Adipose stem cell sheets improved cardiac function in the rat myocardial infarction, but did not alter cardiac contractile responses to β-adrenergic stimulation

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ABSTRACT

Adipose stem cells (ASCs) are a source of regenerative cells available for autologous transplantation to hearts. We compared protective actions of ASC sheets on rat myocardial infarction (MI) in comparison with those of skeletal myoblast cell sheets. Their effects on infarcted hearts were evaluated by biological, histochemical as well as physiological analyses. ASC sheets secreted higher concentrations of angiogenic factors (HGF, VEGF, and bFGF; $P < 0.05$) under normoxic and hypoxic conditions than those of myoblast cell sheets, associated with reduction of cell apoptosis ($P < 0.05$). Like myoblast cell sheets, ASC sheets improved cardiac function ($P < 0.05$) and decreased the plasma level of ANP ($P < 0.05$) in MI hearts. ASC sheets restored cardiac remodeling characterized by fibrosis, cardiac hypertrophy and impaired angiogenesis ($P < 0.05$), which was associated with increases in angiogenic factors ($P < 0.05$). In isolated perfused rat hearts, ASC sheets improved both systolic and diastolic functions, which was comparable to cardiac functions of myoblast cell sheets, while both cell sheets failed to restore cardiac contractile response to either isoproterenol, pimobendan or dibutyryl cAMP. These results indicated that ASC sheets improved cardiac function and remodeling of MI hearts mediated by their paracrine action and this improvement was comparable to those by myoblast cell sheets.

After myocardial infarction (MI), impaired angiogenesis induces cardiac remodeling characterized by cardiac hypertrophy and fibrosis (5). Dysregulation of cardiac β-adrenergic signaling worsens cardiac remodeling and causes terminal heart failure (5). Although left ventricular assist devices or heart transplantation after myocardial infarction (MI) are available for treating patients with terminal heart failure, these therapeutic strategies are limited to a small number of patients and are accompanied with serious complications. Cell-based regenerative medicine could improve blood supply to the damaged heart, and minimize both the area of infarction and cardiac remodeling through secretion of several cytokines such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF). However,

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there have been several adverse effects on injected cells such as loss of living cells (2) and arrhythmogenesis (7). Tissue engineering using a cell sheet has been developed to overcome these disadvantages (18). The cell sheet could improve viability of cells and prolong secretion of several cytokines (8). At first, skeletal myoblast cell sheets have been reported to improve the cardiac function in patients with severe heart failure (16). However, collecting skeletal myoblasts from patients requires invasive procedures, and it takes a long time to obtain a sufficient number of myoblasts in culture. Thus, an alternative source of stem cells is desirable.

Adipose stem cells (ASCs) are an abundant source of regenerative cells for autologous cell-transplantation (22). Furthermore, ASCs secrete multiple angiogenic growth factors that promote neovascularization (15), and ASCs transplanted into an ischemic site improve blood perfusion with increases in tissue capillary density (9). Thus, ASC sheets may be useful for restoration of cardiac function and remodeling of MI hearts to replace myoblast cell sheets (2), but it has never tested whether ASC sheets could improve cardiac function, remodeling, and dysregulation of cardiac β-adrenergic signaling of MI hearts in comparison with myoblast cell sheets. In the present report, we studied effects of ASC sheets on rat MI hearts and compared with those of myoblast cell sheets. We found that ASC sheets could improve cardiac function associated with cardiac remodeling mediated by angiogenic factors like myoblast cell sheets. However, ASC sheets as well as myoblast cell sheets did not improve the cardiac contractile response to β-adrenergic stimulation.

MATERIALS AND METHODS

In vitro study
Engineering of ASC- and myoblast cell-sheets. ASCs were enzymatically isolated from the inguinal subcutaneous fat tissue of rats and cultured as previously described (3). Skeletal myoblasts were isolated from the skeletal muscle of the thigh as described previously (17). To prepare cell sheets, ASCs (2–3 passage) and myoblasts were cultured on 35-mm temperature-responsive culture dishes ($2 \times 10^5/\text{cm}^2$) (UpCell; Cell Seed Inc., Tokyo, Japan) in 37°C incubator. After 24 h, the cultured cells were maintained at 20°C for one hour to release the cultured cells as intact sheets.

Real-time RT-PCR analysis. Cell sheets were incubated under normal (37°C, 5% CO_2) or hypoxic

conditions ($< 2\%$ O_2) for 48 h. Hypoxic conditions were prepared using a Gaspak system (Becton Dickinson, Bedford, MA). Total RNA was extracted from ASC or myoblast cell sheet using RNeasy Mini kit (QIAGEN inc., Valencia, CA). Real-time RT-PCR analysis of HGF, VEGF, basic-fibroblast growth factor (bFGF), and β-actin was performed using 1 μg total RNA employing ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA levels were expressed relative to the levels of β-actin. The primers were designed to span their genomic regions described elsewhere (3). Same condition of RT-PCR was used to quantify mRNA of heart tissues as described in *in vivo* experiments.

Assay of angiogenic factors. Protein levels of angiogenic factors in the culture medium of cell sheets were measured with Enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D, Minneapolis, MN) for HGF, VEGF, and bFGF.

Apoptosis assay. ASC sheet and myoblast cell sheet were incubated under hypoxic conditions for 48 h. Cells were stained by Annexin V-FITC Kit (Miltenyi Biotec Inc., Auburn, CA) and Annexin V-positive cells were measured by flow cytometry.

In vivo study
Transplantation of cell sheets to myocardial infarction model. Adult male syngeneic Lewis rats (200 to 250 g) were obtained from Japan SLC, Inc (Hamamatsu, Japan), which served as cell donors and recipients. The experimental protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Tottori University. Acute MI of rat was created by ligation of the left anterior descending artery as previously described (6). Seven days after creation of acute MI, rats were randomly divided into 3 treatment groups: 1) ASC sheet transplantation (ASC group, n = 6); 2) myoblast cell sheet transplantation (Myoblast group, n = 6); 3) no cell sheet transplantation (control group, n = 6). The cell sheet was applied to the surface of the infarcted area as shown in Fig. S1. Control group underwent the same operative procedures without a cell sheet. To trace cells in the transplanted cell sheets, in some experiments, monolayer cell sheets were stained with PKH26 red fluorescent cell linker kit (Sigma-Aldrich Co., St. Louis, MO) for 5 min before cell seeding.

For quantitative analysis on mRNA of infarcted hearts, mRNA of cardiac tissue in the border zone of myocardium and scar at 4 weeks after transplan-

tation was extracted and subjected to RT-PCR.

Plasma ANP levels. Blood samples were obtained from the femoral vein before and 4 weeks after cell sheet transplantation. Plasma ANP was measured by Rat ANP ELISA Kit (Assaypro, Saint Charles, MO).

Echocardiogram. Cardiac function was evaluated with echocardiography at 6 days after MI (baseline) and 2, 4 weeks after cell sheet transplantation using a 12-MHz transducer (LOGIQ P5J and 12L; GE Healthcare, Fairfield, CT). Short-axis two-dimensional images at the mid-papillary level of the left ventricle were stored as digital loops, and the end-systolic and end-diastolic cavity areas were determined by tracing the endocardia borders to calculate the fractional area shortening. Left ventricular internal diameters at end-systole and end-diastole were measured between the mitral valve and papillary muscles to calculate ejection fraction. Anterior wall thickness was measured at end-diastole phase. All measurements were made in triplicate and averaged by two independent experienced examiners in a blinded fashion.

Measurement of cardiac function in isolated perfused hearts. Rat hearts were isolated and applied to Langendorff apparatus. The left ventricle (LV) pressure, LV end-diastolic pressure, LV dP/dt_{max}, LV dP/dt_{min}, and E_{max} (slope of the end-systolic pressure-volume relation) were measured (14). To estimate the effects of β-adrenergic stimulation, the following agents were used; isoproterenol hydrochloride (TOKYO CHEMICAL INDUSTRY Co., LTD, Tokyo, Japan), pimobendan (Abcam plc, Cambridge, UK), and dibutyryl cyclic-AMP (Santa Cruz Biotechnology, Inc., Texas) were purchased.

Pathological study. At 4 weeks after transplantation, hearts were dissected, and embedded in either paraffin after 10% formalin fixation or OCT compound (Tissue-Tek; Sakura Finetek Japan Co., Ltd, Tokyo, Japan) for frozen sections. The paraffin sections were used for staining with hematoxylin-eosin, Picrosirius-red, and Periodic acid-Schiff (PAS). Picrosirius-red stain was performed to detect interstitial fibrosis, and PAS stain was performed to assess cardiac hypertrophy in the remote area (4). Transverse sections were obtained from lateral wall, posterior wall, and septum. Three randomly selected fields per section (n = 9 per animal) were analyzed by microscope (Eclipse-TE200; Nikon, Tokyo, Japan). Each field was scanned and digital images were an-alyzed by software (Adobe Photoshop and ImageJ). The frozen sections were stained with an antibody against von Willebrand Factor (Abcam plc) to detect capillary vessels in the border zone between myocardium and scar. Five randomly selected fields of transverse sections per animal were analyzed. The number of vessels was counted by light microscopy. The number of capillary vessels in each field was averaged and expressed as the capillary density.

To evaluate the tropomyosin-positive cardiac myocytes, fixed hearts were stained with anti-tropomyosin mouse monoclonal antibody (clone CH1, Sigma). Alexa Flulor 546-labeled goat anti-mouse IgG (Molecular Probes) was used as secondary antibodies.

Data analysis
All data are expressed as mean ± SE. An unpaired Student t test was performed to compare 2 groups. One-way analysis of variance (ANOVA) was used for multiple group comparisons. A probability value of < 0.05 was considered significant.

RESULTS

ASC sheets produced more angiogenic factors than myoblast cell sheets under normoxic and hypoxic conditions to reduce apoptosis
Fig. 1A shows mRNA levels of angiogenic factors in ASC and myoblast cell sheets under normoxic and hypoxic condition. mRNA levels of HGF, VEGF, and bFGF were significantly higher in ASC sheets than those in myoblast cell sheets under both conditions. Fig. 1B shows concentrations of angiogenic factors in the culture media of ASC and myoblast cell sheets under normoxic and hypoxic condition. Under normoxic condition, the protein levels of HGF and VEGF were significantly higher in the culture medium of ASC sheets than those of myoblast cell sheets, while there was no significant difference in the protein levels of bFGF between them. Under hypoxic conditions, the protein levels of HGF, VEGF, and bFGF were significantly higher in the culture medium of ASC sheets than those of myoblast cell sheets. The number of annexin V-positive apoptotic cells was significantly less in ASC sheets (n = 6; 2.3 ± 0.2%, $P < 0.05$) than those in myoblast cell sheets (n = 6; 5.8 ± 0.8%) as shown in Fig. S2.

Improvement of cardiac function and remodeling by ASC is comparable to myoblast cell sheets
Fig. 2A shows effects of ASC and myoblast cell sheets on fractional area shortening of MI hearts at 2 and 4 weeks after their transplantation. Both
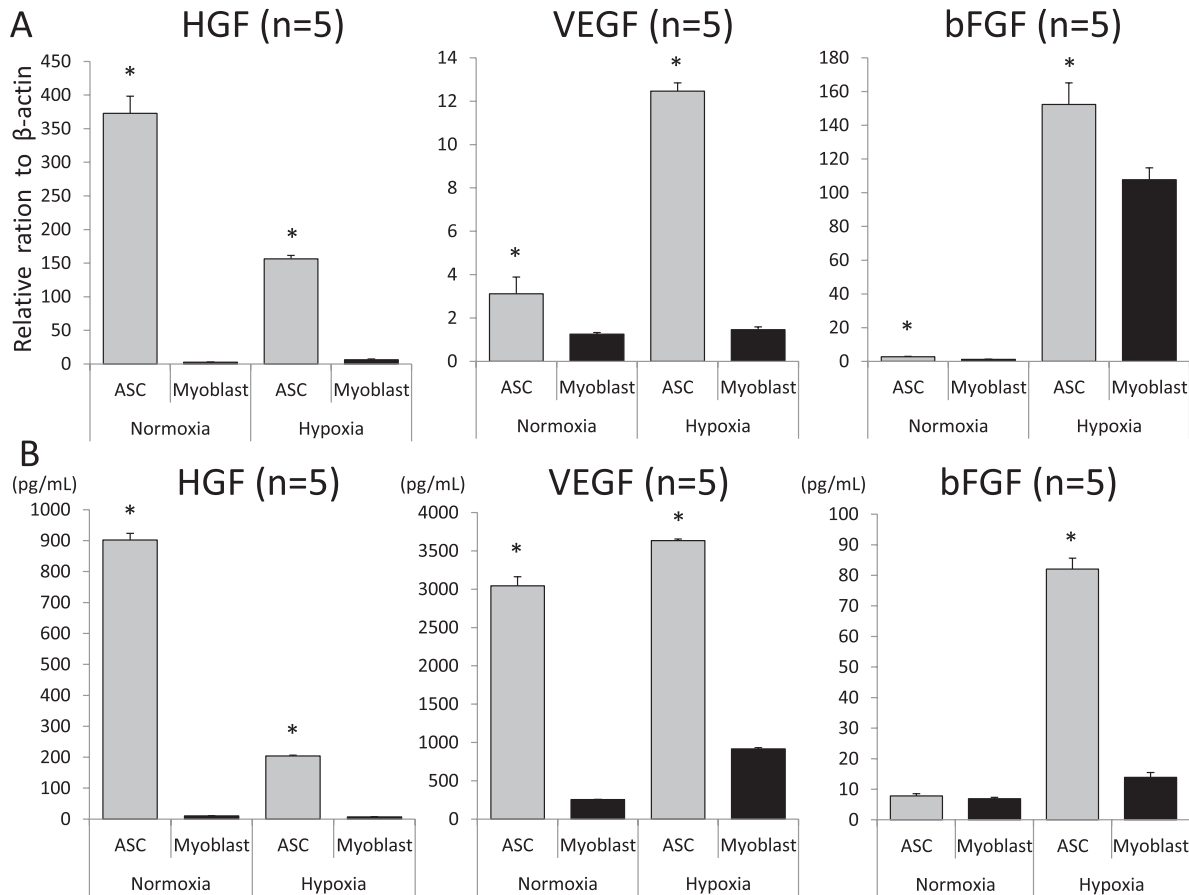
A

B

Fig. 1 Comparison of expression levels of angiogenic factors between ASC and myoblast cell sheets under normoxia and hypoxia conditions. **A** Comparison of mRNA levels of angiogenic factors between ASC and myoblast cell sheets under normoxia and hypoxia conditions. Semi-quantitative RT-PCR analysis of HGF, VEGF, and bFGF mRNA expression levels, which are expressed as the ratio to β-actin mRNA for copy number of each mRNA. *, $P < 0.05$ ASCs vs. myoblasts. **B** Comparison of protein levels of secreted angiogenic factors between ASC and myoblast cell sheets under normoxia and hypoxia conditions. Concentrations of HGF, VEGF, and bFGF in culture medium were measured by ELISA assay. *, $P < 0.05$ ASC sheet vs. myoblast sheet.

sheets significantly improved their values on 2 and 4 weeks after their transplantation. Both the ejection fraction and anterior wall thickness had the same trend as the fractional area shortening as shown in Fig. 2B and C. Two weeks after cell sheet transplantation, ASC and myoblast cell sheets were confirmed to exist on the infarcted areas as layers (Fig. S3). Along with the improvement of cardiac function, the plasma level of ANP was significantly reduced by transplantation of either sheet after 4 weeks (Fig. 2D). Tropomyosin-positive cardiac cells were not observed in ASC sheets on MI heart but were detected in the intact region of MI heart, excluding the possibility of cardiac differentiation of ASCs (data not shown). Fig. 3A shows effects of ASC and myoblast cell sheets on fibrosis in the remote zone. ASC and myoblast cell sheets signifi-

cantly improved cardiac fibrosis after 4 weeks in comparison with control group, while there were not significant differences between two transplantation groups. Fig. 3B shows effects of cell sheets on the diameter of cardiomyocytes in the remote zone of MI hearts. ASC and myoblast cell sheets attenuated cardiac hypertrophy after 4 weeks in comparison with control group, while there were not significant differences between them. Fig. 3C shows effects of cell sheets on the capillary density in the border zone of MI. ASC and myoblast cell sheets significantly increased the capillary density of MI hearts after 4 weeks in comparison with control group, while there were not significant differences between them. Fig. 3D shows effects of cell sheets on production of angiogenic factors at the border zone of MI hearts. ASC cell sheets significantly increased
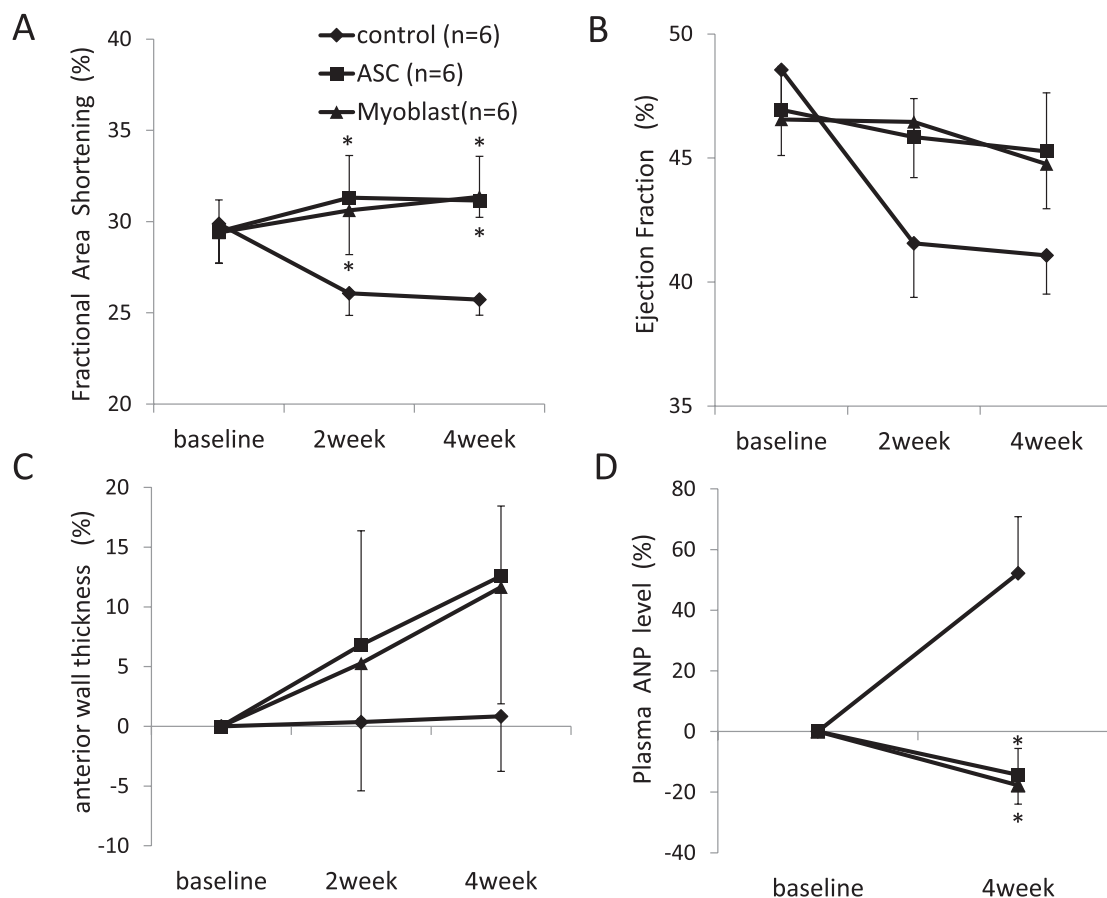
Fig. 2 Comparison of cardiac function and plasma ANP of MI heart transplanted with ASC or myoblast cell sheets to those without cell sheets. **A–C** Fractional area shortening, ejection fraction and anterior wall thickness of MI heart transplanted with ASC sheets or myoblast cell sheets are compared to untreated heart at baseline, 2 and 4 weeks after transplantation (n = 6, each group). * denotes $P < 0.05$ vs. control. **D** Changes in plasma ANP levels of rats with MI hearts transplanted with ASC sheets or myoblast cell sheets are compared to control rats at baseline and 4 weeks after cell sheet transplantation. (n = 6, each group). * denotes $P < 0.05$ vs. control

mRNA levels of both HGF and VEGF with trend of increases in bFGF after 4 weeks in comparison with control group, although myoblast cell sheets significantly increased HGF alone.

ASC and myoblast cell sheets improved cardiac function but failed to alter cardiac contractile responses to β-adrenergic stimulation of MI hearts ex-vivo

Fig. 4A shows effects of transplantation of ASC and myoblast cell sheets on the parameters of cardiac function of MI hearts evaluated by Langendorff techniques. Both cell sheets significantly improved systolic cardiac function expressed by LV dP/dt_{max} and E_{max} as well as diastolic cardiac function expressed by LV dP/dt_{min} without changes in heart rate (HR) in comparison with control group. Fig. 4B showed effects of either β-adrenergic stimulation treated with isoproterenol, pimobendan or dibutyryl

c-AMP on the HR, systolic blood pressure (sBP), dP/dt_{max} and dP/dt_{min} in MI hearts transplanted with ASC (ASC group) or myoblast cell sheets (Myoblast group) as well as in MI hearts without cell sheets (control group), comparing with those of sham operation (sham group). In sham group, either isoproterenol (1 μM), pimobendan (1 μM) or dibutyryl c-AMP (200 μM) significantly increased all of HR, sBP, dP/dt_{max}, and dP/dt_{min}. MI hearts in control group showed the significantly impaired responses of all of HR, sBP, dP/dt_{max}, and dP/dt_{min} to treatment with either isoproterenol, pimobendan or dibutyryl c-AMP in comparison with those of sham group. Like control group, either ASC or myoblast cell sheet group did not improve the response of HR, sBP, dP/dt_{max}, and dP/dt_{min} to treatment with either isoproterenol, pimobendan, or dibutyryl c-AMP.

A Interstitial fibrosis

B Cell diameter

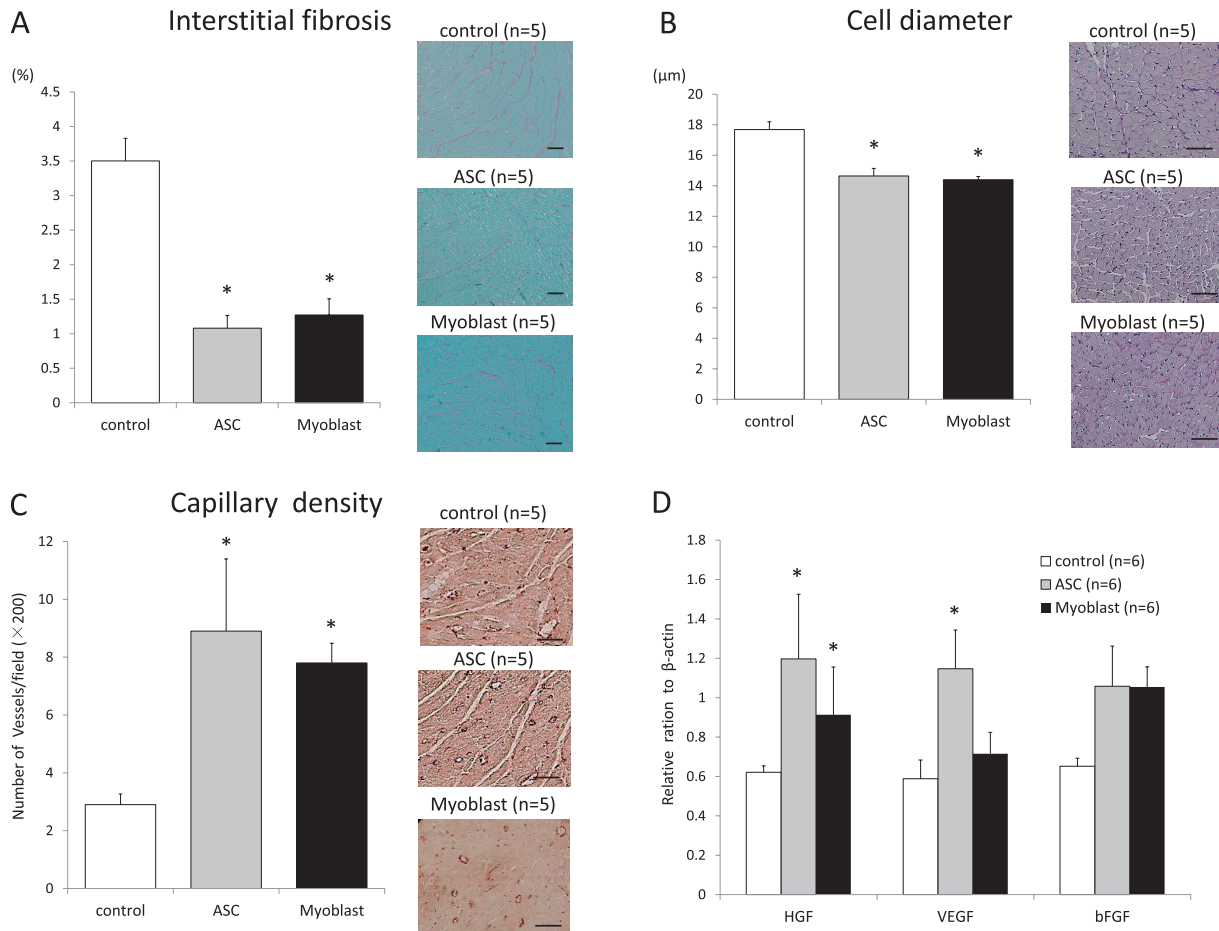**C** Capillary density

D

Fig. 3 Comparison of cardiac remodeling parameters in MI hearts transplanted with ASC sheets or myoblast cell sheets to those of untreated hearts. **A** The extent of interstitial fibrosis in the remote zone of MI hearts transplanted with ASC sheets or myoblast cell sheets comparing to untreated hearts. Summary of the prevalence of the interstitial fibrosis in the remote zone of MI hearts (n = 5 independent experiments). Insets: Representative pictures of the interstitial fibrosis in remote zone of MI hearts transplanted with either ASC sheets, myoblast cell sheets or without cell sheets (control). Picrosirius-red stain was performed to detect interstitial fibrosis. Scale bar = 100 μm. **B** The cardiac cell diameter in remote zones of MI hearts transplanted with ASC sheets or myoblast cell sheets comparing to untreated hearts. Summary data on the diameter of the cardiac myocytes in the remote zone of MI hearts (n = 5 independent experiments). Insets: Representative pictures of cardiomyocytes in the remote zones of MI hearts transplanted with either ASC sheets, myoblast cell sheets or without cell sheets. PAS stain was performed to assess cardiac hypertrophy in the remote area. Scale bar = 100 μm. **C** The capillary density in border zones of MI hearts transplanted with ASC sheets or myoblast cell sheets comparing to untreated hearts. Summary of the numbers of vWF-positive vessels in border zone of myocardium in MI hearts (n = 5 independent experiments). The average of number of capillary vessels stained with an antibody against vWF was expressed as the capillary density in 5 randomly selected fields per animal. Insets: Representative pictures of capillary vessels stained with an antibody against vWF in the border zone of myocardium in MI hearts transplanted with either ASC sheets, myoblast cell sheets or without cell sheets. Scale bar = 100 μm. **D** mRNA levels of angiogenic factors in border zone of MI hearts transplanted with ASC sheets or myoblast cell sheets comparing to untreated hearts (control). Real-time RT-PCR analysis of HGF, VEGF, and bFGF mRNA expression levels, which are expressed as the ratio to β-actin mRNA for copy numbers of each mRNA. *, $P < 0.05$ vs. control. Each data of three groups were obtained from 6 independent experiments.

DISCUSSION

ASC sheets improved cardiac function associated with reduction of ANP levels in MI, indicating their protective action on damaged hearts. This could be attributable to their suppression of cardiac remodel-ing after MI, since ASC sheets attenuated cardiac fibrosis as well as cardiac hypertrophy. We found that ASC sheets increased mRNA levels of VEGF and HGF in the border area of MI *in vivo*, which were associated with increases of capillary density in the MI hearts. VEGF is widely accepted as one of the
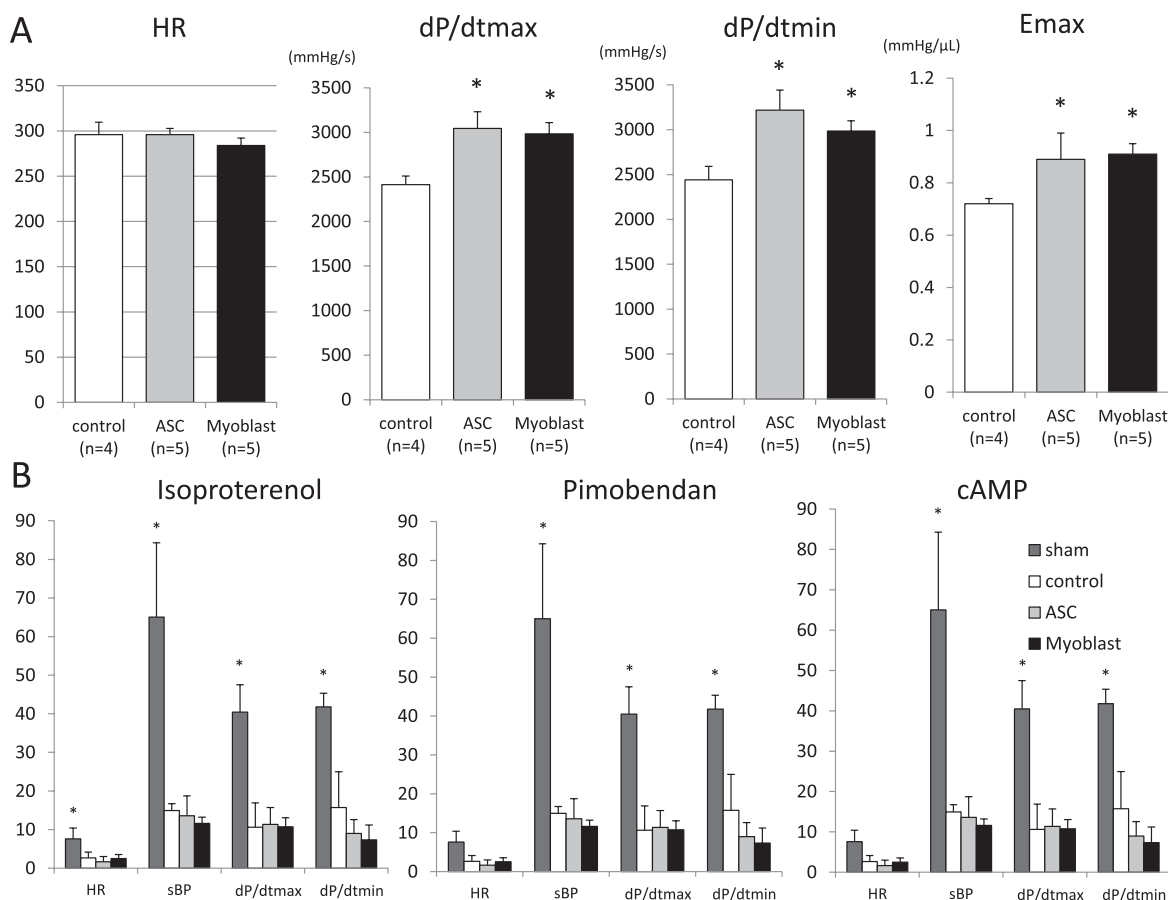
Fig. 4 Comparison of the parameters of cardiac systolic and diastolic functions of MI hearts transplanted with ASC sheets or myoblast cell sheets to untreated hearts estimated by Langendorff techniques in the absence and presence of β-adrenergic stimulants. **A** Comparison of heart rate (HR), dP/dt_{max}, dP/dt_{min} and E_{max} of MI hearts transplanted with ASC sheets or myoblast cell sheets to untreated hearts (control) (n = 5 independent experiments). * denotes $P < 0.05$ vs. control. **B** Effects of isoproterenol (1 μM), pimobendan (1 μM) and dibutyryl cAMP (200 μM) on HR, sBP, dP/dt_{max} and dP/dt_{min} of infarcted hearts transplanted with ASC sheets, myoblast cell sheets, or untreated hearts (control) in comparison with sham operated hearts (normal). (n = 5; each group). * denotes $P < 0.05$ vs. control.

critical factors that promote angiogenesis and inhibit cell apoptosis (11). HGF has been reported to enhance angiogenesis by stimulating migration and proliferation of endothelial cells and inhibiting their apoptosis (21). Cultured ASCs secreted both HGF and VEGF as reported previously (12). Both HGF and VEGF secreted by ASC sheets may play a pivotal role for angiogenesis and inhibition of cell apoptosis in MI hearts.

Interestingly, ASC sheets expressed significantly higher mRNA and protein levels of HGF, VEGF, and bFGF than myoblast cell sheets under hypoxic condition, associated with the less prevalence of cell apoptosis in ASC sheets than myoblast cell sheets. bFGF also augments neovascularization and inhibits cellular apoptosis (3, 21). Since preventing apoptosis of transplanted cells leads to improvement of

cell survival and ventricular function (13), ASC sheets were expected to have a higher tolerance for ischemia and hypoxia than myoblast cell sheets, when they were transplanted into MI hearts. However, in *in vivo* study, there were not any significant differences in restoration of cardiac function and cardiac remodeling between ASC and myoblast cell sheets, suggesting that secretion of higher concentration of angiogenic factors from ASC sheets might not overcome the benefit of myoblast cell sheets (20). Myoblast cell sheets have been reported to minimize myocardial injury and improve cardiac function (18) through the several mechanisms such as paracrine effects of cytokines, hematopoietic stem cell recruitment, relief of myocytes stretching, and formation of myotubules due to their differentiation (10). In the present study, paracrine effects of myo-

blast sheets were less than those of ASC sheets, thus relief of myocytes stretching and formation of myotubules due to their differentiation in myoblast cell sheets might compensate for the secretion of higher concentration of angiogenic factors in ASC sheets.

The most prominent findings were that ASC sheets improved systolic and diastolic functions of MI hearts under an *ex-vivo* condition like myoblast cell sheets. Since it has been reported that myoblasts maintain the potential of regenerating skeletal muscle during periods of stress, it suggests that myotubules could directly contribute to improvement of cardiac contraction (19). ASC sheets also improved systolic and diastolic functions of the MI hearts without their differentiation to cardiac myocytes. These results indicated that paracrine effects, especially, these of VEGF secreted from ASC sheets could be responsible for improvement of the cardiac contraction, since VEGF-dependent angiogenesis has been reported to contribute to cardiac contractile reserve after MI (13).

Unfortunately, ASC as well as myoblast cell sheets failed to restore cardiac contractile responses to β-adrenergic stimulation. Cardiac remodeling and the heart failure lead to β-adrenoceptor down-regulation as well as desensitization/uncoupling of β-adrenoceptor (5). This impaired β-adrenergic signaling is reported to be due to either 1) reduced β-receptors in MI hearts, 2) impaired adenylate cyclase activity to reduce cAMP production, or 3) impaired protein kinase A (PKA) downstream effects (1). In the present study, isoproterenol, pimobendan, and dibutyryl c-AMP did not restore cardiac contractile response to β-adrenergic stimulation, indicating that cell sheets could not improve impaired PKA-mediated downstream effects of β-adrenergic stimulation. Further investigation might be necessary to elucidate this mechanism. Although ASC sheets improved cardiac function and remodeling after MI through secretion of angiogenic factors, ASC cell sheets did not improve their β-adrenoceptor down-regulation and their desensitization of MI hearts like myoblast cell sheets. Thus, combination of pharmacological intervention to restore β-adrenoceptor down-regulation/desensitization with ASC sheets might be necessary to improve cardiac function after MI hearts.

REFERENCE

1. Georget M, Mateo P, Vandecasteele G, Jurevicius J, Lipskaia L, Defer N, Hanoune J, Hoerter J and Fischmeister R (2002) Augmentation of cardiac contractility with no change in L-type Ca^{2+} current in transgenic mice with a cardiac-directed expression of the human adenylyl cyclase type 8 (AC8). *Faseb J* **16**, 1636–1638.

2. Hamdi H, Planat-Benard V, Bel A, Puymirat E, Geha R, Pidial L, Nematalla H, Bellamy V, Bouaziz P, Peyrard S, Casteilla L, Bruneval P, Hagege AA, Agbulut O and Menasche P (2011) Epicardial adipose stem cell sheets results in greater post-infarction survival than intramyocardial injections. *Cardiovasc Res* **91**, 483–491.

3. Harada Y, Yamamoto Y, Tsujimoto S, Matsugami H, Yoshida A and Hisatome I (2013) Transplantation of freshly isolated adipose tissue-derived regenerative cells enhances angiogenesis in a murine model of hind limb ischemia. *Biomed Res (Tokyo)* **34**, 23–29.

4. Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T and Sawa Y (2012) Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation* **126**, Suppl 1: 29–37.

5. Leosco D, Rengo G, Iaccarino G, Golino L, Marchese M, Fortunato F, Zincarelli C, Sanzari E, Ciccarelli M, Galasso G, Altobelli GG, Conti V, Matrone G, Cimini V, Ferrara N, Filippelli A, Koch WJ and Rengo F (2008) Exercise promotes angiogenesis and improves beta-adrenergic receptor signaling in the post-ischaemic failing rat heart. *Cardiovasc Res* **78**, 385–394.

6. Matsubayashi K, Fedak PW, Mickle DA, Weisel RD, Ozawa T and Li RK (2003) Improved left ventricular aneurysm repair with bioengineered vascular smooth muscle grafts. *Circulation* **108**, Suppl 1, 219–225.

7. Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M and Hagege AA (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. *Circulation* **117**, 1189–1200.

8. Memon IA, Sawa Y, Fukushima N, Matsumiya G, Miyagawa S, Taketani S, Sakakida SK, Kondoh H, Aleshin AN, Shimizu T, Okano T and Matsuda H (2008) Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. *J Thorac Cardiovasc Surg* **130**, 1333–1341.

9. Miranville A, Heeschen C, Sengenes C, Curat C.A, Busse R and Bouloumie A (2004) Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* **110**, 349–355.

10. Miyagawa S, Roth M, Saito A, Sawa Y and Kostin S (2011) Tissue-engineered cardiac constructs for cardiac repair. *Ann Thorac Surg* **91**, 320–329.

11. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL and Isner JM (2011) Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* **101**, 2567–2578.

12. Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, Kikuchi Y, Saito Y, Tamai K, Ogihara T and Kaneda Y (2005) Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol* **25**, 2542–2547.

13. Nakamura Y, Yasuda T, Weisel RD and Li RK (2006) Enhanced cell transplantation: preventing apoptosis increases cell survival and ventricular function. *Am J Physiol Heart Circ Physiol* **291**, H939–947.

14. Ogino K, Burkhoff D and Bilezikian JP (1995) The hemody-

namic basis for the cardiac effects of parathyroid hormone (PTH) and PTH-related protein. *Endocrinology* **136**, 3024–3030.

15. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV and March KL (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **109**, 1292–1298.

16. Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, Shimizu T and Okano T (2012) Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *Surg Today* **42**, 181–184.

17. Sekiya N, Matsumiya G, Miyagawa S, Saito A, Shimizu T, Okano T, Kawaguchi N, Matsuura N and Sawa Y (2009) Layered implantation of myoblast sheets attenuates adverse cardiac remodeling of the infarcted heart. *J Thorac Cardiovasc Surg* **138**, 985–993.

18. Shimizu T, Yamato M, Kikuchi A and Okano T (2003) Cell sheet engineering for myocardial tissue reconstruction. *Biomaterials* **24**, 2309–2316.

19. Shudo Y, Miyagawa S, Ohkura H, Fukushima S, Saito A, Shiozaki M, Kawaguchi N, Matsuura N, Shimizu T, Okano T, Matsuyama A and Sawa Y (2014) 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**, 728–739.

20. Sun Y, Zhang J, Zhang JQ and Weber KT (2001) Renin expression at sites of repair in the infarcted rat heart. *J Mol Cell Cardiol* **33**, 995–1003.

21. Yamamoto Y, Matsuura T, Narazaki G, Sugitani M, Tanaka K, Maeda A, Shiota G, Sato K, Yoshida A and Hisatome I (2009) Synergistic effects of autologous cell and hepatocyte growth factor gene therapy for neovascularization in a murine model of hind limb ischemia. *Am J Physiol Heart Circ Physiol* **297**, H1329–1336.

22. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P and Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–4295.
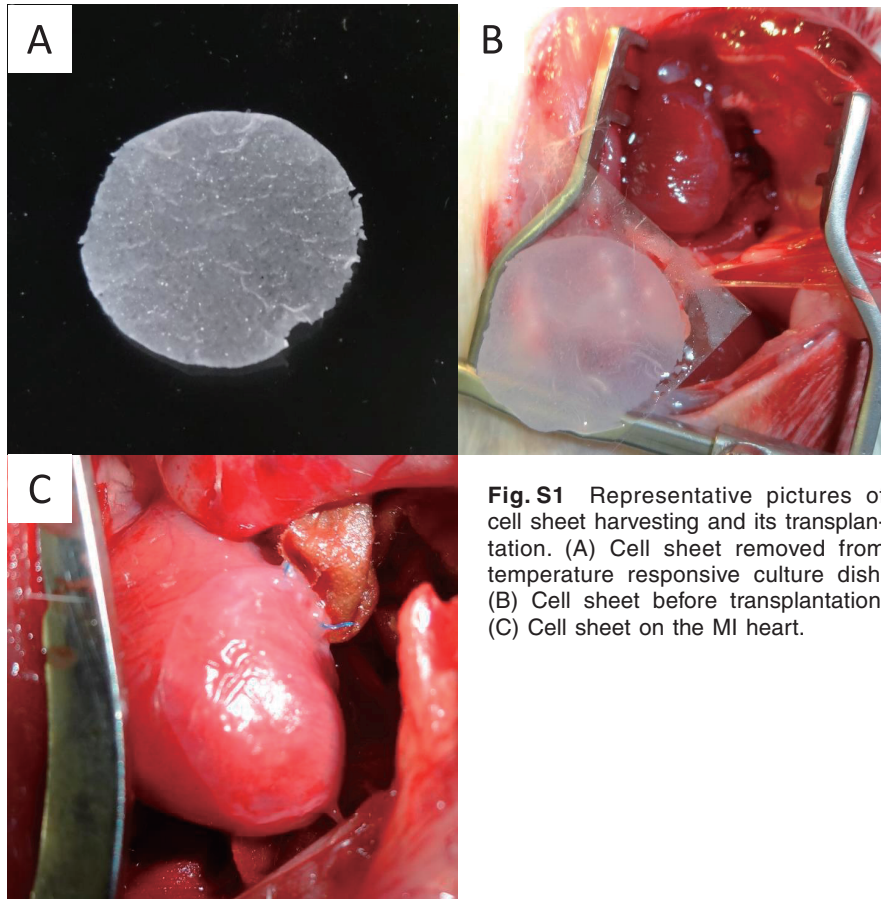
Fig. S1 Representative pictures of cell sheet harvesting and its transplantation. (A) Cell sheet removed from temperature responsive culture dish. (B) Cell sheet before transplantation. (C) Cell sheet on the MI heart.
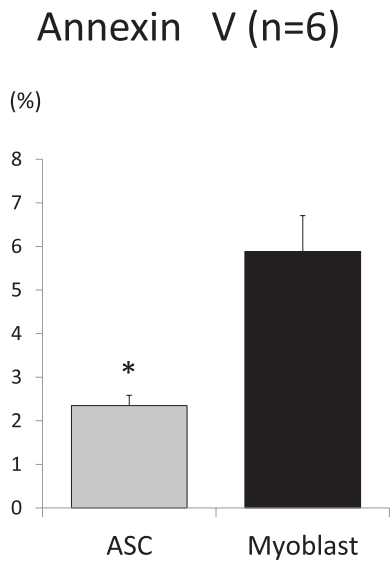
Annexin V (n=6)

Fig. S2 Prevalence of apoptosis marker protein, annexin V expression in cells of ASC (n = 6) and myoblast cell sheets (n = 6) under hypoxic conditions. * denotes $P < 0.05$ ASC vs. myoblast sheets.
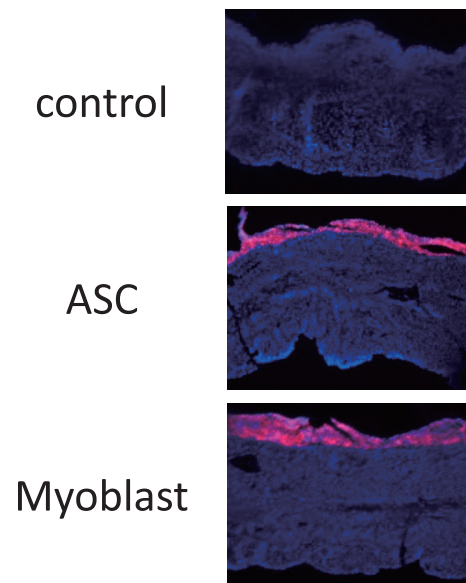

Fig. S3 Representative pictures of the PKH stained transplanted cell sheet (red signal) at 2 weeks after transplantation.