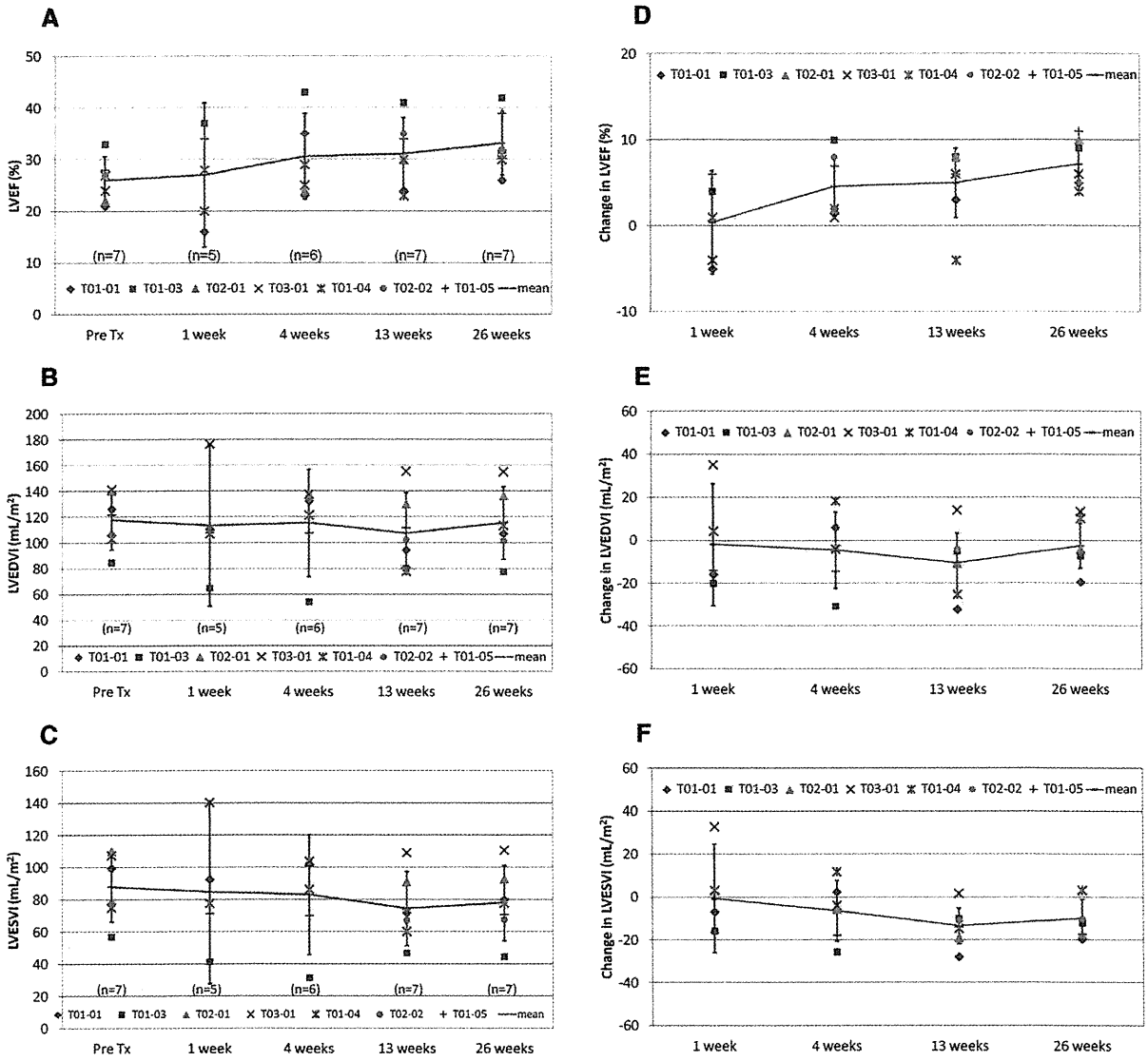


Figure 3. Serial change in echocardiographic parameters from baseline to 26 weeks after cell sheet transplantation: (**A**) left ventricular ejection fraction (LVEF), (**B**) left ventricular end-diastolic volume index (LVEDVI) and (**C**) left ventricular end-systolic volume index (LVESVI). Change from pretransplantation to 26 weeks posttransplantation in (**D**) LVEF, (**E**) LVEDVI and (**F**) LVESVI. Error bar are expressed as mean ±95% confidence interval.

HF and renal impairment). The other subject (subject T01-03) became unwell at the time of LVEF measurement before transplantation, and decreased blood pressure associated with administration of a tracer was likely to have affected the LVEF values. These 2 patients showed an increase in LVEF on echocardiography.

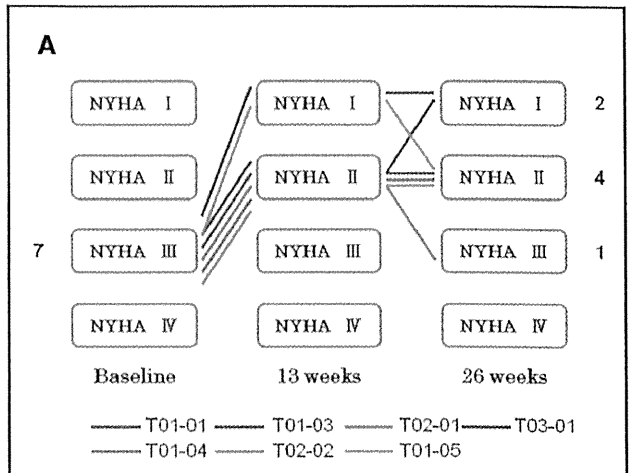
Secondary Efficacy Endpoints Results of the echocardiography performed before and at 1, 4, 13, and 26 weeks after transplantation showed LVEF values of 26.0±4.1%, 27.0±8.9%, 30.6±7.4%, 31.0±6.3%, and 33.1±5.5%, respectively (**Figure 3A**). This is consistent with a trend toward improvement over time, with the changes in LVEF values from pretransplantation to 1, 4, 13, and 26 weeks posttransplantation being 0.4±4.8%, 4.6±3.6%, 5.0±4.4%, and 7.1±2.8%, respectively (**Figure 3D**). In addition, an increase in LVEF values from pretransplantation was observed in all subjects, with an increase >5% from pretransplantation values in 4 subjects. The change in LVEF from pretransplantation to 26 weeks posttransplantation was 2.0±2.6% in 6 subjects in whom cardiac CT was feasible. No LVEF values could be obtained in 1 subject (subject T01-04) with worsened LVEF by gated equilibrium blood-pool scintigraphy at 26 weeks posttransplantation. In the other subject (subject T01-03), LVEF was unchanged.

Changes in NYHA class over time are shown in **Figure 4A**. Before transplantation, all subjects were in NYHA class III, but 2, 4, and 1 of the subjects were in NYHA class I, II, and III, respectively, at 26 weeks posttransplantation, indicating improvement in NYHA class by at least 1 class in 6 of the 7 subjects who received a transplant; 2 of the 6 subjects experienced a substantial improvement from class III to class I.

SAS results showed an improvement of at least 1 metabolic equivalent (MET) in 3 subjects and no change in 4 subjects, from pretransplantation (**Figure 4B**). Subject T01-01 had an increase of at least 2 METs, from 3–4 METs pretransplantation to 6–7 METs at 26 weeks posttransplantation. Subject T01–03 had an increase of at least 2 METs, from 4–5 METs pretransplantation to 6–7 METs at 26 weeks posttransplantation.

The results of the 6MWD and CPX are shown in **Table 4**. The 6MWD was 410.1±136.1 m pretransplantation and 455.4±103.7 m at 26 weeks posttransplantation. Subjects T01-03 and T03-01 experienced increases of 170 and 214 m, respectively, whereas subject T02-02 had a decrease of 110 m from pretransplantation. Peak $\dot{V}O_2$ was 13.4±5.3 ml·kg^{-1}·min^{-1} pretransplantation and 14.6±4.9 ml·kg^{-1}·min^{-1} at 26 weeks posttransplantation. AT was 8.7±2.3 ml·kg^{-1}·min^{-1} pretransplantation and 9.3±2.2 ml·kg^{-1}·min^{-1} at 26 weeks posttransplantation. In the 5 subjects in which a comparison before and after transplantation could be made, peak $\dot{V}O_2$ and AT showed a trend toward worsening in 2 subjects and no change or a trend toward improvement in 3 subjects from pretransplantation.

Subject T02-01 was diagnosed with colon cancer at the time of examination at 26 weeks posttransplantation and had physical examination findings such as shortness of breath on exertion and staggering, which may have affected the results of NYHA classification, SAS, 6MWD, and CPX.



B

Patient ID	Baseline	13 weeks	26 weeks
T01-01	3-4 METs	3-4 METs	6-7 METs
T01-03	4-5 METs	6-7 METs	6-7 METs
T02-01	4 METs	5 METs	4 METs
T03-01	4 METs	4 METs	4 METs
T01-04	4-5 METs	4-5 METs	4-5 METs
T02-02	4 METs	6 METs	5 METs
T01-05	4-5 METs	4-5 METs	4-5 METs

Figure 4. Serial change in New York Heart Association (NYHA) class and specific activity scale (SAS) from baseline to 26 weeks after cell sheet transplantation.

Discussion

Overview of Study Results

This study was an exploratory study designed to evaluate the efficacy and safety of TCD-51073 in subjects presenting with severe chronic HF due to ischemic heart disease who were at risk of worsening HF despite maximal therapy. Despite the small sample size, the results demonstrated the following: (1) harvesting of the skeletal muscle and then cell culture were successfully performed in all enrolled subjects; (2) transplantation of TCD-51073 was safe and feasible in multiple medical institutions; (3) no evidence of serious arrhythmia and no other possible SAEs were observed; (4) LVEF was maintained in 5 subjects on gated equilibrium blood-pool scintigraphy and showed improvement over time on echocardiography; (5) 6 subjects showed improvement in NYHA class and almost all subjects showed symptomatic improvement. Some subjects showed improvement in exercise tolerance.

Efficacy Evaluation

This study demonstrated that 5 of the 7 subjects were responders in the primary endpoint. Of the 2 subjects with worsened LVEF, 1 subject became unwell at the time of LVEF measurement before transplantation, and the decreased blood pressure associated with administration of a tracer was likely to have affected the LVEF values. The other subject had a variable degree of mitral regurgitation and thus was not suitable for the assessment of changes in systolic function based on LVEF. In addition, the secondary endpoint of LVEF showed improvements in cardiac function over time on echocardiography, suggesting that this product might at least be effective in maintaining cardiac function in patients with severe HF who are at risk of worsening. Overall improvement in the secondary

Table 4. Change in Exercise Tolerance From Baseline to 26 Weeks Transplantation Among Patients Undergoing Cell Sheet Transplantation

Patient ID	6-min walk distance (m)		Peak $\dot{V}O_2$ (ml·kg^{-1}·min^{-1})		AT (ml·kg^{-1}·min^{-1})	
	Baseline	26 weeks	Baseline	26 weeks	Baseline	26 weeks
T01–01	485	520	19.4	16.5	10.5	8.6
T01–03	400	570	20.4	21.1	11.3	12
T02–01	486	462*	12.6	7.6*	10.1	7
T03–01	264	478	9.1	13.3	6.7	7.7
T01–04	285	291	7.8	–*	5.4	–*
T02–02	640	530	11	14.5	8.3	11
T01–05	311	337	–*	–*	–	–*

*No examination was performed.

endpoint (NYHA classification) was noted, and a clear improvement in exercise tolerance was observed in some subjects, as measured by 6MWD or Peak $\dot{V}O_2$. As the magnitude of clinically relevant change in these measures has, in some cases, been reported to be ±5% for LVEF, ±1 class for NYHA classification, ±50 m for 6MWD, and ±1.5 ml·kg^{-1}·min^{-1} for Peak $\dot{V}O_2$,[13] not a few subjects experienced a clinically relevant improvement in this study.

Validity of Efficacy Endpoints LVEF is the most common measure used in clinical practice and has been commonly used as an outcome measure in clinical studies. However, patients with severe HF are more likely to have comorbid mitral insufficiency or renal impairment and changes in preload and afterload are caused by various triggers despite administration of diuretics. Assessment of HF status by LVEF alone, a measure that is affected by preload and afterload, is therefore difficult. In addition, the possibility of measurement bias by an assessor cannot be ruled out.

In consideration of the current situation that patients with symptoms of HF in the absence of decreased LVEF, such as patients with HF with preserved EF, account for half of all patients with HF,[14] assessment of cardiac function in patients with HF should include changes in diastolic function. Data from a clinical study conducted by Osaka University, Japan, showed that a review of symptoms and exercise tolerance in patients without LV reverse remodeling found an improvement in pulmonary arterial hypertension and a decrease in pulmonary vascular resistance, as determined by cardiac catheterization. However, data on measures to assess cardiac function needs to be accumulated. Conventional endpoints for regenerative medicine products include improvement in LV contractility as measured by various modalities, which now serves as an efficacy endpoint.[3] In recent years, the US Food and Drug Administration has stated that LV contractility cannot be used as an efficacy endpoint in confirmatory studies, which should use endpoints such as mortality and cardiovascular or HF hospitalizations.[15] In addition, the Study Group of the Japan's Ministry of Health, Labour and Welfare reported that improvement in survival outcomes and quality of life should be used as efficacy outcome measures.[16] With regard to study design, whether a double- or single-arm design should be used for evaluation is controversial. However, based on the abovementioned suggestions and the results of this clinical study, the efficacy of regenerative medicine products likely cannot be adequately evaluated using LV contractility alone as an efficacy outcome measure. Therefore, efficacy assessment from diversified perspectives is considered necessary.[17]

As described earlier, improvement in survival outcomes or prevention of HF hospitalizations is important in terms of maintenance or improvement of HF status. The previous clinical study conducted by Osaka University reported a survival rate 91.7% at 3 years after transplantation in 17 patients who received transplantation with the skeletal myoblast sheet. The event-free rate at 3 years after transplantation based on Kaplan-Meier curves using the endpoints of death, LVAS implantation for worsening cardiac function, and dependence on catecholamine agents was 85.7%, suggesting a higher event-free rate than that in patients who are registered for heart transplantation at Osaka University and waiting for transplantation as Status 2 patients (54.3%), and these data suggest the efficacy of the skeletal myoblast sheet. Although the evaluation period of 26 weeks in this study still makes any discussion difficult, we await the results from an ongoing follow-up study of up to 2 years after transplantation.

Safety

In this study, no deaths occurred during the 26-week evaluation period and none of the subjects withdrew from the study because of ADRs after transplantation of TCD-51073. In contrast, SAEs included prolonged HF in all the subjects at 31 days after transplantation and, additionally, aggravated HF in 1 subject at 49 days after transplantation. The events of prolonged and aggravated HFs were attributed to the transplantation procedure and underlying disease, respectively. Both were reported during hospitalization after transplantation, suggesting that surgical indication in patients with severe HF should be carefully considered, including postoperative management.

Arrhythmia

Since cases of serious arrhythmia were reported in a clinical study of suspension of skeletal myoblasts injected into the myocardium,[18] the potential arrhythmogenic effects of skeletal myoblast transplantation have been discussed. In contrast, animal experiments have demonstrated that skeletal myoblast sheets are free of such side effects.[19] In the present study, no events of serious arrhythmia were observed and no significant change in the incidence of ventricular extrasystole was found on Holter ECG monitoring. Therefore, the arrhythmogenic risk from using TCD-51073 was considered low.

Study Design

As transplantation of TCD-51073 involves invasive surgery, inclusion of a concurrent control group in clinical studies that include subjects with severe disease, such as the present study, is difficult for ethical reasons. Patients with severe symptoms in the present study had already received maximum therapies and had no further treatments for HF. Such patients expect the possible efficacy of this treatment and so all want to receive this treatment and none want to enter the control group. Although a placebo effect is expected to occur when evaluating efficacy before and after therapy in study patients in single-arm efficacy studies, the future development of new efficacy outcome measures that are unaffected by the placebo effect may promote the establishment of an efficacy assessment system that is ethically appropriate and provides highly reliable data.

Significance of TCD-51073 in the Treatment of HF

Although findings from previous clinical research studies and clinical trials suggest that TCD-51073 is unlikely to achieve a marked improvement in cardiac contractility in patients with severe HF who are receiving maximal standard-of-care therapy, this product may be a potential alternative therapy for HF, other than the currently available standardized therapies, such as drug therapy, LVAS implantation, and heart transplantation, if improvement of clinical symptoms, improved exercise tolerance, prolonged survival, and consequent improved cardiac diastolic function are demonstrated in future studies. A current concern in the treatment of HF is the absolute shortage of donors, and we believe that using this product as a bridge to LVAS or heart transplantation to suppress the progress of HF and delay the timing of LVAS implantation while applying various measures to increase donors will contribute to the resolution of the donor shortage. In addition, a reduction in the number of HF hospitalizations and the length of hospital stay because of HF with the use of this product will be beneficial in reducing the escalation of medical costs.

Study Limitations

This study has the following limitations: (1) a small sample size, which is inadequate for statistical analysis; (2) transplantation of TCD-51073 occurred in the absence of other concomitant therapies, so an add-on effect is unlikely to occur in the before-and-after comparisons; however, comparison with a group of untreated patients is necessary; (3) single-arm, open-label study, so the possibility of assessment bias cannot be ruled out; an independent analysis should be considered; and (4) the evaluation period of 26 weeks after transplantation is limited for demonstrating the efficacy of TCD-51073 in improving survival outcomes and preventing hospitalizations because of HF, thus requiring further mid- and long-term follow-up data.

Conclusions

This study demonstrated the feasibility and safety of the transplantation of TCD-51073 in patients with severe chronic HF due to ischemic heart disease, suggesting that TCD-51073 might maintain or improve cardiac function, symptoms, and physical function.

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Disclosures

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Supplementary Files

Supplementary File 1

Table S1. Inclusion and exclusion criteria for study of cell sheet transplantation

Table S2. Observation and test items and schedule of study of cell sheet transplantation

Figure S1. Serial change in B-type natriuretic peptide from baseline to 26 weeks after cell sheet transplantation.

Please find supplementary file(s);
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Addition of Mesenchymal Stem Cells Enhances the Therapeutic Effects of Skeletal Myoblast Cell-Sheet Transplantation in a Rat Ischemic Cardiomyopathy Model

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Introduction: Functional skeletal myoblasts (SMBs) are transplanted into the heart effectively and safely as cell sheets, which induce functional recovery in myocardial infarction (MI) patients without lethal arrhythmia. However, their therapeutic effect is limited by ischemia. Mesenchymal stem cells (MSCs) have prosurvival/proliferation and antiapoptotic effects on co-cultured cells *in vitro*. We hypothesized that adding MSCs to the SMB cell sheets might enhance SMB survival post-transplantation and improve their therapeutic effects.
Methods and Results: Cell sheets of primary SMBs of male Lewis rats (r-SMBs), primary MSCs of human female fat tissues (h-MSCs), and their co-cultures were generated using temperature-responsive dishes. The levels of candidate paracrine factors, rat hepatocyte growth factor and vascular endothelial growth factor, *in vitro* were significantly greater in the h-MSC/r-SMB co-cultures than in those containing r-SMBs only, by real-time PCR and enzyme-linked immunosorbent assay (ELISA). MI was generated by left-coronary artery occlusion in female athymic nude rats. Two weeks later, co-cultured r-SMB or h-MSC cell sheets were implanted or no treatment was performed ($n=10$ each). Eight weeks later, systolic and diastolic function parameters were improved in all three treatment groups compared to no treatment, with the greatest improvement in the co-cultured cell sheet transplantation group. Consistent results were found for capillary density, collagen accumulation, myocyte hypertrophy, Akt-signaling, STAT3 signaling, and survival of transplanted cells of rat origin, and were related to poly (ADP-ribose) polymerase-dependent signal transduction.
Conclusions: Adding MSCs to SMB cell sheets enhanced the sheets' angiogenesis-related paracrine mechanics and, consequently, functional recovery in a rat MI model, suggesting a possible strategy for clinical applications.

Introduction

A RECENT LARGE-SCALE clinical trial, in which autologous skeletal myoblasts (SMBs) were directly injected into the heart by needle, reported only modest therapeutic benefits and a substantial risk of ventricular arrhythmias, due at least partly to the delivery method.[1,2] The major drawbacks of SMB delivery by needle injection are poor cell survival in the heart, leading to insufficient paracrine effects, and mechanical myocardial injury, potentially causing lethal arrhythmia.[1–3] In contrast, cell-sheet techniques, which we developed, deliver SMBs more effectively with minimal myocardial injury, enhanced paracrine effects, and consequently better cardiac function than attained by needle injection.[4–8]

The mechanism by which damaged myocardium is restored by transplanted SMB cell sheets is complex, involving many pathways.[4–8] Recent reports show beneficial effects of SMB cell-sheet transplantation in several animal experimental models and patients with heart failure, which are primarily attributed to cytokine secretion from the transplanted cell sheets (i.e., a paracrine effect).[4–9]

However, SMB cell sheets attached to the surface of the infarcted myocardium are poorly supported by the vascular

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network of the native myocardium, which limits the survival of the SMBs and, consequently, their therapeutic effects.[7] Thus, conventional SMB cell-sheet transplantation might be insufficient to repair severely damaged myocardium, which has poor viability. Mesenchymal stem cells (MSCs) are used as feeder cells to support the survival, proliferation, and differentiation of co-cultured stem/progenitor cells *in vitro*.[10–12] Moreover, MSCs are advantageous for cellular therapy because they are multipotent, potentially immune privileged, and expand easily *ex vivo*. MSCs also proliferate rapidly and induce angiogenesis.[13,14]

We hypothesized that adding MSCs to the SMB cell sheets *in vitro* might enhance their survival and function after transplantation, which might enhance the benefits of SMB cell-sheet transplantation therapy. Here, we investigated whether co-culturing SMBs with MSCs would enhance the SMBs' cytokine production *in vitro*. We also examined the therapeutic effects on chronic ischemic heart failure of transplanting cell sheets created from co-cultured SMBs and MSCs, compared with SMB-only and MSC-only cell sheets.

Materials and Methods

This study was approved by the Institutional Ethics Committee of the Osaka University. Humane animal care was used 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 Animal Resources and published by the National Institutes of Health (Publication No. 85–23, revised 1996). All procedures and evaluations, including assessments of cardiac parameters, were carried out in a blinded manner. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the article as written.

Isolation of SMBs and adipose tissue-derived mesenchymal cells, and cell-sheet preparation

Primary skeletal myoblasts of rat origin (r-SMBs) were isolated from Lewis rats (3 weeks old, male; CLEA Japan, Inc.) and expanded *in vitro* as described previously[7,8]: more than 70% of the isolated cells were actin positive and 60–70% were desmin positive, as determined by flow cytometry (data not shown). To detect r-SMBs, we used GFP transgenic Lewis rats.[15] Primary human MSCs (h-MSCs) were isolated from female subcutaneous adipose tissue samples as described.[12] h-MSCs exhibit mesenchymal morphology (Fig. 1A). Cell sheets consisting of r-SMBs or h-MSCs were prepared using temperature-responsive culture dishes (UpCell®; CellSeed), as described.[12] Cell sheets containing both r-SMBs and h-MSCs were prepared by co-culturing these cells in temperature-responsive culture dishes.

Rat myocardial infarction model and cell-sheet implantation

A proximal site of the left anterior descending coronary artery (LCA) of athymic nude rats (F344/NJcl-rnu/rnu, 8-week-old, female, 120–130 g; CLEA Japan) was permanently occluded using a thoracotomy approach. The animals were then kept in temperature-controlled individual cages for 2 weeks to generate a subacute ischemic heart failure model.[7,8,12] The rats were then divided into 4 experimental groups ($n=10$ in each) as follows: (1) transplantation of triple-layer h-MSC cell sheets (7.5×10^5 cells per sheet), (2) transplantation of triple-layer r-SMB cell sheets (3.0×10^6 cells per sheet), (3) transplantation of triple-layer co-cultured r-SMB (3.0×10^6 cells per sheet) and h-MSC (7.5×10^5 cells per sheet) sheets, and (4) no treatment (control) (Fig. 1B). Thereafter, the rats were kept in individual cages for 4 weeks.

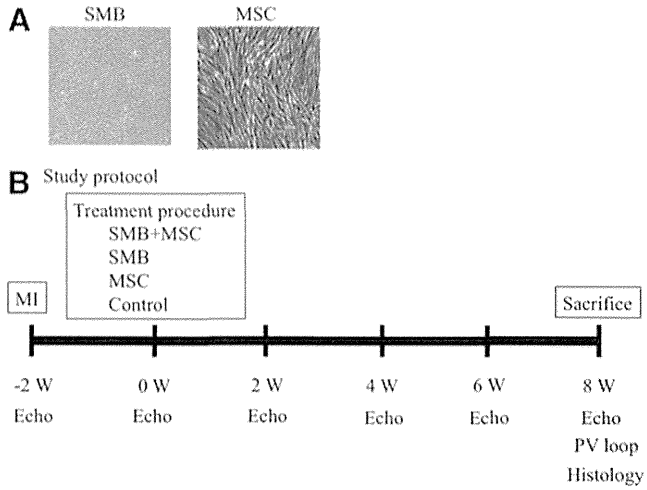

FIG. 1. **(A)** Morphology of SMB and MSC. **(B)** Study protocol used for the assessment of cardiac function and histology. Athymic nude rats (F344/NJcl-rnu/rnu) underwent induction of myocardial infarction by occluding the LAD permanently, followed by the treatment procedure 2 weeks later. Cardiac function was assessed by echocardiography just before 2, 4, 6, and 8 weeks after the treatment procedure. Eight weeks after the treatment procedure, invasive hemodynamic analysis and histological examination were performed following the sacrifice. SMB+MSC, co-culture of SMBs and MSCs; SMB, skeletal myoblast; MSC, derived mesenchymal stem cell; Echo, echocardiography; PV loop, invasive hemodynamic analysis. Color images available online at www.liebertpub.com/tea

Echocardiography

Echocardiography was performed under general anesthesia using 1% isoflurane just before, and 2, 4, 6, and 8 weeks after the treatment procedure (SONOS 7500; Philips Medical Systems) (Fig. 1B). Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and end diastolic anterior wall thickness at the level of the papillary muscles were measured for at least three consecutive cardiac cycles, following the American Society for Echocardiology leading-edge method. Fractional shortening (FS) and ejection fraction (EF) were calculated as parameters of systolic function, as follows:

$$FS\ (\%) = (LVEDD - LVESD)/LVEDD$$

$$EF\ (\%) = [(LVEDD^3 - LVESD^3)/LVEDD^3].^4$$

Cardiac catheterization

To assess systolic and diastolic cardiac function, cardiac catheterization was performed under general anesthesia using 1% isoflurane, 8 weeks after the treatment procedure. A MicroTip catheter transducer (SPR-671; Millar Instruments, Inc.) and conductance catheters (Unique Medical

Co.) were placed longitudinally in the left ventricle (LV) from the apex and connected to an Integral 3-signal conditioner-processor (Unique Medical Co.). End-systolic pressure-volume relationships (ESPVR) were determined by transiently compressing the inferior vena cava. Data were recorded as a series of pressure–volume loops (~20), which were analyzed using Integral 3 software (Unique Medical Co.). The maximal and minimal rates of change in LV pressure (dP/dt max and dP/dt min, respectively) were obtained from steady-state beats using custom-made software. We assessed the early active part of the relaxation using the relaxation time constant (τ), which was determined from the LV pressure decay curve. After the hemodynamic assessment, the heart was removed for further biochemical and histological analyses.

Real-time quantitative PCR

Total RNA was extracted from cultured cell sheets or cardiac muscle tissue 8 weeks post-transplantation using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). Subsequently, real-time PCR assays were performed using an ABI PRISM 7700 machine.[4,7,8] Hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin growth factor (IGF), and thymosin β were assayed using rat-specific primers and probes (Applied Biosystems). The average copy number of gene transcripts for each sample was normalized to that for GAPDH.

Survival of grafted donor cells

The presence of grafted male cells in the female heart was quantitatively assessed by real-time PCR for the Y chromosome-specific gene *sry*. Four weeks after cell-sheet transplantation, genomic DNA was extracted from the entire LV walls using the QIAmp genomic DNA purification system (Qiagen). The signals for the autosomal single-copy gene were normalized to the amount of total DNA.[7] The primers were *sry*: forward, 5′ GCCTCAGGACATATTAATCTCTGGAG-3′; reverse, 5′-GCTGATCTCTGAATTCTGCATGC-3′.

Protein analysis

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure proteins, such as HGF (Institute of Immunology) and VEGF (Quantikine; R&D) of rat origin, secreted from the cultured cell sheets *in vitro*, according to the manufacturers' suggested protocols. Values were calibrated for the extracted total proteins ($n=5$ in each group). The ELISA kits were also used to quantitatively analyze HGF (r-HGF) and VEGF (r-VEGF) of rat origin in heart tissue lysates ($n=5$ in each group).

Cytokine/chemokine multiplex immunology assay

The amount of each protein secreted from the cultured cell sheets *in vitro* was measured by Milliplex Rat Cytokine/Chemokine Panel Premixed 32Plex (Millipore), according to the manufacturer's instructions.[4] In this procedure, we applied human SMBs (h-SMBs) isolated and cultured from the patient (age 53 years, male) and expand *in vitro* as described previously.[6]

Histological analyses

Eight weeks after cell-sheet implantation, the hearts were dissected, fixed in 4% paraformaldehyde, and embedded in either optimum cutting temperature compound for 5-μm-thick cryosections or paraffin for 5-μm-thick sections ($n=5$ in each group) (Fig. 1). The paraffin-embedded sections were used for routine hematoxylin–eosin (HE) staining to assess the myocardial structure. Masson's trichrome staining was performed to assess cardiac fibrosis in the remote myocardium. The fibrotic cardiac area was calculated as the percentage of myocardial area. The data were collected from 10 individual views per heart at a magnification of ×200. The heart sections were also stained with an antibody to von Willebrand Factor (vWF) to assess capillary density, which was calculated as the number of positively stained capillary vessels that were 5–10 μm in diameter in 10 randomly selected fields in the peri-infarct area, per heart. To determine the extent of apoptosis, sections from frozen tissue samples were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with an *in situ* apoptosis detection kit (Apoptag; Chemicon). Image J software was used for quantitative morphometric analysis.

To detect r-SMBs, we used GFP transgenic Lewis rats.[15] Cryosections were stained with an anti-HGF antibody (1:50 dilution; LifeSpan BioSciences). To detect h-MSCs and differentiation of the transplanted cell sheet, sections were stained with an antibody to human leukocyte antigen (1:50 dilution; Dako). The secondary antibody was Alexa Fluor 555 goat anti-mouse (1:200 dilution; Molecular Probes). Cell nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI; Invitrogen). The images were examined by fluorescence microscopy (Keyence).

Western blotting

Tissue homogenates from LV samples in the cell-sheet transplanted site ($n=3$ in each group, on day 1) were prepared using lysis buffer (100 mM Tris pH 7.4, 20% SDS, 10 mM EDTA. 10 mM NaF, 2 mM sodium orthovanadate). The equivalent total protein was loaded onto SDS-polyacrylamide gel electrophoresis gels. Antibodies obtained from Cell Signaling were antiphosphorylated STAT3 (#9145), antiphosphorylated Akt (#4051), anti-Bcl_2 (#2876), and anti-poly (ADP-ribose) polymerase (PARP) (#9542). The labeled membrane was stripped and then re-probed with anti-STAT3 (#9132), anti-AKT (#9272), and anti-cleaved PARP (#9545) antibodies. Blots were scanned, and quantitative analysis was performed using Image J software. The relative proportion of the phosphorylated STAT3 was referred to that of the STAT3. The relative proportion of the phosphorylated Akt was referred to that of the Akt. The relative proportion of the PARP, cleaved PARP, Bcl_2 was referred to that of the control group.

Statistical analysis

Continuous variables are expressed as the mean ± SD. The significance of differences was determined using a two-tailed multiple *t*-test with Bonferroni correction following repeated-measures analysis of variance for individual differences. A *p*-value less than 0.05 was considered to be statistically significant. All statistical calculations were performed using the SPSS software (version 11.0; SPSS, Inc.).

Results

Production and release of cytokines/chemokines by cell sheets

Both h-SMBs and h-MSCs, as analyzed by cytokine antibody array, released abundant angiogenic factors *in vitro*, with distance profiles (Fig. 2A). Co-cultures of h-SMBs and h-MSCs showed significantly enhanced levels of HGF, VEGF, Leptin, and PECAM-1, but not of follistatin, G-CSF, IL-8, or PDGF-BB from the h-SMBs.

The seeding ratio of 4:1 r-SMBs:h-MSCs elicited the greatest *in vitro* mRNA expression of rat HGF and VEGF by real-time PCR (Fig. 2B). The mRNA levels of SMB-derived r-HGF and r-VEGF, analyzed by real-time PCR using rat-specific primers, were significantly greater in the co-cultured cell sheets than r-SMB-only ones (Fig. 2C), whereas the mRNA levels of IGF-1, bFGF, SDF-1, and TMSB4 were essentially the same (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). No mRNAs for cytokines of rat origin were detected in h-MSC-only cell sheets. Rat HGF and VEGF in the culture supernatants, analyzed by ELISA with rat-specific primary antibodies, were significantly higher in the co-culture supernatants than the r-SMB-only ones, and no rat cytokines were detected in the h-MSC-only supernatants (Fig. 2D).

Cardiac functional recovery after cell-sheet transplantation

The effects of cell-sheet transplantation on cardiac function were assessed in a rat chronic ischemic heart-failure model. Two weeks after permanent occlusion of the LCA, the LV developed echocardiographic features typical of chronic ischemic heart failure, including decreased FS, EF, and anterior wall thickness, and increased end-diastolic and systolic diameter (EDD and ESD, respectively). Following myocardial

A Cytokine/Chemokine multiplex immunology assay

	SMB+MSC	SMB	MSC	Medium only
Follistatin (pg/ml)	276±35*,†	350±56†	127±8.0	3.3
G-CSF (pg/ml)	333±44*,†	487±89†	7.7±0.7	1
HGF (pg/ml)	1190±256*,†	167±28†	695±44	3.8
IL-8 (pg/ml)	805±36*,†	1079±138†	579±29	0.6
Leptin (pg/ml)	1335±690*,†	262±40	195±13	59
PDGF-BB (pg/ml)	51±11*	25±2†	51±3	3.3
PECAM-1 (pg/ml)	438±70*	239±71†	386±16	64
VEGF (pg/ml)	5673±812*,†	1402±272	2019±184	1.9

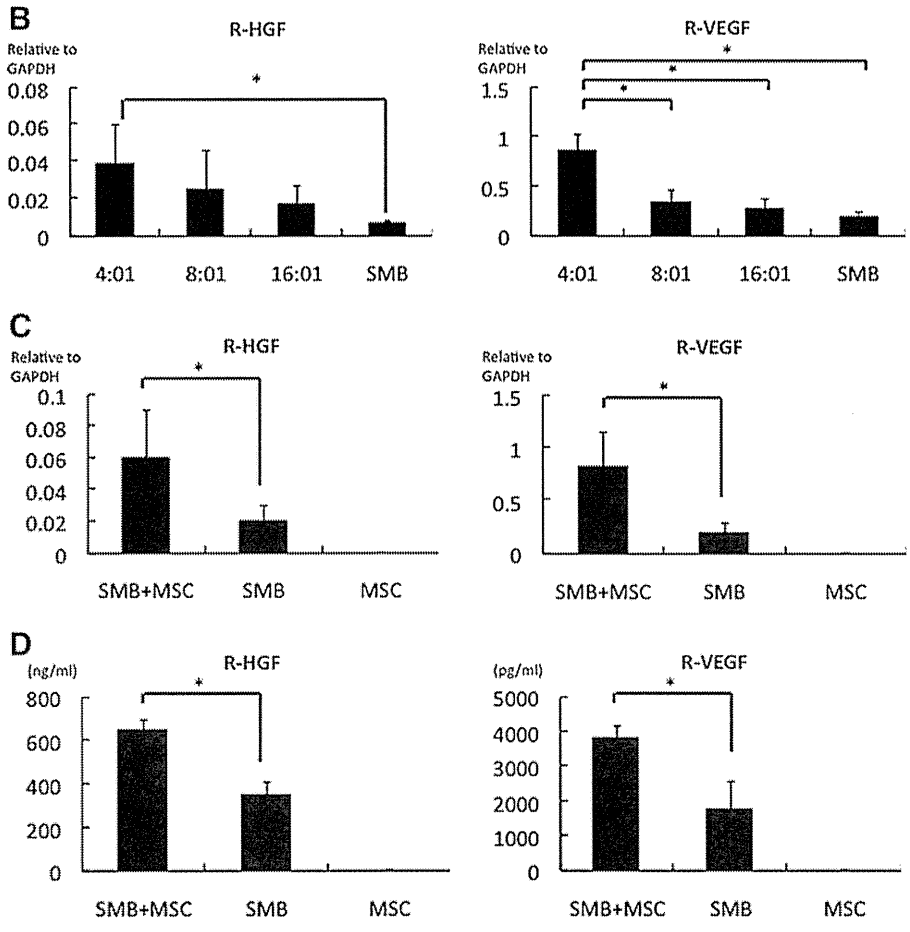

FIG. 2. Production and release of angiogenic factors by cell sheets. **(A)** Cytokine/chemokine multiplex immunology assay results from cultured cell sheets *in vitro*, prepared from human SMBs, human MSCs (h-MSCs), or both. SMB+MSC showed significantly enhanced the release of HGF, VEGF, leptin, and PECAM-1. $N=4$ in each group. $^{*}p<0.05$ versus SMB. $^{\dagger}p<0.05$ versus MSC. **(B)** Optimal seeding ratio of rat SMBs (r-SMBs) to h-MSCs. The *in vitro* mRNA levels of rat HGF and VEGF, analyzed by real-time PCR, were highest at 4:1 r-SMBs:h-MSCs. $N=4$ in each group. $^{*}p<0.05$. **(C)** mRNA levels in cultured cell sheets determined by real-time PCR using rat-specific primers. The SMB+MSC sheets expressed significantly more HGF and VEGF than the SMB-only ones. $N=5$ in each group. $^{*}p<0.05$. **(D)** Secretion of cytokines into the culture medium determined by enzyme-linked immunosorbent assay (ELISA) kits. The SMB+MSC sheets secreted significantly more HGF and VEGF than the SMB-only sheets. $N=5$ in each group. $^{*}p<0.05$. G-CSF, granulocyte-colony stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor. Error bars=SD.

infarction (MI), FS, EF, and anterior wall thickness showed steady reductions, whereas EDD/ESD showed steady increases, suggesting progressive LV remodeling.

Following either SMB-only or MSC-only cell-sheet transplantation, the heart showed mild recovery, including increases in FS, EF, and anterior wall thickness. At 2, 4, 6, and 8 weeks after treatment, FS, EF, and anterior wall thickness were significantly greater following SMB-only or MSC-only cell-sheet transplantation than the control, and significantly better recovery was obtained using the co-cultured cell sheets than either single cell-type sheet (Fig. 3A).

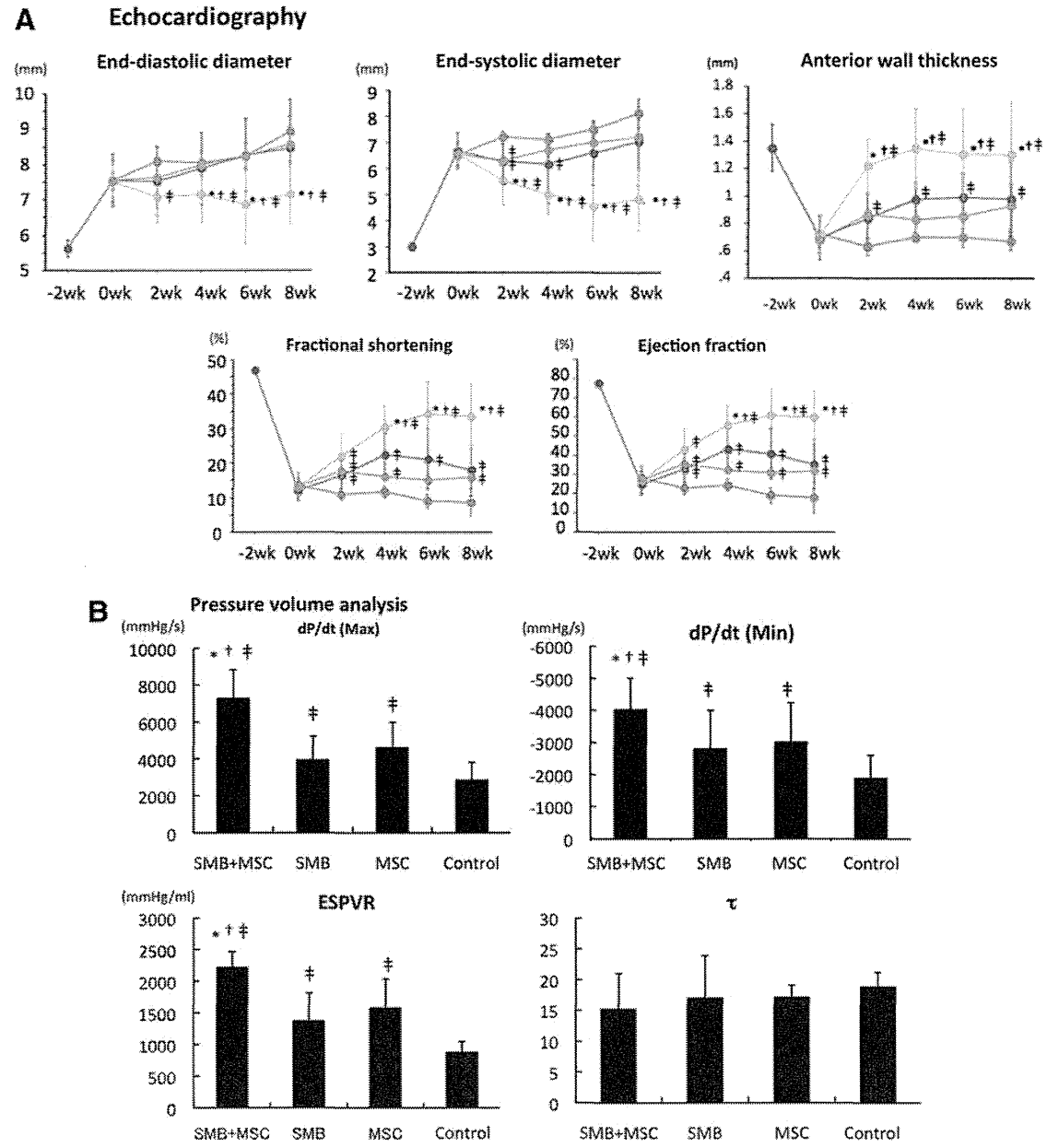

FIG. 3. Cardiac functional recovery after cell-sheet transplantation. **(A)** Echocardiographic analysis. Fractional shortening, ejection fraction, and anterior wall thickness were significantly improved 2, 4, 6, and 8 weeks after cell-sheet transplantation in the SMB+MSC sheet group, compared with the other three groups. Left ventricular end-diastolic and end-systolic diameters in the SMB+MSC sheet group were significantly decreased 4, 6, and 8 weeks after cell-sheet transplantation, compared with the other three groups ($N=10$ in each group. SMB+MSC group, green line; SMB group, blue line; MSC group, pink line; control group, red line). **(B)** Hemodynamic measurements determined by cardiac catheterization ($n=10$ in each group). Max. and min. dP/dt and ESPVR significantly improved in the SMB+MSC group, compared with the other three groups. Max. dP/dt, maximal rate of change in left ventricular pressure; min. dP/dt, minimal rate of change in left ventricular pressure; ESPVR, end-systolic pressure-volume relationship; EDPVR, end-diastolic pressure-volume relationship; τ, active part of relaxation shown by the relaxation time constant. $N=10$ in each group. $^{*}p<0.05$ versus SMB-only cell sheet. $^{\dagger}p<0.05$ versus MSC-only cell sheet. $^{\ddagger}p<0.05$ versus control. n.s., not significant. Error bars=SD. Color images available online at www.liebertpub.com/tea

Assessment by LV catheter showed a similar trend. Eight weeks after transplantation, the maximal and minimal rate of change in LV pressure (max. dP/dt and min. dP/dt, respectively) and end-systolic pressure-volume relationship (ESPVR) were significantly greater following either single-cell-type cell-sheet transplantation than the control, but τ was significantly different. After the co-culture cell-sheet transplantation, the max. dP/dt, min. dP/dt, and ESPVR improved further, with no significant difference in EDPVR or τ (Fig. 3B).

Reverse remodeling after co-culture cell-sheet transplantation

The LV structure was better maintained after SMB-only or MSC-only cell-sheet transplantation, compared to the control, in which the LV cavity was severely enlarged with a thin anterior wall, as assessed by HE staining (Fig. 4A). The LV structure was even better maintained after the co-culture cell-sheet transplantation. In the control, abundant collagen accumulations were observed in the infarct area, and diffuse fibrotic changes were induced in the remote area, whereas collagen accumulation was attenuated in both the remote area with the single cell-type sheet transplants, as assessed by Masson's trichrome staining (Fig. 4B, C). Fibrotic changes in the remote area were further attenuated by transplantation of the co-cultured cell sheet (Fig. 4D).

A greater number of vWF-positive blood vessels was detected in the peri-infarcted myocardium following the transplantation of either single-cell-type cell sheet, compared to the control (Fig. 5A), and even more vWF-positive blood vessels were seen with transplantation of the co-cultured cell sheet. The capillary density in the peri-infarcted myocardium, which was semi-quantitatively assessed in 10 randomly selected individual fields, was significantly greater following the transplantation of either single-cell-type cell sheet, compared to the control (Fig. 5B), and it was further increased after the co-cultured cell-sheet transplantation.

Major intercellular signaling molecules relevant to angiogenesis and cell survival were analyzed by western blotting. The ratio of p-STAT3 over total STAT3 was greatly increased after co-cultured cell-sheet transplantation (Fig. 5C).

Survival of transplanted cells in the heart

Four weeks after the cell-sheet transplantation, significantly more transplanted rat cells survived in co-cultured sheets than SMB-only sheets, as analyzed by PCR assays for the Y-chromosome-specific *Sry* gene (Fig. 6A).

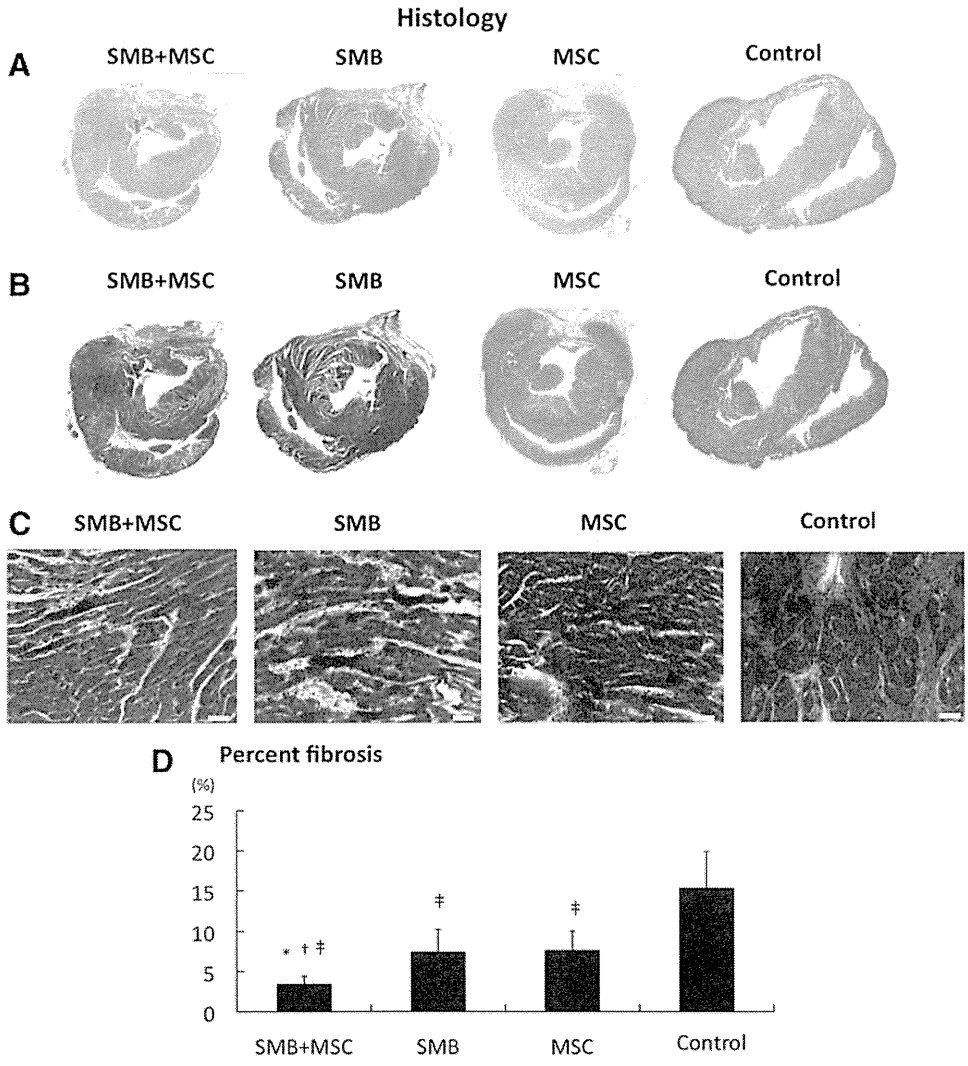

FIG. 4. Histological reverse remodeling after cell-sheet transplantation. **(A)** Macroscopic (×40) views of the heart stained by hematoxylin–eosin. **(B)** Macroscopic (×40) views of the heart stained by Masson's trichrome. **(C)** Microscopic (×200) representative Masson's trichrome staining at the remote myocardium (white bar = 40 μm). **(D)** Quantification of percent fibrosis at the remote area. Significant suppression of fibrosis was found after SMB + MSC sheet transplantation compared with the other three groups. $N = 5$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD. Color images available online at www.liebertpub.com/tea

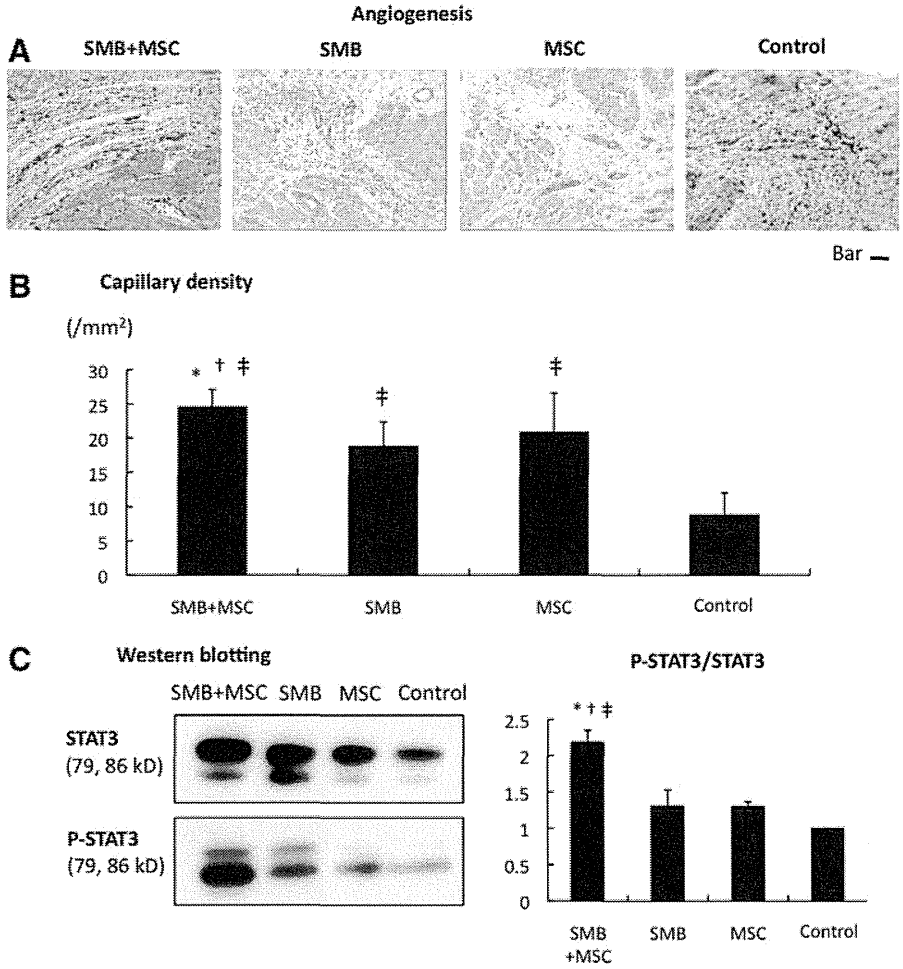

FIG. 5. Angiogenesis. **(A)** Microscopic (×100) views of sections of the peri-infarct border-zone region stained with anti-von Willebrand factor antibody (factor VIII) in the four groups (bar = 50 μm). **(B)** Capillary density: the SMB + MSC group showed significant improvement in capillary density as assessed by immunostaining for von-Willebrand factor-positive blood vessels. $N = 5$ in each group. **(C)** Western blotting showing enhanced STAT3 phosphorylation over total STAT3 in the SMB + MSC sheet group. $N = 3$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD. STAT3, signal transducer and activator of transcription 3. Color images available online at www.liebertpub.com/tea

The percentage of TUNEL-positive myocytes was significantly lower following the transplantation of the co-cultured cell sheet compared to the control (Fig. 6B).

Akt-1 and Bcl-2 were highly expressed in the heart following transplantation of the SMB-only or co-cultured cell sheet, compared with the control, as analyzed by real-time quantitative PCR using rat-specific primers (Fig. 6C).

Notably, among apoptosis-signaling molecules, Bcl_2 and cleaved PARP were increased 1 day after the co-culture cell-sheet transplantation. There was no significant difference in the ratio of phosphorylation of Akt over Akt (Fig. 6D).

Upregulation of cardioprotective factors in the myocardium after cell-sheet transplantation

The mRNA expression of cardioprotective factors, such as HGF, VEGF, IGF-1, and bFGF, in the infarct and infarct-remote areas of the myocardium was analyzed by real-time PCR using rat-specific primers, which detected factors released by transplanted SMB or the native myocardium. The expression of these factors was not significantly different after transplantation of either single-cell-type cell sheet or no treatment, except for HGF expression in the infarct area, which was significantly greater after the SMB-only sheet transplantation (Fig. 7A, B). In contrast, following transplantation of the co-cultured cell sheet, the HGF and VEGF levels in the infarct area were significantly greater than after transplantation of either single cell-type cell sheet or control, although the levels of IGF-1 and bFGF were unchanged (Fig. 2A). The intramyocardial protein levels of HGF and VEGF, analyzed by ELISA, were significantly greater after transplantation of the co-cultured cell sheet than of either single-cell-type cell sheet or no treatment (Fig. 7C).

Immunoconfocal microscopy showed that HGF was found in the transplanted SMBs from the co-cultured cell sheet (Fig. 8A).

MSCs differentiate into new vessels in situ

The differentiation capacity of the transplanted h-MSCs was assessed by immunoconfocal microscopy. As expected, no human-derived cells were seen in either the r-SMB-only transplantation group or the control group. However, human vWF-positive staining was observed in the host vessels in both the co-cultured cell-sheet group and the h-MSC-only cell-sheet transplantation group. Thus, the h-MSCs could differentiate into vessel walls *in vivo* (Fig. 8B).

Discussion

Here, we demonstrated that SMB cell sheets abundantly synthesized and extracellularly released multiple cytokines and chemokines, and adding MSCs enhanced the SMB cell

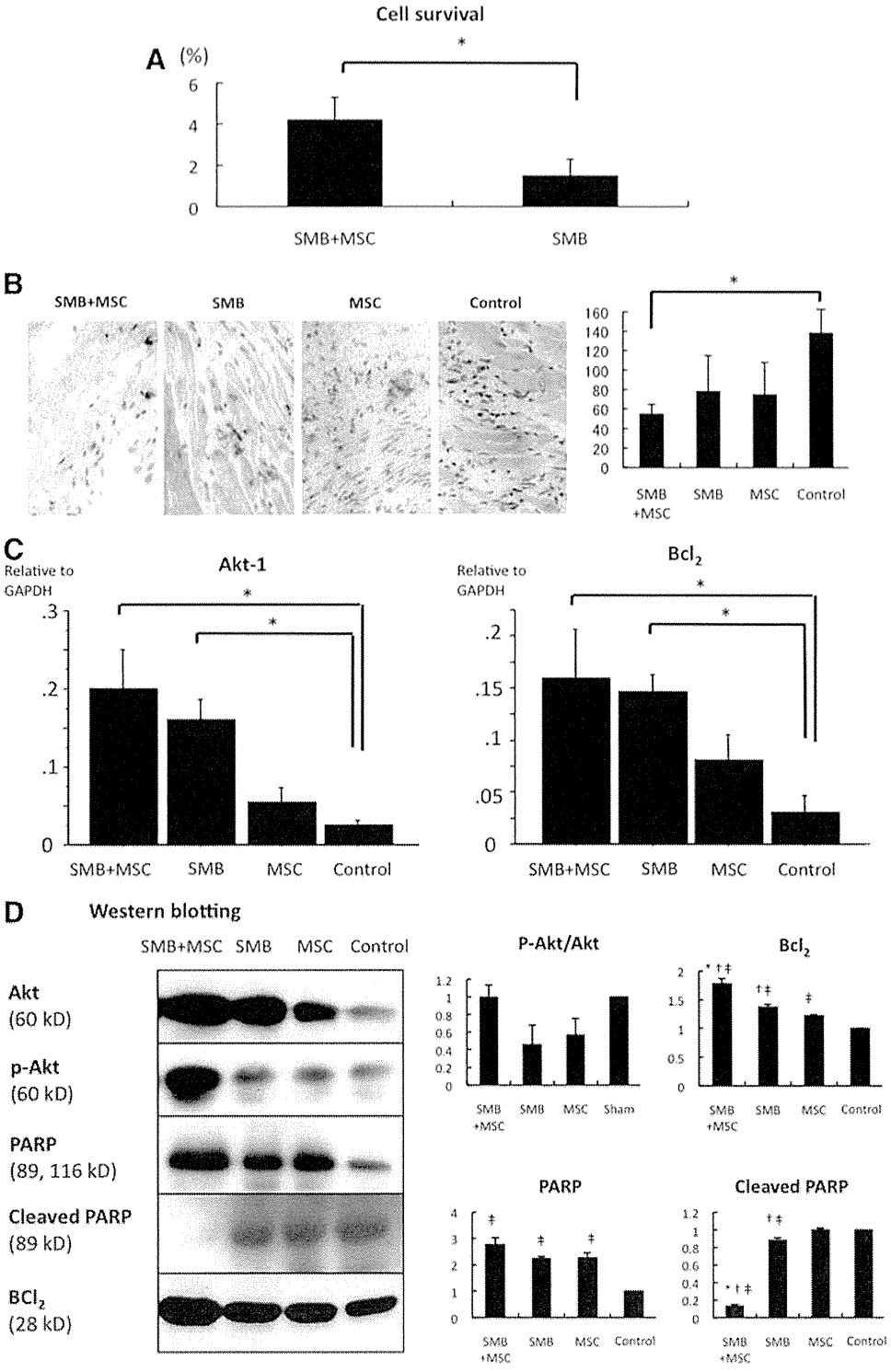

FIG. 6. Cell survival. **(A)** Survival of transplanted cells of rat origin was significantly greater in the SMB+MSC sheet group than in the SMB sheet group. $N = 4$ in each group. $^{*}p < 0.05$. **(B)** The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive myocytes was significantly lower in SMB+MSC group than in control. $N = 4$ in each group. $^{*}p < 0.05$. **(C)** Expressions of mRNA in the transplanted infarct area of hearts were determined by real-time PCR using rat-specific primers. The expressions of Akt-1 and Bcl_2 mRNA were significantly increased in the SMB+MSC sheet group compared with the other groups. $N = 4$ in each group. $^{*}p < 0.05$. **(D)** Western blotting showed that Bcl_2 was much more enhanced, and cleaved PARP was significantly downregulated in the SMB+MSC group. There was no significant difference in the ratio of phosphorylation of Akt over Akt. $N = 3$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD. Color images available online at www.liebertpub.com/tea

sheets' release of HGF and VEGF but not of IGF-1, bFGF, or SDF-1, *in vitro*. The transplantation of SMB-only cell sheets into the chronically ischemic failing rat heart resulted in reversed LV remodeling, including increased capillaries, attenuated collagen accumulation, and prolonged cell survival, which increased global functional recovery, mediated by the paracrine effects of upregulated HGF and VEGF in the myocardium.

Recent studies, including ours,[3–9] have suggested that a paracrine effect mediated by cytokines secreted from the transplanted cell sheets is a likely mechanism for the therapeutic effects on the myocardium, which was a focus of the present study. Here, we added h-MSCs to the cell sheets to enhance the potential performance of the transplanted r-SMB sheets. Our *in vitro* findings, that h-MSCs enhanced rat mRNA levels and the secretion of cytokines such as r-HGF

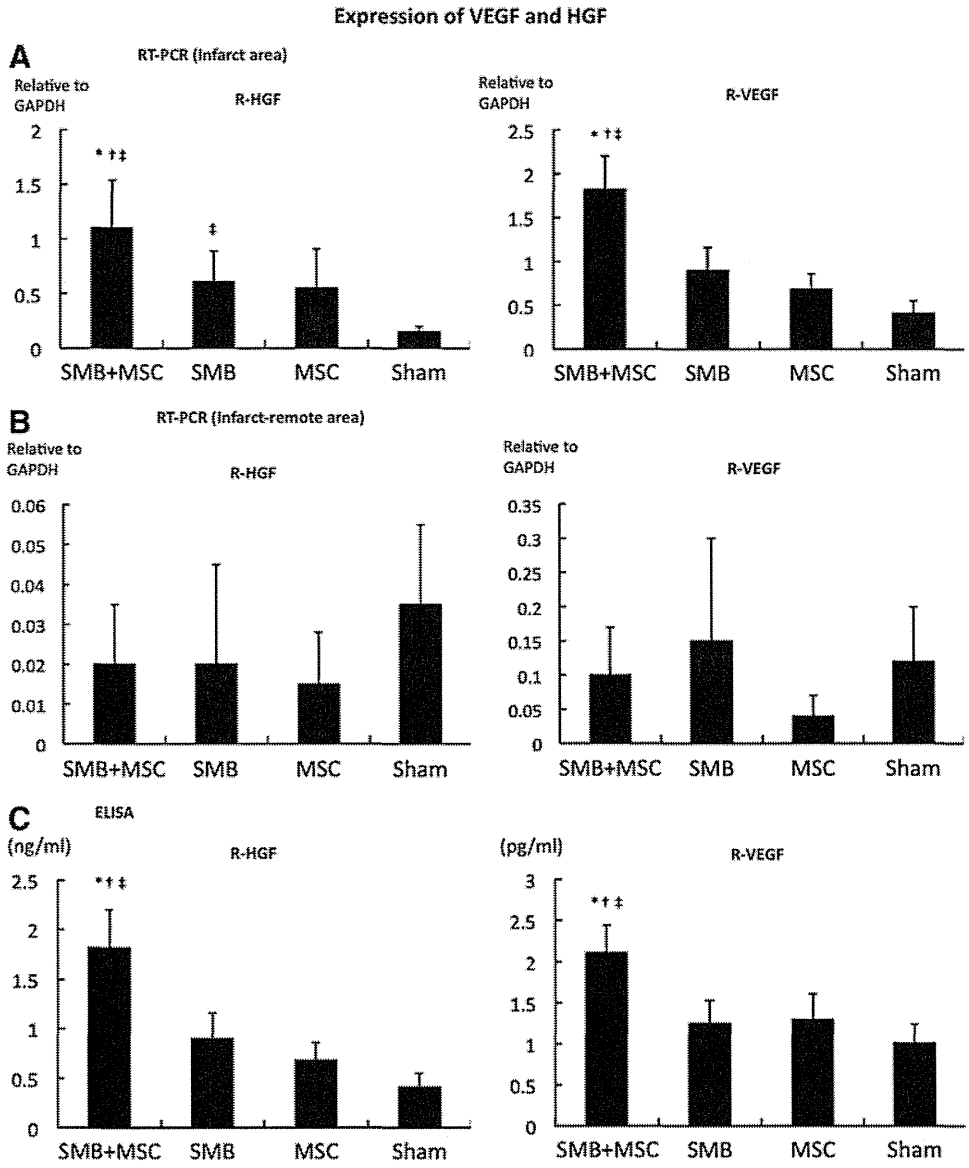

FIG. 7. Expression of VEGF and HGF is higher at the infarct area. **(A, B)** Levels of mRNA in the transplanted infarct and infarct-remote heart areas by real-time PCR using rat-specific primers. The HGF and VEGF mRNA expressions within the transplanted infarct area of the hearts were significantly increased in the SMB + MSC sheet group compared with the other groups. $N = 4$ in each group. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus sham. **(C)** Intramyocardial protein levels of HGF and VEGF, analyzed by ELISA, were significantly greater in the heart in the SMB + MSC sheet group compared with the other groups. $^{*}p < 0.05$ versus SMB. $^{\dagger}p < 0.05$ versus MSC. $^{\ddagger}p < 0.05$ versus control. Error bars = SD.

and r-VEGF from r-SMBs, suggested that transplanted co-cultured cell sheets would secrete r-HGF and r-VEGF *in vivo*. Although the exact mechanisms by which "feeder layers" support cell growth have not been elucidated, it is possible that h-MSCs enhance the r-SMBs directly (via cellular interaction) or indirectly (via secreted cytokines from the h-MSCs).[16] A more comprehensive examination aimed at differentiating these effects might help reveal how feeder layers work.

HGF and VEGF participate in many complex molecular and cellular mechanisms, and their signaling pathways have been intensively investigated *in vivo*.[3,9] SMBs or MSCs act as the natural supplier of both HGF and VEGF and provide feasible and safe sources for cell therapy in clinical applications. Indeed, SMBs and bone marrow-derived mesenchymal stem cell sheets can secrete growth factors (e.g., HGF and VEGF) into the myocardium and accelerate neovascularization in the damaged area.[5–8] More recent reports have revealed that angiogenesis induced by HGF or VEGF, an antifibrotic effect promoted by HGF, or the migration and survival of SMBs supported by VEGF,[17] could be beneficial to an impaired heart.[7,8] In addition, our data from a cytokine/chemokine multiplex immunology assay indicate that leptin may also be beneficial (e.g., by inducing angiogenesis though the Jak/STAT pathway).[18] Other cytokines may also contribute to the improvement of cardiac function by single-cell-type cell sheets in as-yet-undiscovered ways.

The mechanism by which the implanted cell sheet attenuates ventricular remodeling and improves cardiac function seems to depend on the cell sheet being placed over the scarred area of the myocardium and leads to repair of the anterior wall thickness, reduction of LV wall stress, and the improvement of ejection performance.[3] Previous studies indicated that the surviving myocardium and implanted cell sheet attenuate complex cellular and molecular events, including hypertrophy, fibrosis, apoptosis of the myocardium, and the pathological accumulation of extracellular matrix.[9] Similarly, the greater cellularity observed after cell-sheet

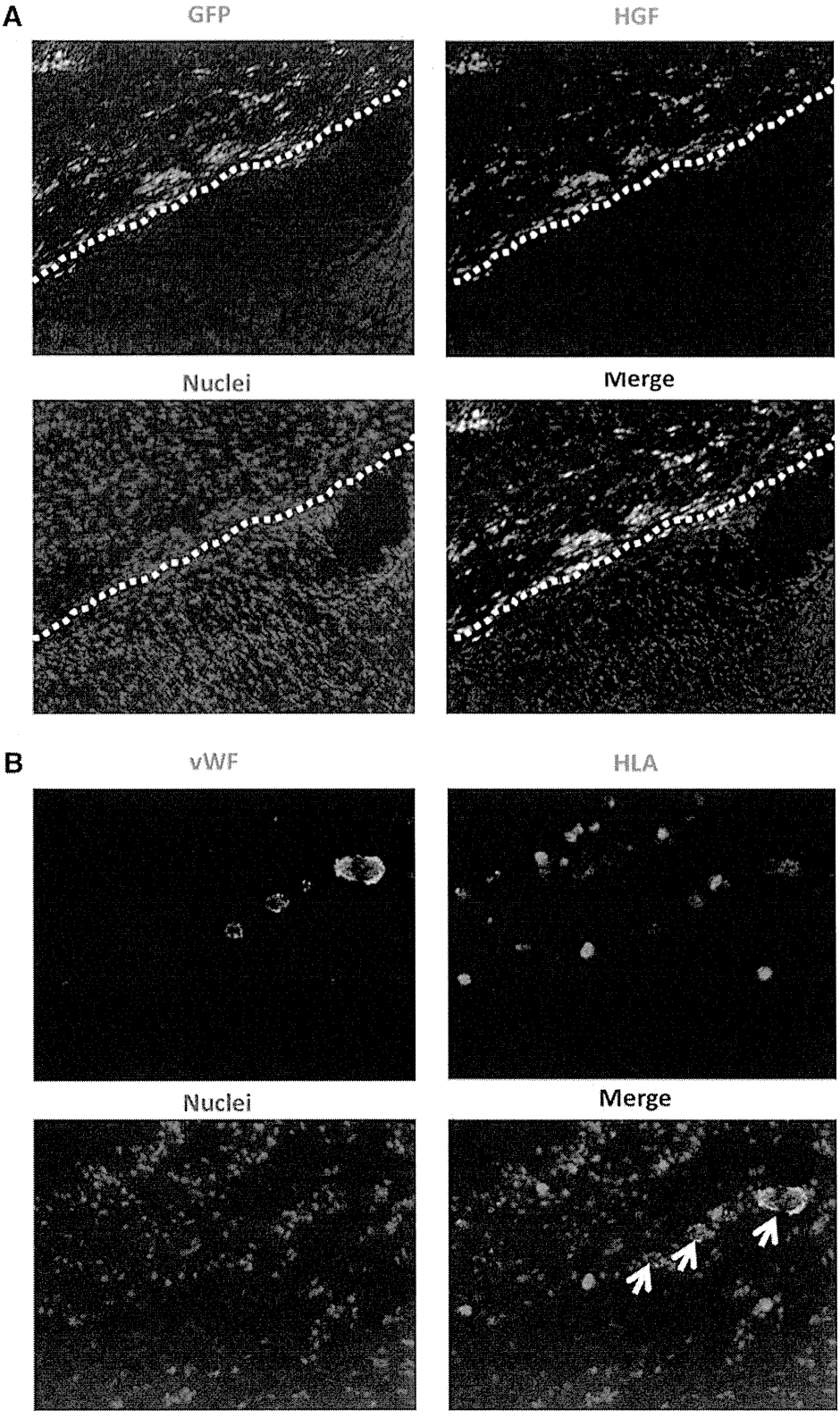

FIG. 8. Characterization of transplanted cells. **(A)** Cryosections were stained with an antibody to HGF to detect the distribution of SMB and HGF in the heart. HGF expressions and GFP-positive cells were found in the myocardium after transplantation of the SMB+MSC sheet. White broken line shows the border between the transplanted cell sheet and the host heart. Green indicates GFP; red, HGF; blue, nuclei. **(B)** Cryosections were stained with antibodies to human leukocyte antigen (HLA) and to von Willebrand factor (vWF). Human vWF-positive (white arrows) staining was observed in the host vessels in the h-MSC-transplanted group. Green indicates vWF; red, HLA; blue, nuclei. Color images available online at www.liebertpub.com/tea

treatment might have resulted from released SDF-1, which is related to cell migration, adhesion, and proliferation, by the transplanted cell sheet[19,20]

In this study, we performed additional investigations on the paracrine mechanism from a new perspective, by analyzing signaling pathways within the myocardium following cell-sheet transplantation because the signals induced by released paracrine mediators presumably activate phosphorylation cascades of signaling molecules. We found that STAT3 and Akt phosphorylations were significantly increased, and cleaved PARP was significantly downregulated, 24 h after the co-cultured cell-sheet implantation. Together