Esm1 and Stc1 as Angiogenic Factors Responsible for Protective Actions of Adipose-Derived Stem Cell Sheets on Chronic Heart Failure After Rat Myocardial Infarction

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Background: Although adipose-derived stem cell (ADSC) sheets improve the cardiac function after myocardial infarction (MI), underlying mechanisms remain to be elucidated. The aim of this study was to determine the fate of transplanted ADSC sheets and candidate angiogenic factors released from ADSCs for their cardiac protective actions.

Methods and Results: MI was induced by ligation of the left anterior descending coronary artery. Sheets of transgenic (Tg)-ADSCs expressing green fluorescence protein (GFP) and luciferase or wild-type (WT)-ADSCs were transplanted 1 week after MI. Both WT- and Tg-ADSC sheets improved cardiac functions evaluated by echocardiography at 3 and 5 weeks after MI. Histological examination at 5 weeks after MI demonstrated that either sheet suppressed fibrosis and increased vasculogenesis. Luciferase signals from Tg-ADSC sheets were detected at 1 and 2 weeks, but not at 4 weeks, after transplantation. RNA sequencing of PKH (yellow-orange fluorescent dye with long aliphatic tails)-labeled Tg-ADSCs identified mRNAs of 4 molecules related to angiogenesis, including those of Esm1 and Stc1 that increased under hypoxia. Administration of Esm1 or Stc1 promoted tube formation by human umbilical vein endothelial cells.

Conclusions: ADSC sheets improved cardiac contractile functions after MI by suppressing cardiac fibrosis and enhancing neovascularization. Transplanted ADSCs existed for >2 weeks on MI hearts and produced the angiogenic factors Esm1 and Stc1, which may improve cardiac functions after MI.

Key Words: Adipose-derived stem cell; Cell sheet; Esm1; RNA sequencing; Stc1

The efficacy of pharmacological and surgical treatments for chronic heart failure after myocardial infarction (MI) is limited. Cell-based regenerative therapy (cell transplantation) improved the blood supply to the damaged heart, and minimized the area of infarction.^{1–3}

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These beneficial effects are mediated, in part, by cytokines, such as hepatocyte growth factor (HGF) and

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vascular endothelial growth factor (VEGF), secreted by transplanted cells.^{4–6} However, direct injection of cells into the heart was problematic because of a significant loss of live cells and proarrhythmic effects.^{7,8} Tissue engineering using cell sheets has been developed to overcome these disadvantages; cell sheets could improve viability of cells^{9,10} and prolong secretion of cytokines.¹¹

Adipose-derived stem cells (ADSCs) are expected to be a useful cell source and ADSC sheets improve the cardiac function after experimental MI.¹² ADSC sheets created by magnetite tissue engineering have been reported to improve the function of MI hearts.¹³ ADSCs secrete cytokines such as VEGF, HGF and fibroblast growth factor (FGF), and prevent cardiac dysfunction and remodeling after MI^{14,15} by inducing neovascularization.^{16,17} We have previously reported that ADSC sheets using temperature-responsive culture dishes decreased interstitial fibrosis, induced neovascularization, and prevented remodeling in rat MI hearts.¹² Pretreatment with either the angiotensin receptor blocker (ARB), irbesartan,¹⁸ or a β -blocker, carvedilol,¹⁹ abolished neovascularization in hearts transplanted with ADSC sheets. These findings suggest that ADSC sheets could facilitate angiogenesis by secreting certain angiogenic factors, contributing to the improvement of MI heart function. However, it remains to be elucidated what types of angiogenic factors are expressed in ADSC sheets and how long transplanted ADSCs exist and secrete angiogenic factors.

In the present study, we determined the life-span of ADSC sheets transplanted into MI hearts and encompassed gene expressions of angiogenic factors in transplanted ADSC sheets. We found that transplanted ADSCs exist for more than 2 weeks and express 2 candidate molecules, endothelial cell specific molecule-1 (Esm1) and stanniocalcin-1 (Stc1), as angiogenic factors.

Methods

Animal Care

Adult male syngeneic Lewis rats weighing 200–250 g (Japan SLC Inc., Hamamatsu, Japan) served as both donors and recipients of ADSC sheets. To determine the life-span of transplanted ADSC sheets, we developed dual reporter transgenic (Tg) rats in which green fluorescence protein (GFP) and luciferase are expressed through P2A alliance.²⁰ The experimental protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Tottori University.

Engineering of ADSC Sheets

ADSCs were isolated from the inguinal subcutaneous fat tissue of 8-week-old wild-type (WT) Lewis rats and Tg rats of the same strain, and cultured as described previously.^{18,19} To prepare cell sheets, ADSCs were cultured on 35-mm temperature-responsive culture dishes (UpCell, Cell Seed Inc., Tokyo, Japan) in an incubator at 37°C for 48 h. They were then maintained at 20°C for 1 h and were detached as an intact sheet.

Creation of a Rat MI Model and Transplantation of ADSC Sheets

Rats were assigned to 4 groups: (1) no treatment after MI (Control MI group, n=7); (2) Tg-ADSC sheet transplantation 1 week after MI (Tg-ADSC group, n=9); (3) WT-ADSC sheet transplantation 1 week after MI (WT-ADSC

group, n=12); and (4) sham operation without MI (sham-operated non-MI group, n=10). For the 3 MI groups, acute MI was induced by ligation of the left anterior descending artery, as described previously.^{12,18,19} Sheet transplantation, as described in our previous report,¹² was performed for the Tg-ADSC and WT-ADSC group rats 7 days after MI.

Echocardiography

Cardiac function was evaluated by echocardiography just before and 1, 3 and 5 weeks after MI (7 days before, just before, and 2 and 4 weeks after ADSC sheet transplantation) using a 12-MHz transducer (LOGIQ P5J and I2L; GE Healthcare, Fairfield, CT, USA). Short-axis 2-dimensional images at the mid-papillary level of the left ventricle (LV) were taken. The end-systolic and end-diastolic cavity areas were determined by tracing endocardial borders to calculate the fractional area shortening (FS). Left ventricular internal diameters at the end-systole (LVESD) and end-diastole (LVEDD) were measured between the mitral valve and papillary muscles to calculate the ejection fraction (EF).

Histopathology

Five weeks after MI (4 weeks after transplantation), hearts were dissected, fixed in 10% formalin, and embedded in paraffin. Transverse sections obtained from the lateral wall, posterior wall and septum were subjected to Masson's trichrome staining to detect interstitial fibrosis. We determined the percentage of stained areas to the total sectional area by using a microscope (BZ9000; Keyence, Osaka, Japan). Each field was scanned, and digital images were analyzed by using Keyence analysis software. The sections were also stained with an anti-von Willebrand factor (vWF) antibody (Abcam plc, Cambridge, UK) to detect capillaries in the border zone between the myocardium and scar. The border zone is defined as the fibrotic area between the normal and infarcted areas,^{12,21} whereas the remote zone as the normal area was localized on the opposite side of the infarction area. Capillaries were counted in 2 fields of transverse sections per animal. The number of capillaries in each field was averaged and expressed as capillary density.

Ex Vivo Imaging of Langendorff Rat MI Hearts Transplanted With Tg-ADSC Sheets

To see how long transplanted Tg-ADSC sheets exist on the surface of hearts, MI hearts were excised and subjected to the Langendorff experiment at 1, 2 and 4 weeks after transplantation. Isolation and coronary perfusion of hearts were conducted by using a Langendorff perfusion system (Model 7523-40; Masterflex, Barrington, IL, USA), as described previously.¹⁸ Coronary arteries were perfused with a modified Tyrode's solution (NaCl 144, KCl 5, CaCl₂ 1.5, MgCl₂ 0.9, HEPES 6, and D-glucose 5 [in mmol/L]; pH 7.4 with NaOH) equilibrated with 100% oxygen. D-luciferin (Toyobo, Osaka, Japan) of 25 μ mol (1 mL of 25 mmol/L solution) was injected into coronary arteries of MI hearts through the aorta. Ten minutes after the D-luciferin injection into coronary arteries, bioluminescence images of Tg-ADSC sheet-transplanted MI hearts were obtained using the Xenogen IVIS 200 imaging system (Sumitomo, Tokyo, Japan).

RNA Sequencing Analysis Using PKH (Yellow-Orange Fluorescent Dye With Long aliphatic Tails)-Positive ADSCs

RNA sequence library preparation, sequencing, mapping,

Figure 1. Effects of sheet transplantation of either Tg-ADSCs or WT-ADSCs on cardiac functions after MI. The ejection fraction (EF) and fractional area shortening (FS) of the hearts excised from rats are shown for the 4 groups. EF and FS were measured by echocardiography just before MI (0 w), and 1 week (1 w), 3 weeks (3 w) and 5 weeks (5 w) after MI; that is, 1 week before, just before, 2 weeks after and 4 weeks after transplantation of either a Tg- or WT-ADSC sheet for MI hearts. The numbers of experiments are given in the parentheses (n=4–11). **P<0.01 (vs. Control MI group). Tg, transgenic; ADSC, adipose-derived stem cell; WT, wild type; MI, myocardial infarction.

gene expression analysis, and gene ontology (GO) enrichment analysis were performed by using DNAFORM (Yokohama, Japan).²² Qualities of total RNA were assessed by using a Bioanalyzer (Agilent, RNA 6000 kit; Tokyo, Japan) to ensure the RNA integrity number (RIN). RNA-seq libraries, which consist of double-stranded cDNA, were prepared by using a SMARTer Stranded Total RNA-seq kit-Pico Input Mammalian (Takara Bio, Shiga, Japan), following the manufacturer's instructions. The size and purity of cDNA libraries were confirmed by an Agilent 2100 Bioanalyzer (High Sensitivity DNA kit), and the libraries were quantified by quantitative polymerase chain reaction (PCR) using primer pairs that have complementary sequences of Illumina adapters (Illumina Inc., Tokyo, Japan). After denaturation, sequencing was conducted on Illumina HiSeq utilizing 150-bp paired-end reads. Obtained raw reads were trimmed and quality-filtered using Trim Galore! (ver. 0.4.4; Babraham Bioinformatics, Cambridge, UK), Trimmomatic (ver. 0.36), and cutadapt (ver. 1.16) software. Trimmed reads were then mapped to the rat genome (Rnor 6.0) using STAR (ver. 2.6.1a). Reads on annotated genes were counted using featureCounts (ver. 1.6.1). Fragments per kilobase of exon per million reads mapped (FPKM) values were calculated from mapped reads by normalizing to total counts and transcripts. Differentially expressed genes were detected by using the DESeq2 package (ver. 1.18.0). The list of differentially expressed genes detected by the DESeq2 package (basemean >5 and fold-change <0.25, or basemean >5 and fold-change >4) was used for GO enrichment analysis by the clusterProfiler package (ver. 3.6.0).

Pathway Analysis

The data obtained by RNA sequencing were loaded into Ingenuity pathway analysis software (Qiagen, Hilden, Germany) to identify functional relationships between

molecules.

Real-Time Reverse Transcription (RT)-PCR Analysis

ADSCs were incubated at 37°C under normal (21% O₂) or hypoxic (<2% O₂) conditions for 48 h using an AnaeroPack system (MITSUBISHI GAS CHEMICAL Inc., Tokyo, Japan). Total RNA was extracted from ADSCs using the RNeasy Mini kit (QIAGEN inc., Valencia, CA, USA). Real-time RT-PCR analysis of VEGF, Esm1, Stc1, Ccbe1, Gng8, and β -actin was performed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Their mRNA levels were expressed as ratios to that of β -actin. The primers used are shown in the **Supplementary Table**. The mRNA levels were determined by using the comparative Ct method, and expressed as $2^{-\Delta\Delta C_t}$.

Tube Formation Assay

Matrigel matrix growth factor reduced (GFR; BD Biosciences Japan, Tokyo, Japan) was thawed, dispensed in 24-well plates, and allowed to solidify at 37°C for 30 min. Human umbilical vein endothelial cells (HUVECs) were seeded at a density of 5×10^4 cells per well, and incubated at 37°C for 1 h. Then, a cell culture insert (0.8 μ m pore; BD Biosciences Japan) was set on each well. HUVECs were resuspended in Dulbecco's Modified Eagle Medium (DMEM) and the inserts were removed after 18 h. The network-like structures of HUVECs were examined under a microscope. Angiogenic activities were assessed by determining the total length of each tube.

Data Analysis

All the data are expressed as mean $\pm$ SEM. Student's t-test and ANOVA with Tukey's post-hoc test were used for comparisons of the 2 groups and multiple (≥ 3) groups, respectively. A probability value of <0.05 was considered significant.

Figure 2. Effects of sheet transplantation of either Tg-ADSCs or WT-ADSCs on interstitial fibrosis in the border zone of MI hearts. **(A)** Representative pictures of interstitial fibrosis in MI hearts isolated from the Control MI **(a)**, Tg-ADSC **(b)** and WT-ADSC **(c)** group rats 4 weeks after transplantation (5 weeks after MI). Picrosirius-red staining was performed to detect interstitial fibrosis. Scale bar=100 μ m. **(B)** Summary of the areas (%) of interstitial fibrosis in the border zone of MI hearts from the 3 groups. The numbers of experiments are given in the parentheses for each group (n=3–9). * P <0.05, ** P <0.01. Tg, transgenic; ADSC, adipose-derived stem cell; WT, wild type; MI, myocardial infarction; n.s., not significant.

Figure 3. Effects of sheet transplantation of either Tg-ADSCs or WT-ADSCs on neovascularization in the border zone of MI hearts. **(A)** Representative images of capillary vessels stained with an antibody against von Willebrand factor (vWF) in the border zone of MI hearts isolated from the Control MI **(a)**, Tg-ADSC **(b)** and WT-ADSC **(c)** group rats 5 weeks after MI; that is, 4 weeks after ADSC sheet transplantation (orange ellipses; scale bar=100 μ m). **(B)** Summary of the numbers of vWF-positive vessels in the border zone of MI hearts from each group. The number of capillary vessels was determined for 2 selected fields in the border zone per animal and expressed as an averaged value. The numbers of experiments are given in the parentheses (n=4–8). * P <0.05. Tg, transgenic; ADSC, adipose-derived stem cell; WT, wild type; MI, myocardial infarction; n.s., not significant.

Table. Top 15 Maximally Upregulated Genes in PKH-Positive Cells Determined 1 Week After Transplantation					
Gene ID	Name	baseMean	log2FoldChange	lfcSE	padj
ENSRNOG00000024536	Ccbe1*	1,225.483	13.643	1.216	1.57203E-27
ENSRNOG00000010797	Esm1*	3,217.316	13.195	1.055	4.92392E-34
ENSRNOG00000024843	Prl8a9	300.43	11.615	1.251	4.59753E-19
ENSRNOG00000049695	Myh2	277.556	11.501	1.226	1.96668E-19
ENSRNOG00000015781	Ndst3	265.65	11.438	1.229	3.66231E-19
ENSRNOG00000006653	Slc38a4	246.443	11.329	1.254	4.38509E-18
ENSRNOG00000020030	Crlf1	226.377	11.207	1.241	4.54854E-18
ENSRNOG00000002537	Wnk3	214.111	11.126	1.289	1.39559E-16
ENSRNOG000000021441	Reln	179.018	10.868	1.261	1.58955E-16
ENSRNOG00000015075	Stc1*	148.039	10.594	1.609	5.74405E-10
ENSRNOG00000016701	Gng8*	143.069	10.544	1.265	1.62042E-15
ENSRNOG000000008706	Tbx3	141.839	10.532	1.263	1.59222E-15
ENSRNOG00000014686	Kcnd3	140.893	10.523	1.303	1.33301E-14
ENSRNOG00000016099	Id4	134.904	10.46	1.268	3.35369E-15

baseMean, average of the normalized count values taken over all samples; log2FoldChange, log2 fold change between the groups; lfcSE, the standard error of the log2FoldChange estimate; padj, adjusted P value by using the Benjamini-Hochberg method. *Candidates for angiogenic molecule genes are indicated.

Results

Effects of Tg-ADSC Sheets on Cardiac Function, Fibrosis and Neovascularization of MI Hearts in Comparison to Those of WT-ADSC Sheets

We first studied effects of Tg-ADSC and WT-ADSC sheets on cardiac functions and remodeling after MI in vivo. EF was significantly lower in the control MI group than in the sham-operated non-MI group. Two and four weeks after ADSC sheet transplantation (3 and 5 weeks after MI), EF and FS in the Tg-ADSC and WT-ADSC groups were significantly higher than those in the control MI group (**Figure 1**). There was no significant difference in the cardiac functions between the Tg-ADSC group and the WT-ADSC group. No rats died in any group during experiments (control MI group,

0/7; WT-ADSC group, 0/5; Tg-ADSC group, 0/11).

Cardiac fibrosis and neovascularization were evaluated by using histological analyses 4 weeks after transplantation (5 weeks after MI). In the control MI group, extensive fibrosis occurred around the infarcted region. This fibrosis was reduced in both the Tg-ADSC and WT-ADSC groups (**Figure 2A**). **Figure 2B** shows the summary data obtained from 3 to 9 experiments. The extent of cardiac fibrosis was significantly lower in both the Tg-ADSC and WT-ADSC groups than in the control MI group. There was no difference in cardiac fibrosis between the Tg-ADSC group and WT-ADSC group. The capillary density was remarkably increased in both the Tg-ADSC and WT-ADSC groups, as compared with that in the control MI group (**Figure 3A**). As summarized in **Figure 3B**, the capillary density was

significantly higher in both the Tg-ADSC and WT-ADSC groups than in the control MI group. There was no difference in the capillary density between the Tg-ADSC group and WT-ADSC group. Thus, Tg-ADSC sheets enhanced neovascularization of MI hearts with a reduction of cardiac fibrosis to the same extent as WT-ADSC sheets.

Tracking the Luciferase Signal of Tg-ADSC Sheets After Transplantation Into MI Hearts

To evaluate survival of transplanted Tg-ADSC sheets, the

signal of luciferase was determined by the IVIS imaging system 1, 2 and 4 weeks after Tg-ADSC sheet transplantation. Injection of D-luciferin into the coronary artery enabled visualization of the Enhanced Luc (ELuc) signal at the engraftment site by in vivo imaging (Figure 4). The ELuc signal was found in Tg-ADSC sheets in MI hearts at 1 and 2 weeks, but not 4 weeks after transplantation. This result suggests that transplanted Tg-ADSC sheets existed for more than 2 weeks (but disappeared within 4 weeks).

Figure 6. mRNA expression of candidate angiogenic molecules in ADSCs determined by RNA sequence analysis. Levels of Esm1, Stc1, Ccbe1 and Gng8 mRNAs as well as VEGF mRNA expressed in ADSCs were determined after incubation for 6, 12, 24 and 48 h under normoxia (21% O₂) or hypoxia (<2% O₂). Semi-quantitative RT-PCR analysis was performed to determine mRNA expression levels, which were normalized to β -actin mRNA levels. The numbers of experiments are given in the parentheses (n=3–6). **P<0.01, *P<0.05 (vs. normoxia). ADSC, adipose-derived stem cell; Esm1, endothelial cell specific molecule-1; RT-PCR, reverse transcription polymerase chain reaction; Stc1, stanniocalcin-1; VEGF, vascular endothelial growth factor.

Figure 7. Effects of Esm1 and Stc1 on tube formation of HUVECs. (A) Representative images of the tube formation of HUVECs in the absence and presence of VEGF (50 ng/well), Esm1 (10 ng/well) or Stc1 (20 ng/well) proteins. (B) The summary data on the total tube length obtained from multiple experiments of tube formation (n=3–6). *P<0.05 (vs. Control). Esm1, endothelial cell specific molecule-1; Stc1, stanniocalcin-1; HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

Encompassing Analysis of Gene Expressions of Tg-ADSC Sheets Transplanted Into MI Hearts

In order to encompass the gene expression of Tg-ADSC sheets, PKH-labeled Tg-ADSCs were enzymatically isolated from transplanted MI hearts at 1 week after transplantation

and PKH-positive cells were sorted by fluorescence-activated cell sorting (FACS). Using PKH-positive cells, RNA sequencing was conducted to identify the mRNA expressed in Tg-ADSCs. As shown in the **Table**, of top 15 genes expressed in PKH-positive cells, the 4 genes Esm1, Stc1, Ccbe1 and Gng8

were picked up as the candidate genes related to angiogenesis. The pathway analysis indicates that both *Esm1* and *Stc1* are related to vascular angiogenesis (**Figure 5**).

To examine whether the upregulation of mRNA expressions of *Esm1* and *Stc1* occurs in the host MI heart (cardiomyocytes) transplanted with a ADSC sheet, we evaluated the mRNA expression of *Esm1* and *Stc1* of the hearts in the control MI group and WT-ADSC group at 2 weeks after MI (1 week after transplantation). There was no significant difference in the mRNA level of *Esm1* or *Stc1* between the control MI group and WT-ADSC group (data not shown). This finding indicates that either *Esm1* or *Stc1* mRNA expression do not increase in the host MI hearts transplanted with ADSC sheets, and that *Esm1* and/or *Stc1* proteins secreted from ADSCs play a pivotal role in promoting angiogenesis and improving cardiac functions after MI.

Expression and Angiogenic Actions of *Esm1* and *Stc1* in ADSCs in Response to Hypoxia

To examine changes in the transcription of candidate genes in response to hypoxia, we conducted a quantitative analysis of *Esm1*, *Stc1*, *Ccbe1* and *Gng8* mRNAs under normal and hypoxic conditions. As shown in **Figure 6**, *Esm1* and *Stc1* mRNAs as well as VEGF mRNA were significantly increased under hypoxic conditions, whereas *Ccbe1* or *Gng8* mRNA was not significantly altered. To determine the role of *Esm1* and *Stc1* in angiogenesis, it was examined whether recombinant *Esm1* or *Stc1* proteins could promote in vitro angiogenesis of HUVECs as VEGF did. **Figure 7A** shows the representative tube formation of HUVECs in the absence and presence of VEGF, *Esm1* or *Stc1* proteins. Like VEGF, both *Esm1* and *Stc1* remarkably enhanced the tube formation of HUVECs. **Figure 7B** shows the summary data obtained from multiple experiments of tube formation. Administration of either *Esm1* or *Stc1* significantly increased the total tube length as the index of tube formation.

Besides, we studied the effect of conditioning medium from ADSCs transfected with siRNA against mRNA of either *Esm1* or *Stc1* on the tube formation of HUVECs. The conditioning medium from ADSCs with siRNA against *Stc1* mRNA led to significantly reduced tube formation of HUVECs compared with that from the normal ADSCs, and that from ADSCs with siRNA against *Esm1* mRNA tended to cause reduced tube formation (data not shown).

Discussion

In the present study, we found that transplantation of Tg-ADSC sheets improved the cardiac function, increased angiogenesis, and reduced fibrosis after MI. Transplanted Tg-ADSC sheets existed at least for 2 weeks. Both *Esm1* and *Stc1* mRNAs were significantly upregulated in ADSCs under hypoxic conditions. Administration of either *Esm1* or *Stc1* protein significantly facilitated the tube formation by HUVECs.

Tg-ADSC sheets improved cardiac functions of MI hearts as WT-ADSC sheets did. This finding indicates that neither GFP nor luciferase influences the cardioprotective actions of ADSC sheets. Tracking of the luciferase signal showed that the transplanted Tg-ADSC sheets existed for more than 2 weeks after transplantation, suggesting that the angiogenic factors released from ADSC sheets in the early stage after transplantation protect MI hearts against functional failure. It has been reported that transplanted ADSCs can differentiate into cardiomyocytes and endo-

thelial cells in a MI model.²³ However, immunofluorescence analyses indicated that implanted ADSCs did not differentiate into either endothelial cells or cardiac myocytes.¹² ADSC therapy for MI repair has improved cardiac functions after MI through enhancement of angiogenesis via paracrine factors,²⁴ and cardiac remodeling attenuation.²⁵ A previous study using ¹²⁴I-HIB-labeled ADSCs visualized by positron emission tomography (PET) indicated that ADSCs transplanted into MI hearts survived only for 3 days after transplantation.²⁶ Under a harsh microenvironment due to hypoxia in MI hearts, the viability of transplanted stem cells reduced due to apoptosis.²⁷ In the present study, however, the luciferase signal of transplanted ADSCs obtained from the Tg-rat harboring the luciferase gene was observed in MI hearts for more than 2 weeks, which is far longer than the lifetime of the ¹²⁴I-HIB signal of ADSCs.²⁸ Taken together, transplanted ADSC sheets improved cardiac functions after MI through enhancement of angiogenesis and prevention of fibrosis, but not via their differentiation into cardiovascular cells. Because an ARB¹⁸ and a β -blocker¹⁹ abolished the ADSC sheet effects to restore cardiac functions of MI hearts, the angiogenic actions of ADSC sheets via the angiotensin receptor and β -adrenergic receptor play a pivotal role in the functional improvement of MI hearts. RNA sequence analysis for PKH-positive ADSCs collected from ADSC sheet-transplanted MI hearts showed the involvement of 4 candidate angiogenic factors, *Ccbe1*, *Esm1*, *Stc1* and *Gng8* in enhanced angiogenesis. Pathway analysis predicted *Esm1* and *Stc1* as angiogenic factors.

Esm1 named as Endocan is a dermatan sulfate proteoglycan expressed in endothelial cells,²⁹ which is upregulated via either angiogenic factors such as VEGF-A or inflammatory cytokines such as tumor necrosis factor (TNF)- α .³⁰ *Esm1* secreted from endothelial cells binds to VEGF on the cell surface, promoting the interaction of VEGF with VEGFR2 to enhance VEGF signaling. It has been reported that *Esm1* binds to fibronectin and heparan sulfate proteoglycan 2, substitutes for fibronectin-bound VEGF-A165, and accelerates its bioavailability to promote angiogenesis through enhancement of the VEGF signal.³¹ *Esm1* has also been reported to regulate phosphorylation of ERK1 and p38.³²

Stanniocalcin (Stc) is a hypocalcemic glycoprotein secreted from the Staninus body and regulates homeostasis of both Ca²⁺ and phosphate in bony fishes. *Stc1*, a mammalian homologue of Stc, is known to be expressed in various tissues and is a downstream effector molecule of VEGF.³³ It has been reported that *Stc1* facilitates the binding of VEGF to VEGFR2 to enhance neovascularization, and increases VEGFR2 in the retina.³⁴ Taken together with the present RNA sequencing data, *Esm1* and *Stc1* released from transplanted ADSC sheets may act as angiogenic factors to induce neovascularization of MI hearts.

In the present study, hypoxic conditions could upregulate the mRNA expression of both *Esm1* and *Stc1*, as well as VEGF in ADSCs. Both recombinant *Esm1* and *Stc1* significantly increased tube formation of HUVECs in vitro. Thus, hypoxic conditions may increase *Esm1* and *Stc1* released from ADSCs, leading to the tube formation of endothelial cells. Pathway analysis also showed that the hypoxic condition of ADSC sheets could activate expressions of *Esm1* and *Stc1*. Conditioning medium from ADSCs with siRNA against *Stc1* led to significantly decreased tube formation of HUVECs. This result suggests that *Stc1* plays a central role in ADSC-induced angiogenesis,

whereas both Esm1 and Stc1 may be involved in the ADSC sheet enhancement of angiogenesis. In our study, either HGF or FGF2 mRNA in ADSCs did not significantly increase under the hypoxic condition for 24h (data not shown, but see reference 12). These findings in vitro may be extrapolated to the status in vivo, suggesting that ischemic conditions of transplanted ADSC sheets in MI hearts could stimulate the secretion of the angiogenic factors, Esm1 and Stc1.

Reasons for the lack of enhanced expression of Cbcl1 and Gng8 in ADSCs under the hypoxic condition remain unknown. Cbcl1 has been reported to enhance lymphangiogenesis through the VEGF-C – VEGF Receptor 3 (VEGFR3) pathway,³⁵ which may not be involved in the angiogenesis stimulated by ADSCs. Gng8 participates in the multi-cell signal transduction through the cell membrane G-protein phosphorylation and dephosphorylation.³⁶ Although a previous report indicated that Gng8 was associated with the ability of inflammatory zone 1 (FIZZ1) to promote rat aortic endothelial cell (RAEC) in vitro angiogenesis,³⁷ our study suggests that Gng8 is not implicated in the enhanced angiogenesis by ADSCs in MI hearts under hypoxic conditions.

In the present study, there were several limitations. First, we did not measure the infarct size of the control MI, WT-ADSC or Tg-ADSC groups. However, the values of EF in the 3 groups were approximately equal to those found in our previous reports.^{12,18,19} Given that the infarct sizes of the 3 groups were proportional to their values of EF, the infarct sizes of the 3 groups in this study were comparable to those found in our previous studies. Second, the effectiveness of the blank sheet was not examined in the present study. Nevertheless, we previously evaluated the effects of dermal fibroblast sheets as the blank sheet on contractile functions of MI hearts and demonstrated that the function of MI hearts transplanted with dermal fibroblast sheets was comparable to that of the control group MI hearts without transplantation.²¹ Finally, we examined the mRNA expression profile of transplanted ADSC sheets only 7 days after transplantation; the mRNA expression profile could not be determined in an earlier phase of transplantation because of the limited number of collected ADSCs in that phase. Moreover, we did not directly examine whether the transplantation of ADSC sheets that express neither Esm1 nor Stc1 could exert their protective effects on MI hearts as observed in this study.

Despite these limitations, the present study clearly shows that the transplanted ADSC sheet, existing on MI hearts for more than 2 weeks, improves the cardiac function after MI via increased expressions of the angiogenic factors, Esm1 and Stc1.

Disclosures

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IRB Information

The present study was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Tottori University (Reference number: 15-Y-51, 17-Y-16).

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Supplementary Files

Please find supplementary file(s);
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