とに異論はありません。

相談事項1　成人（拡張型心筋症ならびに類縁疾患に対するTCD-51073の探索的試験） 2.　選択基準／除外基準について

【相談内容の解釈】

相談者は、成人DCM治験の被験者組入れに際して、薬事戦略相談　対面助言資料　別紙1p.3に示した選択基準／除外基準の設定を予定している。対象疾患は、DCMに限らず、病理組織学的所見及び臨床症状が類似し、病態の増悪により心機能が悪化し、進行すると内科的及び外科的治療抵抗性となる「拡張型心筋症ならびに類似疾患（虚血性心筋症、拡張相肥大型心筋症、心筋炎後心筋症、周産期心筋症及び薬剤性心筋症など）患者」とすることを予定している。また、試験データの一部を抜粋して示したとおり（平成26年2月24日提出追加資料）、New York Heart Association心機能分類（以下、「NYHA」）Ⅲ度以上の患者を対象に実施した臨床研究の結果から、比較的心機能の保たれている患者では治療効果が見込まれる可能性が示唆されたため、対象患者はNYHAⅡ度以上とすることが妥当と考えている。なお、症例数は抗悪性腫瘍薬の第Ⅰ相試験の考え方及び成人ICM治験を参考に、6例を予定している。相談者の計画する選択基準／除外基準について、機構の意見を聞きたい。

【機構の意見】

機構は、提示された選択基準／除外基準について、以下の点に対応する必要があると考えます。

1)　「拡張型心筋症ならびに類似疾患者」

対象疾患に挙げられたDCMの「類似疾患」は、それぞれ病因、病態及び病気の進行等がDCMと異なるため、本剤が同程度に作用するかどうかは不明であり、疾患ごとに本剤適用後の効果の程度や効果が認められる時期が異なる可能性があるため、予定する症例数で治験を実施した場合、種々の疾患患者により構成される対象集団から得られた試験成績を適切に評価することが困難になることが懸念されます。したがって、予定する症例数で治験をせざるを得ない状況であり、かつ主な対象疾患をDCMと想定しているのであれば、得られる有効性及び安全性成績の解釈をしやすくするために、対象疾患としてまずはDCMのみを選択することが適切と考えます。

今後、DCM以外の疾患も適応に含めて開発を進めていくことについて、疾患特性から安全性上の問題が特に懸念される場合を除き、異論はありませんが、DCM以外の疾患を治験の対象とする場合、疾患ごとに本剤適用により効果が期待できるか精査し、適切な対象疾患を選択した上で、当該疾患に対する治験デザインを検討し、当該試験の目的を達成するために必要な症例数を検討する必要があると考えます。

2)　「NYHA心機能分類がⅡ度以上の心不全が持続している患者」

成人DCM治験の対象患者として、「現状NYHAⅡ度に近い状態であるが、将来的にNYHAⅢ度になる可能性が高い患者」（平成26年3月5日付け照会事項に対する回答書、以下「回答書」p.2）が想定されていますが、NYHAⅡ度とNYHAⅢ度以上の患者では重症度が異なるため既存治療の治療成績が大きく異なることが知られています。また、相談者が想定するNYHAⅡ度の集団を規定する指標が確立されていないこと、提示されている選択基準には「標準的な外科的ならびに内科的治療を施して3ヶ月以上が経過しているにもかかわらず、心不全の悪化が危惧される患者」と明確な判断が困難な基準が含まれていることから、対象をNYHAⅡ度以上とした場合、様々な重症度の患者が組み入れられることが想定されます。さらに、予定症例数は6例とされており、患者背景のバラツキが本剤の有効性及び安全性の評価に影響を及ぼし、試験成績の評価が困難になることが懸念されます。また、提出された臨床研究を抜粋したデータからは、現時点において本剤の効果が広く期待できるとまでは判断できないと考えます。以上の点から、現在の状況において単群で実施する成人DCM治験にNYHAⅡ度の患者を組み入れることは適切ではないと考えます。

3) 「標準的な外科的ならびに内科的治療を施して3ヶ月以上が経過しているにもかかわらず、心不全の悪化が危惧される患者」

対象患者を明確化するため、標準的な外科的治療について回答書p.2で示された「左室縮小術、僧帽弁形成術等」を追記する必要があると考えます。

相談事項1：成人（拡張型心筋症ならびに類縁疾患に対するTCD-51073の探索的試験）
4. 効果安全性評価委員会を設定しないことについて

【相談内容の解釈】

成人DCM治験の対象患者は死亡のおそれのある重篤な患者であることから、治験責任医師等の判断で的確に治験中止の判断が可能となるよう、中止基準を設けることとしている。また、本剤は相談者自身が開発している製剤であるため本剤の安全性を熟知していることに加え、成人DCM治験は単施設で実施し中間解析等を予定していない。効果安全性評価委員会を設置することの意義は、死亡例や死亡のおそれのある有害事象が発生したときに、治験の継続、中止、中断の判断をすることにあると考えており、上記の点を踏まえると成人DCM治験において効果安全性評価委員会を設置することは不要と考えている。以上の考えについて機構の意見を聞きたい。

【機構の意見】

効果安全性評価委員会は、適切な治験の継続、変更又は中止について、治験責任医師から独立した第三者の立場から提言することを目的としています。成人DCM治験では、本剤の新規性が高いこと、対象疾患が重症であること、症例数が限られていることから、治験を適切に実施していくために、効果安全性評価委員会を設置することをお勧めします。

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
Shudo Y, Miyagawa S, Ohkura H, Fukushima S, Saito A, Shiozaki M, Kawaguchi N, Matsuura N, Shimizu T, Okano T, Matsuyama A, Sawa Y.	Addition of Mesenchymal Stem Cells Enhances the Therapeutic Effects of Skeletal Myoblast Cell-Sheet Transplantation in a Rat Ischemic Cardiomyopathy Model.	Tissue Eng Part A	20(3-4)	728-39	2014
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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.

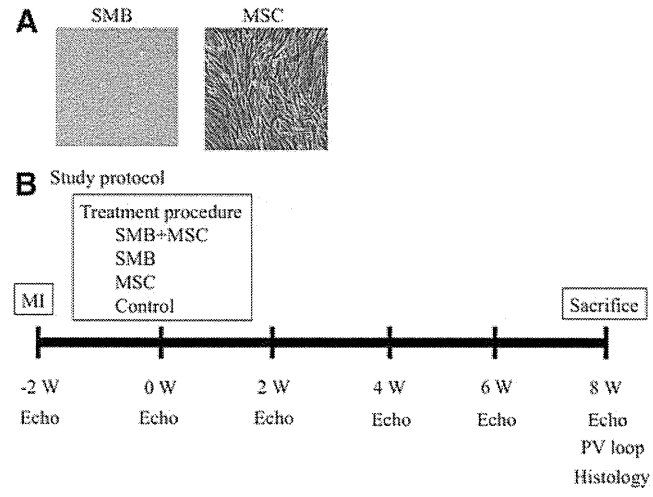

FIG. 1. **(A)** Morphology of SMB and MSC. **(B)** Study protocol used for the assessment of cardiac function and histology. Athymic nude rats (F344/NJcl-rnu/rnu) underwent induction of myocardial infarction by occluding the LAD permanently, followed by the treatment procedure 2 weeks later. Cardiac 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

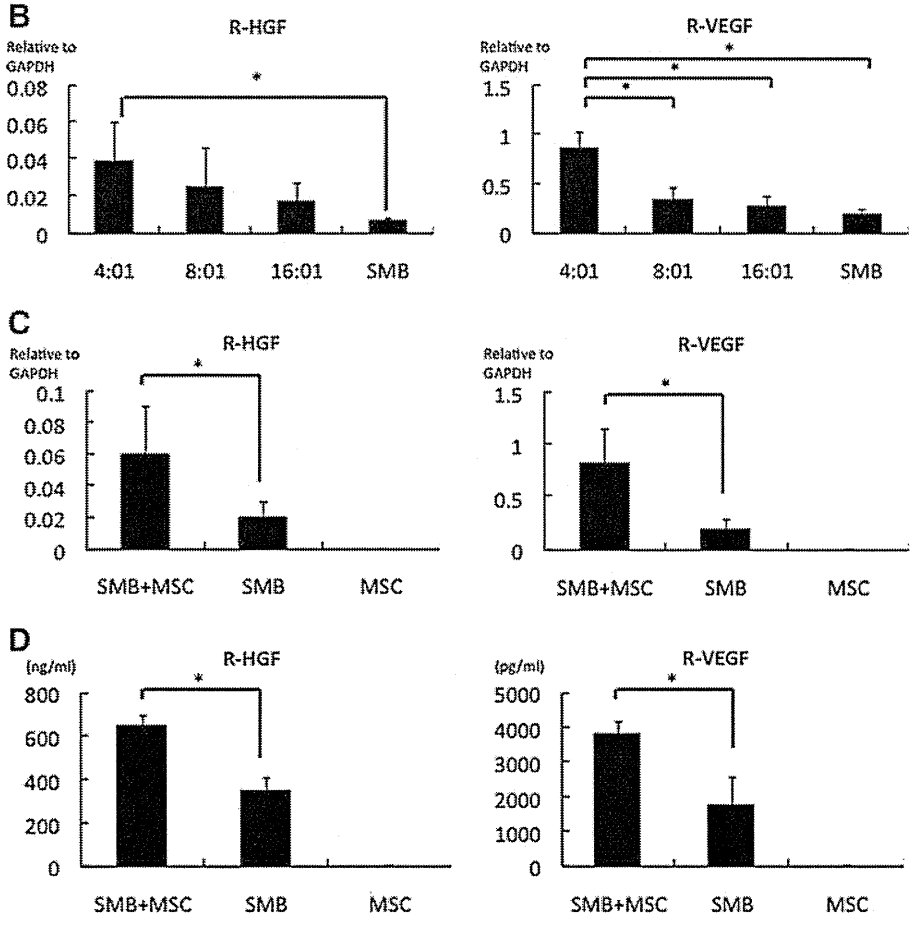

FIG. 2. Production and release of angiogenic factors by cell sheets. **(A)** Cytokine/chemokine multiplex immunology assay results from cultured cell sheets *in vitro*, prepared from human SMBs, human MSCs (h-MSCs), or both. SMB+MSC showed significantly enhanced the release of HGF, VEGF, leptin, and 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).

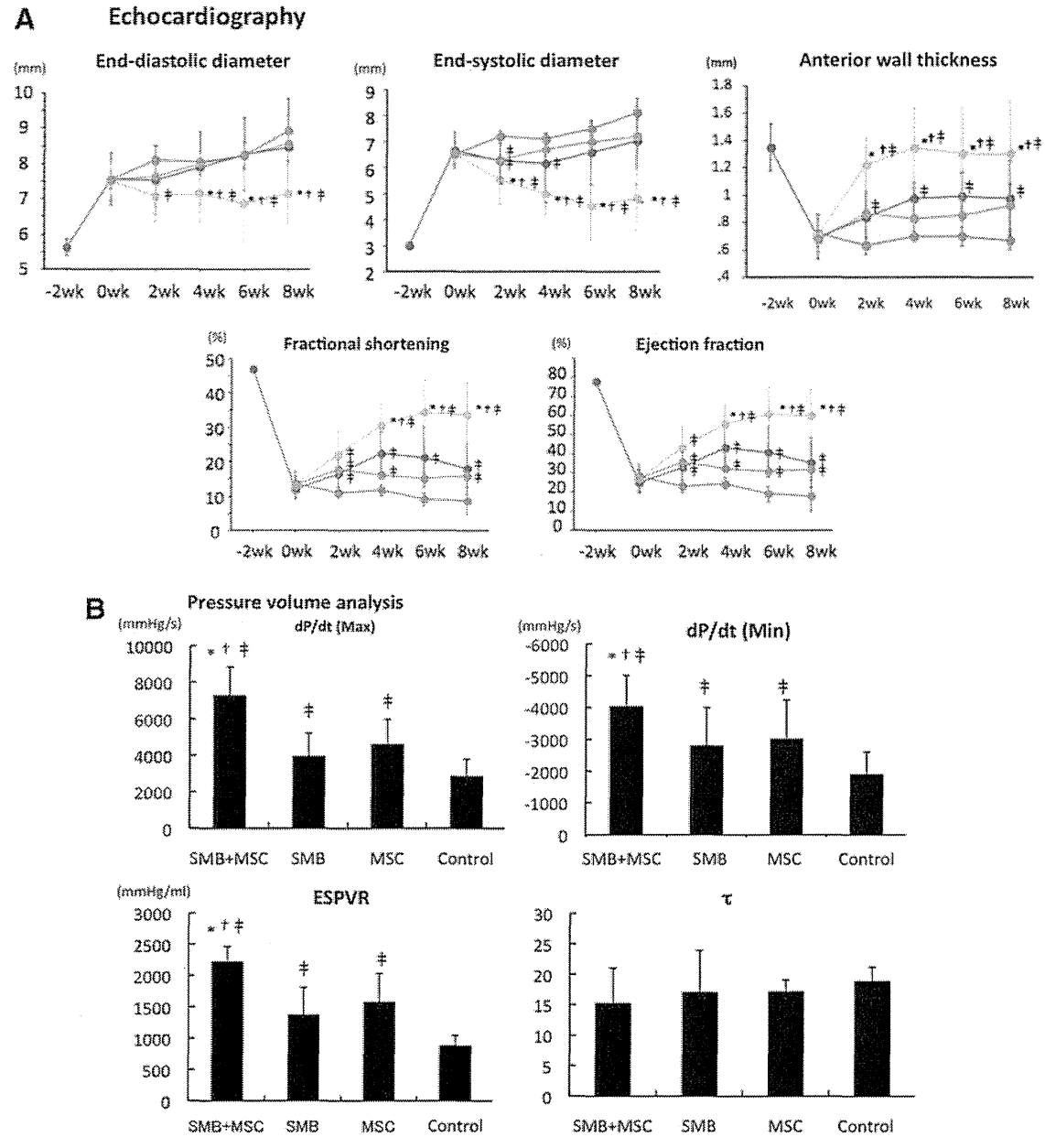

FIG. 3. Cardiac functional recovery after cell-sheet transplantation. **(A)** Echocardiographic analysis. Fractional shortening, ejection fraction, and anterior wall thickness were significantly improved 2, 4, 6, and 8 weeks after cell-sheet transplantation in the SMB+MSC sheet group, compared with the 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).

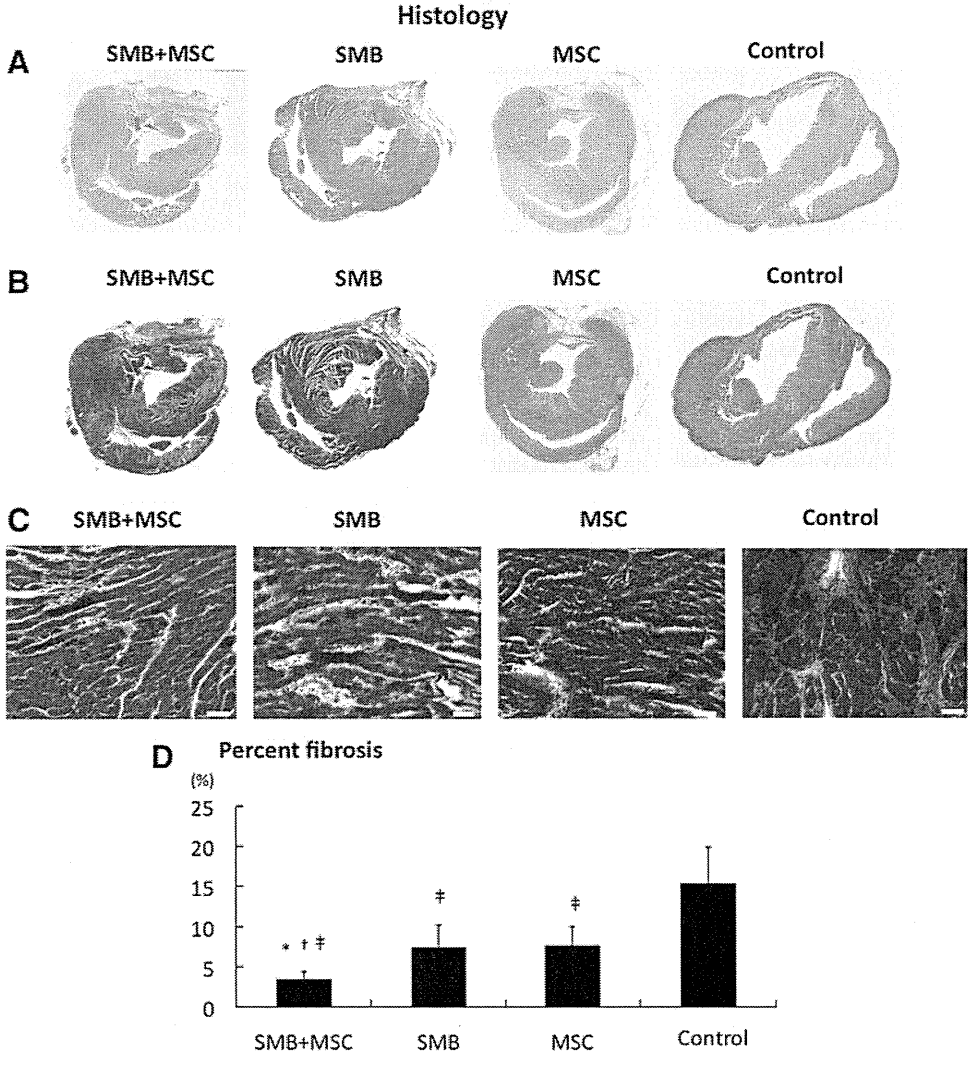

FIG. 4. Histological reverse remodeling after cell-sheet transplantation. **(A)** Macroscopic (×40) views of the heart stained by hematoxylin–eosin. **(B)** Macroscopic (×40) views of the heart stained by Masson's trichrome. **(C)** Microscopic (×200) representative Masson's trichrome staining at the 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

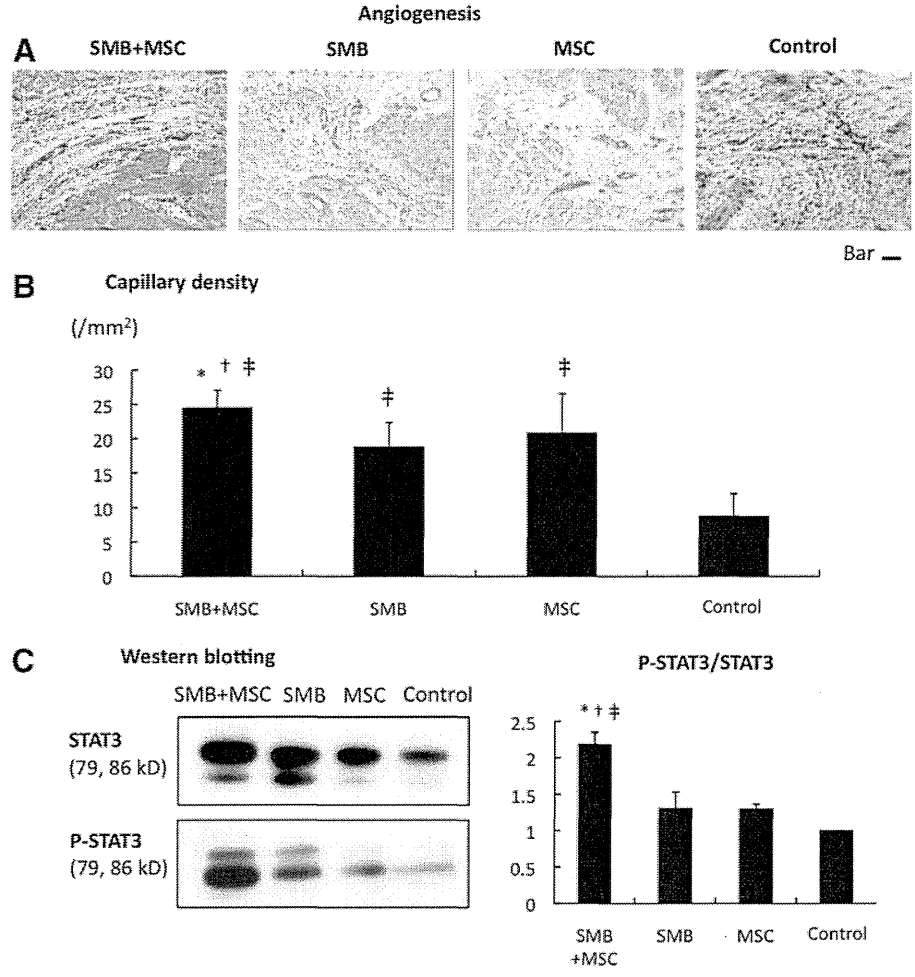

FIG. 5. Angiogenesis. **(A)** Microscopic (×100) views of sections of the peri-infarct border-zone region stained with anti-von Willebrand factor antibody (factor VIII) in the four groups (bar = 50 μm). **(B)** Capillary density: the SMB + MSC group showed significant improvement in capillary density 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

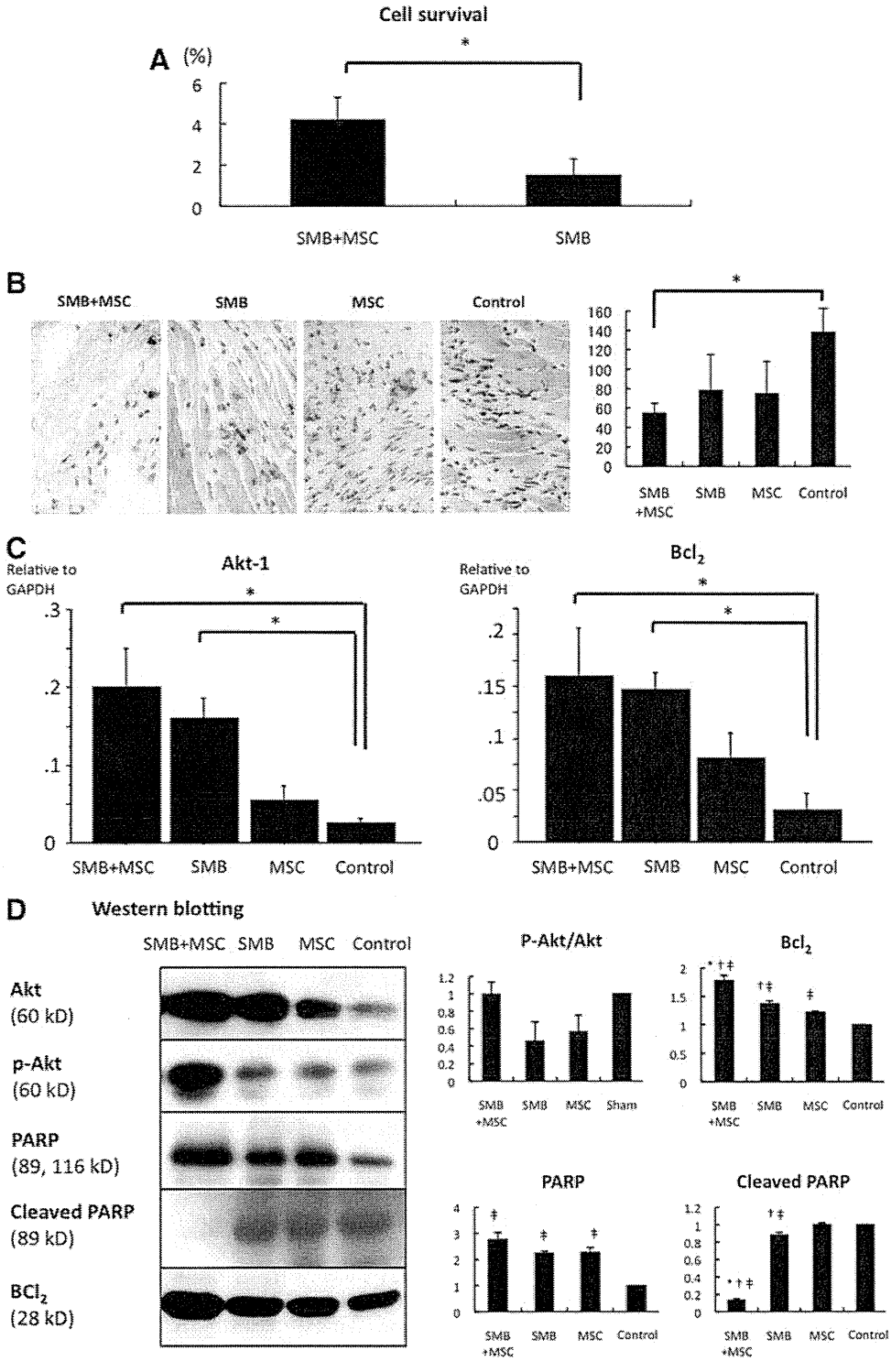

FIG. 6. Cell survival. **(A)** Survival of transplanted cells of rat origin was significantly greater in the SMB+MSC sheet group than in the SMB sheet group. $N = 4$ in each group. $^*p < 0.05$. **(B)** The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive 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

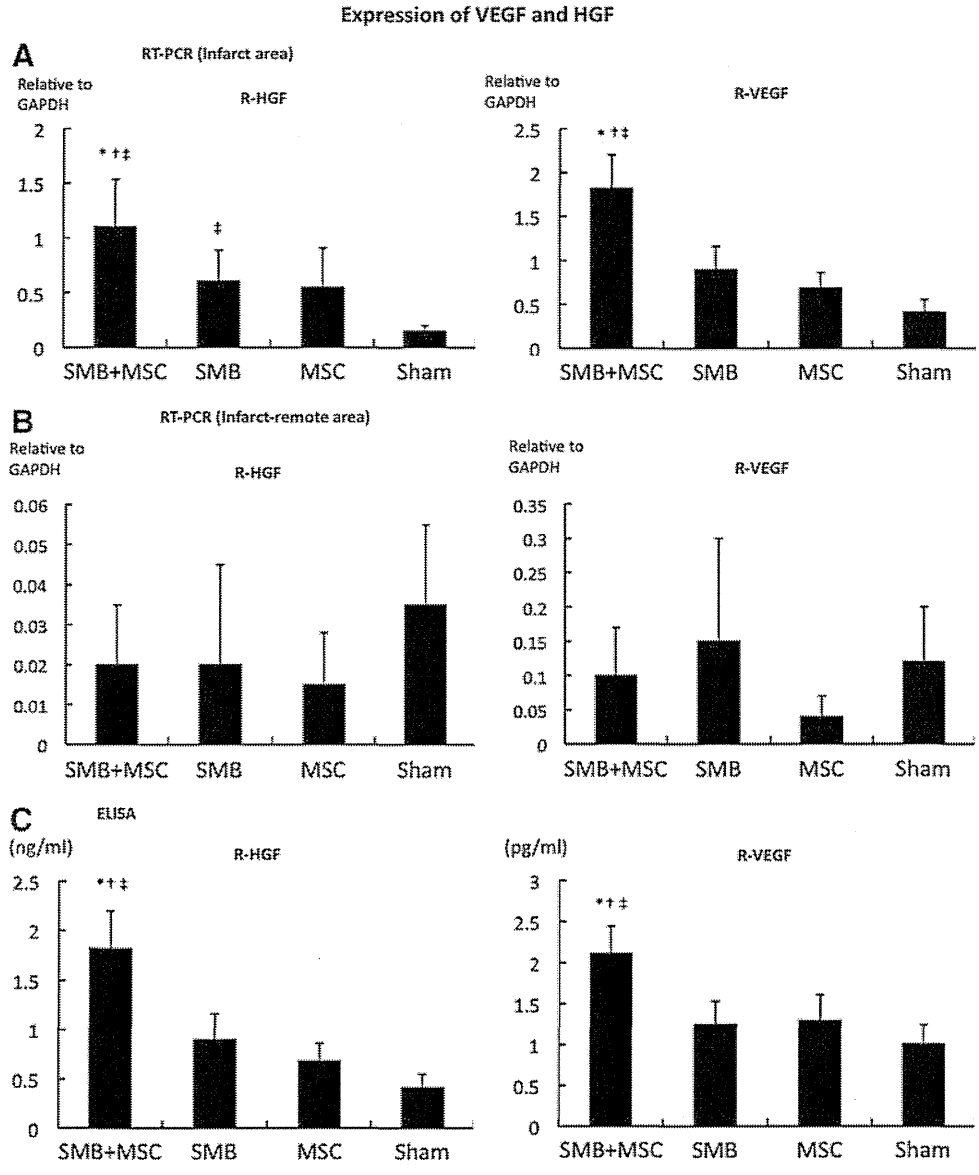

FIG. 7. Expression of VEGF and HGF is higher at the infarct area. **(A, B)** Levels of mRNA in the transplanted infarct and infarct-remote heart areas by real-time PCR using rat-specific primers. The HGF and VEGF mRNA expressions within the transplanted infarct area of the hearts were significantly increased 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

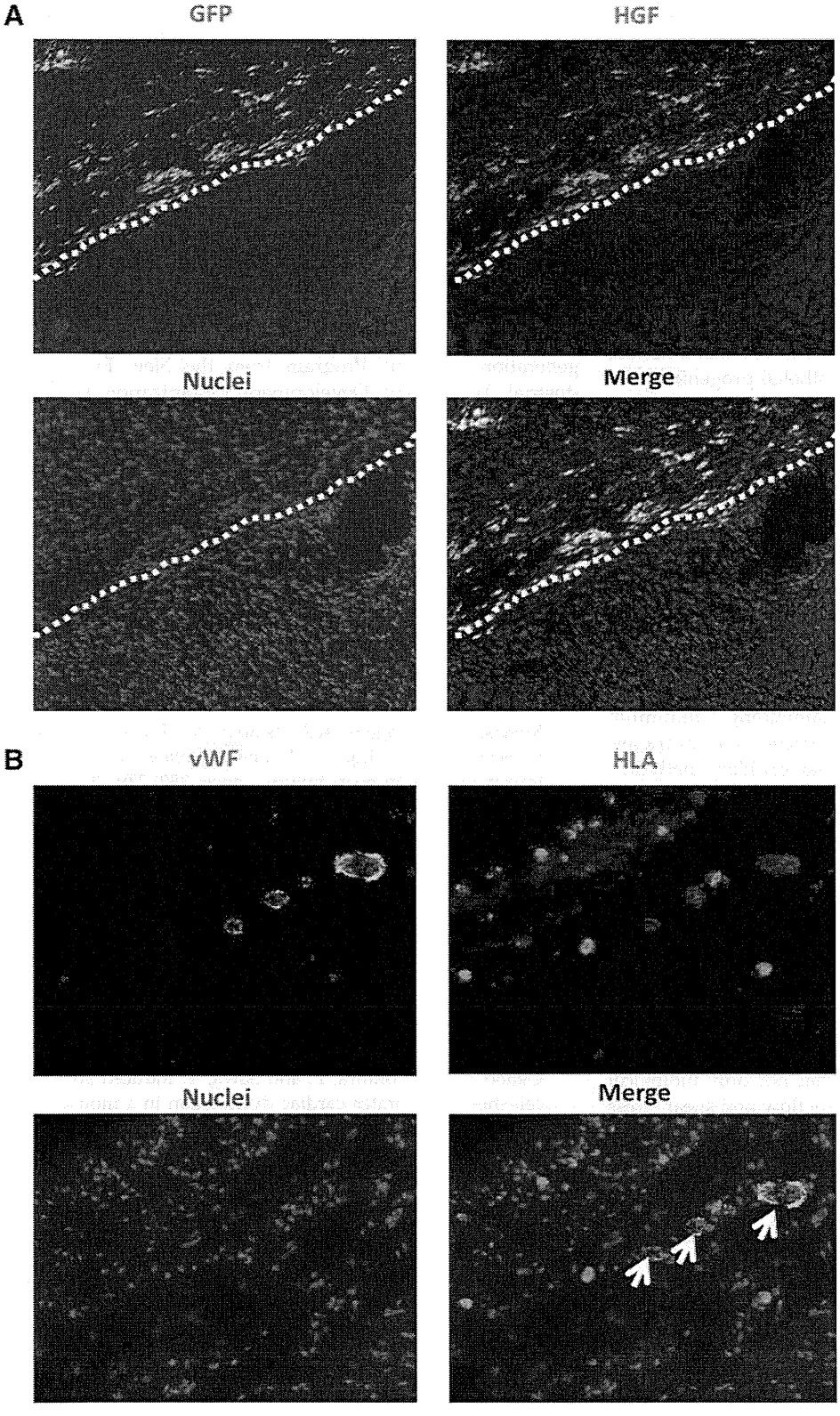

FIG. 8. Characterization of transplanted cells. **(A)** Cryosections were stained with an antibody to HGF to detect the distribution of SMB and HGF in the heart. HGF expressions and GFP-positive cells were found in the myocardium after transplantation of the SMB+MSC sheet. White broken line shows the 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

with our findings that vascular density was significantly enhanced, and myocardial apoptosis and fibrosis was significantly attenuated in the co-cultured group, it is possible that the co-cultured cell-sheet transplantation induced angiogenesis partially through the Jak/STAT signaling pathway[18] and that it prolonged cell survival by preventing apoptosis through PI-3K/Akt-mediated signaling, which is partially modulated by HGF.[21]

Although we emphasized combining SMBs with h-MSCs, some investigators have focused on different combinations of various cell sources. Sekine *et al.*[22] reported that cardiomyocytes co-cultured with endothelial cells induce greater numbers of capillaries, due to increased secretion of angiogenic growth factors.[22] Another report showed that a dermal fibroblast sheet co-cultured with endothelial progenitor cells was more effective than either single cell-type sheet for improving damaged heart function, accompanied by the inhibition of fibrotic tissue formation and the acceleration of neovascularization in the infarcted myocardium.[23] Thus, the paracrine effect may be improved by combining different cell sources; however, further investigation focused on determining the optimal combinations of cell sources is needed.

Regarding h-MSCs as a cell source, bone marrow-derived or adipose tissue-derived stem cells are reported to differentiate into mature endothelial cells and participate in blood vessel formation in the recipient heart.[24] The presence of endothelial capillary networks improves the survival and organization of implanted cells by maintaining a minimum intercapillary distance to provide oxygen and nutrients. Therefore, the presence of endothelial capillary networks may be partially correlated with cardiac function.

For future tissue engineering for cardiac therapy, the creation of thick cell-dense constructs with functional vessels may be essential. Capillary formation occurs via two basic vessel-constructing processes: angiogenesis, that is, the formation of new capillaries via sprouting or intussusception from pre-existing vessels, and vasculogenesis, which occurs in the developing embryo.[25] Here, the morphology of the vessel formation within myocardial tissues, including the diameter, composition, and fragility of vessel walls, suggested that improper vascularization may occur under pathological conditions. It is likely that not only biological factors but also physical stimuli such as flow and shear stress are required to mimic the *in vivo* environment and enable the formation of mature vascular networks.

A potential limitation of this study is that the exact number of transplanted cells was different in each group *in vivo*. Clinically, open-chest surgery is unlikely to gain easy acceptance except in certain situations; however, less invasive methods (e.g., intracoronary catheter-based procedures) might be technically difficult for carefully placing the cell sheets. Additionally, further studies that include longer timeframe than 8 weeks are needed to examine a longer term restoration of heart function post-MI. It is likely that the source of HGF is the transplanted SMB; however, it is unclear whether the source of other therapeutic cytokines is the transplanted cells, such as SMBs, MSCs, or both, or native cardiac cells.

In conclusion, we found that h-MSCs enhanced the paracrine effects of r-SMB sheets, thus enhancing angiogenesis, lowering fibrosis, inhibiting cellular hypertrophy, improving cardiac function, and prolonging cell survival in MI model rats. These observations of improved effects from this co-cultured cell sheet may lead to new regeneration therapies for heart failure following advanced cardiomyopathy that are superior to the conventional SMB-only cell-sheet technique.

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Disclosure Statement

T.S. is a consultant for CellSeed, Inc. T.O. is an Advisory Board Member in CellSeed, Inc., and the inventor/developer designated on the patent for temperature-responsive culture surfaces.

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ORIGINAL ARTICLE

Impact of cardiac stem cell sheet transplantation on myocardial infarction

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Abstract
Purpose Myocardial infarction (MI) remains a major cause of mortality because of the limited regenerative capacity of the myocardium. Transplantation of somatic tissue-derived cells into the heart has been shown to enhance the endogenous healing process, but the magnitude of its therapeutic effects is dependent upon the cell-source or cell-delivery method. We investigated the therapeutic effects of C-Kit positive cardiac cell (CSC) cell-sheet transplantation therapy in a rat model of MI.
Methods and results CSCs of human origin were sorted and cultured to generate scaffold-free CSC cell-sheets. One-layered or 3-layered cell-sheets were transplanted into nude rats 1 h after left coronary artery ligation. We observed a significant increase in the left ventricular ejection fraction and a significant decrease in left ventricular systolic dimension at 2 and 4 weeks in the 3-layer group, but not in the 1-layer or sham groups. Consistently, there was less accumulation of interstitial fibrosis in the 3-layer group than in the 1-layer or sham groups. Moreover, capillary density was significantly greater in the 3-layer group than in the 1-layer or sham groups.
Conclusions The 3-layered cell-sheet improved cardiac function associated with angiogenic and anti-fibrotic effects. Thus, CSC is a promising cell-source to use with the cell-sheet method for the treatment of cardiac failure, as long as a sufficient number of cells are delivered.

Keywords Cardiac · Stem cell · Myocardial infarction

Introduction

The limited regenerative capacity of the myocardium accounts for the fact that cardiac failure related to myocardial infarction (MI) remains a major cause of morbidity and mortality worldwide, despite major advances in medical and/or interventional treatments [1]. The treatment of cardiac failure relies on strategies that are designed to target and/or limit residual or persistent myocardial ischemia, additional myocardial damage, pathological cardiac remodeling, and hemodynamic impairment, including cardiac dyssynchrony [2]. On the other hand, the transplantation of somatic tissue-derived stem/progenitor cells into the heart has been shown to enhance the endogenous healing process of the damaged myocardium, while the magnitude of the therapeutic effects are dependent on the cell-source, cell-number, cell-delivery method, and target cardiac pathology [3–5]. It has been shown that the transplantation of C-kit-positive heart-derived cells into the MI heart yields functional recovery, mediated by proliferation and differentiation into the heart-composing cells in situ, and by releasing cardioprotective factors that activate native healing processes [6]. However, the optimal preparation and delivery method of CSCs into the heart has not been established.

The cell-sheet method, in which aggregated cells in a sheet shape cultured under a thermoresponsive dish are attached to the epicardial surface [7], has been shown to deliver a large scale of cultured cells with minimal damage to the cells or native cardiac tissues [8]. This enhances its therapeutic effects and minimizes inflammation-related

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complications, representing a promising cell-delivery method in CSC transplantation therapy [9]. However, there are concerns about potential ischemia of the implanted cell-sheet, which would limit cellular function, survival, and therapeutic potential. According to a previous study, a 3-layered cell-sheet generated by skeletal myoblasts showed greater therapeutic effects than a 1-layered cell-sheet, while a 5-layered cell-sheet did not enhance the effects, possibly because of ischemia in the implanted cell-sheet [10]. Based on the hypothesis that the therapeutic potential of CSC cell-sheet treatment might be dependent on the number of layers of the cell-sheet, we investigated the therapeutic effects of CSC cell-sheet transplantation therapy on MI hearts using a rat model.

Methods

All studies using human tissues and experimental animals were carried out under approval of the institutional ethical committee. Human tissues were collected only after obtaining written informed consent. This investigation conforms to 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 (US National Institutes of Health Publication No. 85-23, revised 1996). All experimental procedures and evaluations were carried out in a blinded manner.

Isolation and culture of C-Kit-positive human cardiac cells and cell-sheet generation

Discarded cardiac tissue samples were taken from the left ventricular apex of a 31-year-old man with dilated cardiomyopathy, requiring daily cardiovascular procedures in Osaka University Hospital. Cardiac cells were dissociated from the tissues, cultured, and then sorted for C-kit using FACSAria (BD Biosciences) to yield C-Kit positive cardiac cells, which were then cultured for expansion with multiple passages. The cells were then incubated in thermoresponsive dishes (35 mm UpCell, CellSeed, Tokyo, Japan) at 37 °C for 2 days prior to transplantation, when the cells were incubated at 25 °C to induce their spontaneous detachment, to yield a mono-layered scaffold-free CSC cell-sheet that included 1.5×10^6 cells (Fig. 1a). The 3-layered cell-sheet was generated by filling up the mono-layered cell-sheet, as described previously [10].

Generation of AMI model and CSC cell-sheet transplantation

Thirty-nine athymic female nude rats, 8 weeks of age, were subjected to permanent ligation of the left coronary artery (LCA) under general anesthesia with endotracheal intubation and isoflurane inhalation, as previously described [10]. LCA ligation-related death occurred prior to treatment in 16 %. The rats that survived for 50 min after the ligation were randomly assigned to the following three treatment groups: transplantation of a 3-layered cell-sheet ($n = 12$), transplantation of a l-layered cell-sheet ($n = 10$), or a sham operation ($n = 11$). In the two transplantation groups, the cell-sheet was attached directly to the epicardial surface of the ischemic/infarct area (Fig. 1b) [10]. The cell-sheet was large enough to cover all of the ischemic or infarcted area. By 20 min after the transplantation, when the cell-sheets were properly fixed to the cardiac surface, the chest was closed and the rats were allowed to recover in individual temperature-controlled cages until they were killed 28 days after the treatment.

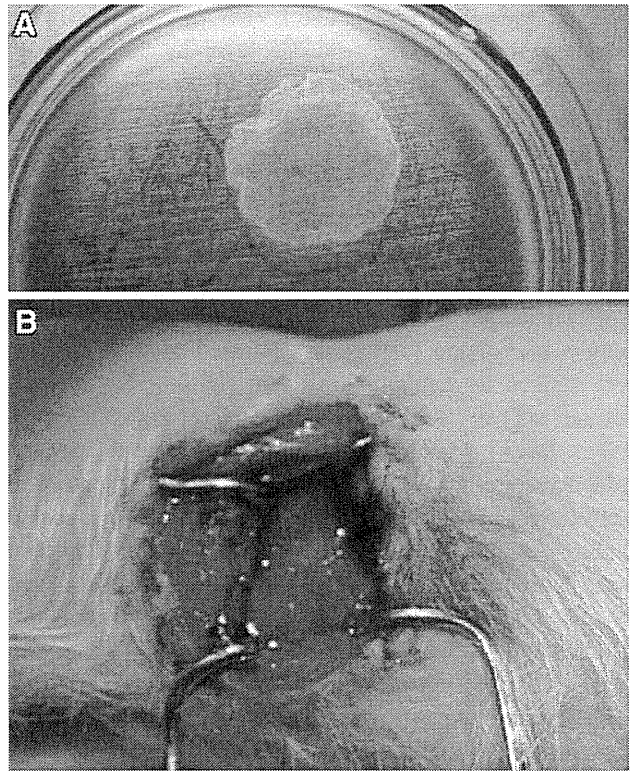

Fig. 1 A mono-layered cell-sheet was generated by c-kit positive cardiac cells of human origin on thermoresponsive dishes in vitro (**a**). A mono-layered or 3-layered cell-sheet was transplanted over the left ventricular free wall of the rat heart, which had been subjected to ischemia by permanent ligation of the corresponding coronary artery, 1 h prior to the treatment (**b**)

Transthoracic echocardiography

Transthoracic echocardiography was performed under isoflurane inhalation, using a system equipped with a 12 MHZ transducer (GE Healthcare). Diastolic and systolic dimensions of the left ventricular diastolic and

systolic dimensions (LVDD and LVDS, respectively) were measured at the papillary muscle level by the M-mode, while the LV ejection fraction (LVEF) was calculated by the following formula: $(LVDD^3–LVDS^3)/LVDd^3 \times 100$ [10, 11].

Histology

The ventricles were immerse-fixed in 4 % paraformaldehyde, embedded in paraffin, and cut into 5 micrometres using a microtome for histological studies. The sections were stained by hematoxylin–eosin (HE) or Masson trichrome (MT) and assessed by optical microscopy (Olympus, Tokyo, Japan). Metamorph software was used to separate stained and non-stained myocardium by MT staining and to quantitatively calculate each area. The sections were labeled immunohistologically by polyclonal anti-von Willebrand factor antibody (vWF, DAKO, Glostrup, Denmark), and visualized by the LSABTM kit (DAKO), which is an automated immunostaining system based on the LSAB Lepto strept avidin–biotin-peroxidase method. The sections were labeled immunohistologically by the anti-human-specific HLA antibody or anti-cardiac troponin (cTn) I antibody, visualized by corresponding secondary antibodies that were counterstained by DAPI, and assessed by confocal microscopy (Olympus).

Statistics

Values are expressed as mean ± SEM. The three groups were compared with 1-way or 2-way ANOVA as appropriate, followed by the Fisher protected least-significant difference test, or the Kruskal–Wallis test, followed by the post hoc pairwise Wilcoxon-Mann–Whitney *U* test, as appropriate. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA).

Results

Functional recovery following CSC cell-sheet transplantation

Scaffold-free CSC cell-sheet was prepared from primary C-kit positive cardiac cells of human origin, cultivated in thermoresponsive dishes. We transplanted the 1-layered or 3-layered cell-sheets onto the epicardial surface of the nude rat 1 h after the permanent LCA ligation. A sham operation was performed for the control group. Cardiac performance was serially assessed by transthoracic echocardiography just after the treatment (baseline), and then 1, 2, and 4 weeks after the treatment.

Before any intervention, the LVEF, LVDD, and LVDS did not differ significantly among the groups (Fig. 2). However, for 4 weeks after treatment, the LVEF showed a significantly progressive reduction, while the LVDD and LVDS showed a significantly progressive increase in the sham group and the 1-layer group. Conversely, in the 3-layer group, the LVEF showed a significant increase, and the LVDS showed a significant decrease 2 and 4 weeks following the transplantation, while the LVDD did not change significantly in this group over the 4 weeks. Notably, the LVEF in the 3-layer group was significantly greater than that in the 1-layer group or sham group, while the LVDS in the 3-layer group was significantly lower than that in the 1-layer group or sham group. The LVDD did not differ significantly among the groups at any time.

Histological reverse LV remodeling following CSC cell-sheet transplantation

We assessed gross structure, interstitial fibrosis and capillary distribution in the myocardium 4 weeks after the CSC cell-sheet transplantation to qualitatively and semi-quantitatively explore the degree of LV remodeling in each group by HE staining, Masson Trichrome staining, and immunohistolabelling for von Willebrand factor, respectively. The infarcted area, in which the cell-sheet was transplanted, was clearly thicker in the 3-layer group than in the 1-layer or sham groups, as assessed by the HE staining (Fig. 3a–c). In addition, the myocardial structure in the peri-infarcted area was better preserved in the 3-layer group than in the 1-layer group or the sham group. There seemed to be less accumulation of interstitial fibrosis in the peri-infarcted and infarct-remote myocardium of the 3-layer group than in the 1-layer group or sham groups (Fig. 3d–f). In fact, computer-based morphometry confirmed significantly less fibrosis in the 3-layer groups than in the 1-layer group or sham group (Fig. 4a). Capillary density in the peri-infarcted myocardium was significantly greater in the 3-layer group than in the 1-layer group or sham group (Fig. 4b).

Phenotypic fate of the transplanted CSCs in the heart

The transplanted CSCs in the heart were phenotypically assessed by immunohistolabelling for human-specific HLA, which clearly dissected the transplanted cells in the native cardiac tissue. While the transplanted cells were rarely present in the 1-layer group 4 weeks after transplantation, the 3-layer group showed abundant human-specific HLA-positive transplanted cells in the tissues epicardially attached to the native cardiac tissue, which were assumed to consist of the remaining transplanted cell sheet and accumulated cells of native origin (Fig. 5a).

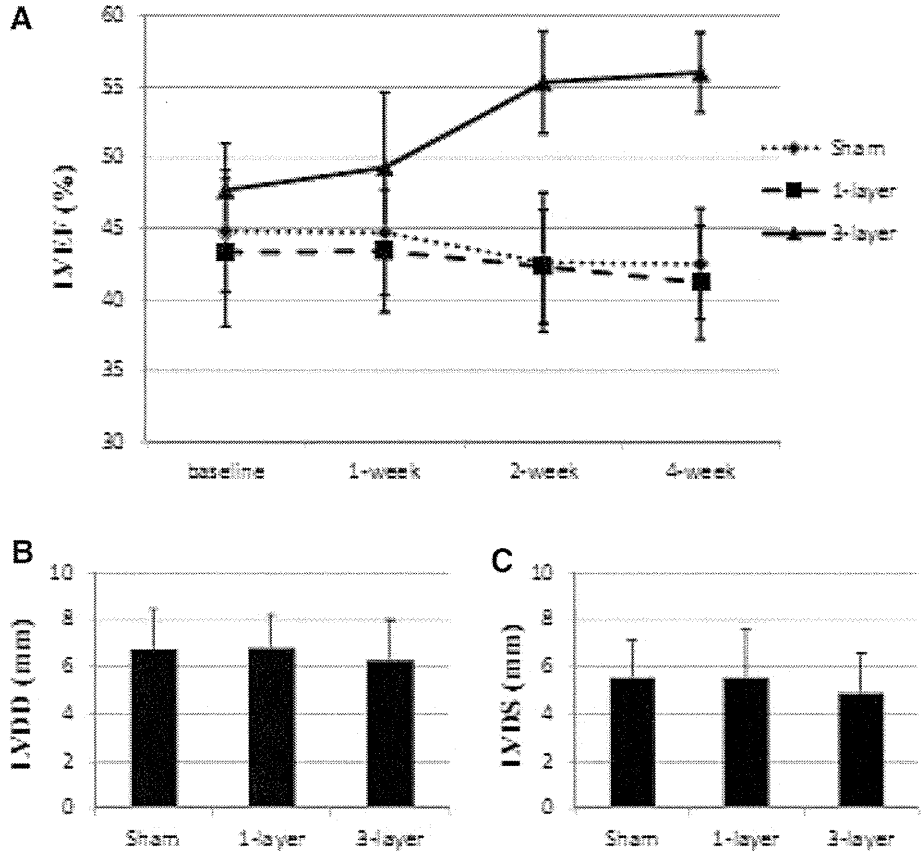

Fig. 2 Cardiac performance measures, such as left ventricular ejection fraction (LVEF) (**a**), LV diastolic dimension (LVDD, **b**), and LV systolic dimension (LVDS, **c**), were assessed echocardiographically immediately after treatment and then 1, 2, and 4 weeks after treatment (sham operation vs. 1-layer cell-sheet transplantation vs. 3-layer cell-sheet transplantation)

Notably, some human-specific HLA-positive transplanted cells were present in the native myocardium, suggesting the migration of transplanted cells into the native cardiac tissue (Fig. 5b–d).

Discussion

This study demonstrated clearly that the transplantation of CSC cell-sheets to treat the MI heart yielded significant recovery of cardiac performance in a cell-sheet layer dependent manner. Consistently, the hearts transplanted with the multi-layered cell-sheet showed significantly more preserved gross myocardial structure, reduced interstitial fibrosis, and increased capillary density than the hearts transplanted with a mono-layered cell-sheet. Moreover, the differentiation of heart-composing cells, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells, was greater in the hearts transplanted with the multi-layered cell-sheet than in those transplanted with the mono-layered cell-sheet.

The transplanted cell-source is known to be a major determinant of the therapeutic effects of cell transplantation therapy for cardiac failure [10–12]. The transplantation of skeletal myoblast transplantation predominates anti-fibrotic effects, whereas that of bone marrow-derived cell transplantation predominates neoangiogenesis in the ischemic/infarcted myocardium. These effects are mediated by indirect effects, in which cell transplantation upregulates a variety of cardioprotective factors to enhance the native healing process, although differentiation of the transplanted cells into the functional heart-composing cells, such as cardiomyocytes or vascular cells rarely occur following the transplantation of skeletal myoblasts or bone marrow-derived cells [13, 14]. In contrast, the transplantation of CSCs has been shown to yield therapeutic effects both directly and indirectly [15, 16]. This study showed that the transplantation of CSCs induced both anti-fibrotic and neoangiogenic effects in a transplanted cell number-dependent manner, indicating that CSCs might have released soluble factors to activate the anti-fibrotic and angiogenic process of the native myocardium following the transplantation. Moreover, the differentiation into the cardiomyocytes and vascular cells, shown in this study, suggests potential direct contribution of these cells to functional recovery, although the magnitude of these direct effects on the global cardiac function remains unclear.

The number of transplanted cells is also an important contributor to the therapeutic effects. Although the cell-sheet method has been shown to deliver more cells into the heart than other delivery methods, such as intramyocardial or intracoronary injection [10], ischemia in the transplanted