SUPPLEMENTAL MATERIALS

MS ID#: CIRCULATIONAHA/2010/009993/R1

MS TITLE: Induced Adipocyte Cell-Sheet Ameliorates Cardiac Dysfunction in Mouse Myocardial Infarction Model - A Novel Drug Delivery System for Heart Failure

Materials and Methods

Preparation of Adipocyte cell-sheet

Stromal-vascular fraction (SVF) cells were enzymatically isolated from adipose tissues.[1] Briefly, inguinal adipose tissue was excised from wild type mice (WT; male C57BL/6J), APN knockout (KO) mice which were generated and backcrossed to C57BL/6J over 6 generations as described previously[2], or from rats (3-week-old, male LEW/Sea). Adipose tissue was digested in Hank's balanced buffered saline (Sigma-Aldrich, MO, USA) containing 0.1% collagenase type II (Life Technologies, CA, USA) at 37°C with shaking vigorously for 1 hour. The adipose cell extracts were passed 100 μm and 70 μm filters, resuspended in Dulbecco's Modified Eagle's Medium (Life Technologies) containing 10% fetal bovine serum (Equitech-bio, TX, USA), 200 μM ascorbic acid (Sigma-Aldrich), and antibiotics (Life Technologies), then cultured on culture dishes (AGC Techno Glass, Chiba, Japan) at 37°C and 5% CO_2. Twenty-four hours after plating, all the non-adherent cells were removed by washing. The SVF cells were cultured for 3 days in

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

the same medium. The SVF cells were then seeded at 7000 cells/cm^2 in an Upcell dish, which is coated with temperature-responsive polymers (CellSeed, Tokyo, Japan). The culture area was 1.9 cm^2 for mouse cells and 8.8 cm^2 for rat cells. Seven days after passage, the SVF cells were induced to differentiate into the adipogenic lineage by adding 0.87 μM insulin, 0.25 μM dexamethasone, 500 μM isobutylmethylxanthine (IBMX), and 5 μM Pioglitazone (Sigma-Aldrich) for 48 hours. Seven days after induction, the adipocytes were induced to spontaneously detach by placing the plates at 20 °C for 1 hour, yielding a scaffold-free sheet-shaped monolayer of induced adipocyte cell-sheet (iACS) that could be used as a graft. Finally, two iACS were layered to make a thicker sheet for grafting. Both WT mouse-derived iACS and APN-KO mouse-derived iACS were either assessed *in vitro* or labeled using a PKH26 red fluorescent linker kit (Sigma-Aldrich) prior to transplantation.

Enzyme-Linked Immunosorbent Assay

To determine the content of the secreted factors, enzyme-linked immunosorbent assays (ELISA) were performed. The collected culture supernatant of the WT-iACS, WT SVC cell-sheet or KO-iACS was centrifuged to remove debris and contaminating cells. For APN, samples were diluted 1:200 and analyzed (W, n=5; K, n=8). APN content of plasma samples from iACS-received APN-KO mice were analyzed with no dilutions (W 1 mo, n=4; W 3 mo, n=3; K

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

1 mo, n=4). An APN ELISA kit was purchased from Otsuka Pharmaceutical (Tokushima, Japan) according to the manufacturer's instructions. Content of HGF, VEGF, leptin, IL-6, IL-10, and TNFα in the culture supernatants were also analyzed by ELISA kit (R&D systems, MN, USA) with no dilutions (W, n=8-12; K, n=6-9).

Animal experiments

Myocardial infarction (MI) model of mice was created by left anterior descending artery (LAD) ligation as described previously.[3] Mice (10-15-weeks old, male C57BL/6J) were anesthetized by inhalation of isoflurane (1.5%, 1L/min, Mylan Inc., Pittsburgh, PA) provided by an anesthetic gas machine (DS Pharma, Osaka, Japan). The anesthetized mice were intubated in an endotracheal manner, and positive pressure ventilation was maintained with a ventilator (room air, 90 cycles/minutes, tidal volume 1 ml, Shinano, Tokyo, Japan). Then, the heart was exposed through a left lateral thoracotomy. With minimal manipulation of the fat pad surrounding the heart, the LAD component could easily be visualized. LAD was ligated with an 8-0 prolene suture (Johnson & Johnson, NJ, USA) at 1 mm distal to the left atrial appendage, immediately after bifurcation of the major left coronary artery. The myocardial ischemic area was visually assessed, to confirm that the LAD ligation had consistent ischemic effects. Procedure-related mortality, which occurred prior to chest closure, was consistently 6% in all the experimental

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

groups, suggesting a consistent level of acute myocardial ischemia. Within 5 minutes after LAD ligation, the mice were randomly allocated into 3 groups; those that underwent transplantation of WT-iACS (W group; n=40), those that underwent transplantation of KO-iACS (K group; n=40) and those that underwent sham transplantation (C group; n=43). The pericardium was closed to prevent the dislocation of iACS. The mice were allowed to recover under care. In the experiment of iACS implantation to APN-KO mice, MI was not induced.

The mice were euthanized at 2 and 28 days after surgery by intravenous injection of pentobarbital (200 mg/kg body weight; DS Pharma) and 30 mM of potassium chloride (Wako Pure Chemical Industries, Osaka, Japan) to cause cardiac arrest in diastole under terminal anesthesia, and the heart was excised.

On day 2, the specimens for RNA analyses were cut transversely, and then the apex-side specimens were dissected to remove the right ventricular free wall, and part in the three pieces of infarction, peri-infarction, and remote, and soaked in RNA Later (Qiagen, Hilden, Germany, W, n=4; K, n=4; C, n=6). The specimens for CD11b staining were cut into 4 segments, embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and snap frozen in liquid nitrogen (W, n=4; K, n=4; C, n=6). The appropriate segments used for gene expression or histological analyses on day 2 were also used for TTC staining (W, n=8; K, n=7; C, n=9). The specimens used for the cytokine-array analysis were snap frozen in liquid nitrogen on day 2 (W,

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

n=4; K, n=5; C, n=6). The remaining mice were used for survival-rate analysis (W, n=24; K, n=26; C, n=25), but cases of accidental death were excluded. Twenty-eight days after treatment, 18 mice from each group were randomly chosen for cardiac performance analysis by echocardiography. Histological analyses were also performed at 28 days (W, n=6; K, n=8; C, n=5).

MI was also created in rats (8-week-old, female LEW/Sea) by the same method described above, except the tidal volume was 4 ml. Five minutes after LAD ligation, either two layers of iACS were transplanted onto the LV (n=9) or a sham transplantation was performed (n=6). Four weeks after the operation, a hemodynamic assessment was performed.

Quantitative real time PCR

Total RNA was isolated from the stored specimens using the RNeasy Mini Kit (Qiagen) and reverse transcribed with Ominiscript Reverse transcriptase (Qiagen). Quantitative PCR was performed with the ABI 7500 Fast Real-Time PCR System (Life Technologies) using Taqman Universal Master Mix (Life Technologies). Measurement copy number of mRNA was performed in triplicate. The primers and probes are shown in the Table. All probes were designed with a 5' fluorogenic probe 6FAM and a 3' quencher TAMURA. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

Determination of infarct size

Freshly excised hearts from the W, K, and C groups 2 days after transplantation were washed in PBS and dissected into four transverse slices. The slices were then stained for 5 min at 37°C with 1% 2,3,5-triphenyltetrrazolium chloride (TTC; Sigma-Aldrich) to determine the infarct area. The stained slices were photographed, and then the infarct area was determined by computerized planimetry using MetaMorph Software (Molecular Device, CA, USA).

Histological analysis

Histological analyses of the hearts were performed 2 and 28 days after transplantation. The hearts and cell-sheets were cut into 8-μm sections. The sections were stained with antibodies for APN (1:1000 dilution; Otsuka Pharama), CD11b (1:100 dilution; Abcam, Cambridge, UK), or CD31 (1:200 dilution; Abcam). The secondary antibody was Alexa 488 goat anti-rabbit (1 μg/ml; Life Technologies). Counterstaining was with 6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Life Technologies). Images were captured by fluorescence microscopy (Keyence, Osaka, Japan). Routine hematoxylin-eosin staining was performed. Masson's trichrome staining was performed to analyze the collagen accumulation. The collagen volume fraction in the peri-infarct area was calculated as the percentage of the myocardium. The data were collected

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

from 10 individual views per heart at a magnification of ×200. Furthermore, the heart sections were stained with Periodic acid-Schiff (PAS) to assess the cardiomyocyte size. Cardiomyocyte size at a magnification of ×400 was average from 50 myocytes per sample. MetaMorph Software was used for quantitative morphometric analysis.

Myocardial echocardiography

Echocardiography examinations were performed 4 weeks after cell transplantation by an investigator blinded to the group identities (n=18 each). Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiography system equipped with a 12-MHz transducer (GE Healthcare, WI, USA). The left ventricular (LV) dimensions were measured following the method of the American Society of Echocardiology from at least 3 consecutive cardiac cycles. Three readings were obtained from each mouse and averaged. The LV ejection fraction (EF) was calculated as $(LVDd^3\text{-}LVDs^3)/LVDd^3 \times 100$, where LVDd is the LV end-diastolic dimension and LVDs is the LV end-systolic dimension.[4]

Hemodynamic analysis

Four weeks after LAD ligation and cell-sheet transplantation, rats (iACS-treated group, n=9; sham-treated group, n=6) were anesthetized and ventilated. A silk thread was placed under the

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

inferior vena cava to change the LV preload. The conductance catheter (Unique Medical, Tokyo, Japan) was inserted through the LV apex toward the aortic valve along the longitudinal axis of the LV cavity, then fixed. A Millar 1.4 F pressure-tip catheter (Millar Instruments, TX, USA) was also inserted from the LV anterior and fixed. The conductance system and the pressure transducer controller (Integral 3; Unique Medical) were set as previously reported.[5] The conductance, pressure, and intracardiac electrocardiographic signals were analyzed with the Integral 3 software. The baseline indices were initially measured under stable hemodynamic conditions, then the pressure-volume loop was drawn during inferior vena caval occlusion, and analyzed. The following indices were calculated: dP/dtmax, dP/dtmin, the time constant of isovolumic relaxation (τ), and the end-systolic pressure-volume relationship (ESPVR).

Evaluation of survival rate after the operation

To evaluate the life-saving effect of the implantation, survival rates were determined. The mice were housed for 50 weeks after the operation. The survival rates of the mice in the W, K, and C groups were evaluated by the Kaplan–Meier method. The comparison among the three groups was analyzed by the overall log-rank test, and the pairwise comparisons were performed by log-rank test with the Benjamin-Hochberg multiplicity correction.

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

Cytokine antibody array

Protein was isolated from the stored specimens of mouse heart at -80°C. The whole heart tissues were homogenized in Tissue Extraction Reaction Reagent I (Life Technologies), centrifuged, and finally passed 0.22-µm filter (Millipore, MA, USA). Protein contents were measured by Milliplex Mouse Cytokine/Chemokine Panel Pre-mixed 32Plex, according the manufacturer's instructions (Millipore).

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

1. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. *Circulation*. 2004;109:656-663
2. Maeda N, Funahashi T, Shimomura I. Metabolic impact of adipose and hepatic glycerol channels aquaporin 7 and aquaporin 9. *Nat Clin Pract Endocrinol Metab*. 2008;4:627-634
3. Imanishi Y, Saito A, Komoda H, Kitagawa-Sakakida S, Miyagawa S, Kondoh H, Ichikawa H, Sawa Y. Allogenic mesenchymal stem cell transplantation has a therapeutic effect in acute myocardial infarction in rats. *J Mol Cell Cardiol*. 2008;44:662-671
4. Miyagawa S, Sawa Y, Taketani S, Kawaguchi N, Nakamura T, Matsuura N, Matsuda H. Myocardial regeneration therapy for heart failure: Hepatocyte growth factor enhances the effect of cellular cardiomyoplasty. *Circulation*. 2002;105:2556-2561
5. Sato T, Shishido T, Kawada T, Miyano H, Miyashita H, Inagaki M, Sugimachi M, Sunagawa K. Espvr of in situ rat left ventricle shows contractility-dependent curvilinearity. *Am J Physiol*. 1998;274:H1429-1434

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

Table. Primers and probes used in this study.

	Forward primer	Reverse Primer	Probe
GAPDH	GCATGGCCTTCCGTGTTC	GATGCCTGCTTCACCACCTT	CCGCCTGGAGAAACCTGCCAAGTATG
TNF-α	CCACCACGCTCTTCTGTCTACT	TTGGTGGTTTGCTACGACGT	CCCAGACCCTCACACTCAGATCATCTTC

Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

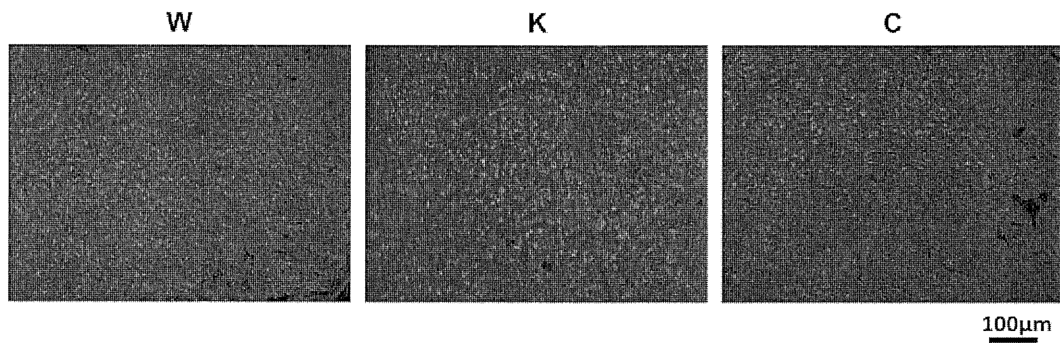
Supplementary figure legends

Supplementary figure 1. Capillary formation in the iACS-treated heart. Four weeks after infarction and iACS transplantation, CD31 staining was performed to assess angiogenesis. Representative images from each group are shown. The density of CD31-positive capillaries was the same among the groups. Green, CD31; blue, nuclei.

Supplementary figure 2. Hemodynamic effects of WT-iACS transplantation in rat AMI. In the iACS-treated group, the dP/dt_{max} was significantly higher and the dP/dt_{min} significantly lower than in the control ($P<0.05$ v.s. sham-treated group, *unpaired t* test). The τ value was not significantly different between the groups, although it was smaller in the iACS-treated group ($P=0.12$ v.s. sham-treated group, *unpaired t* test). The ESPVR value was significantly higher in the iACS-treated group. tx, treatment.

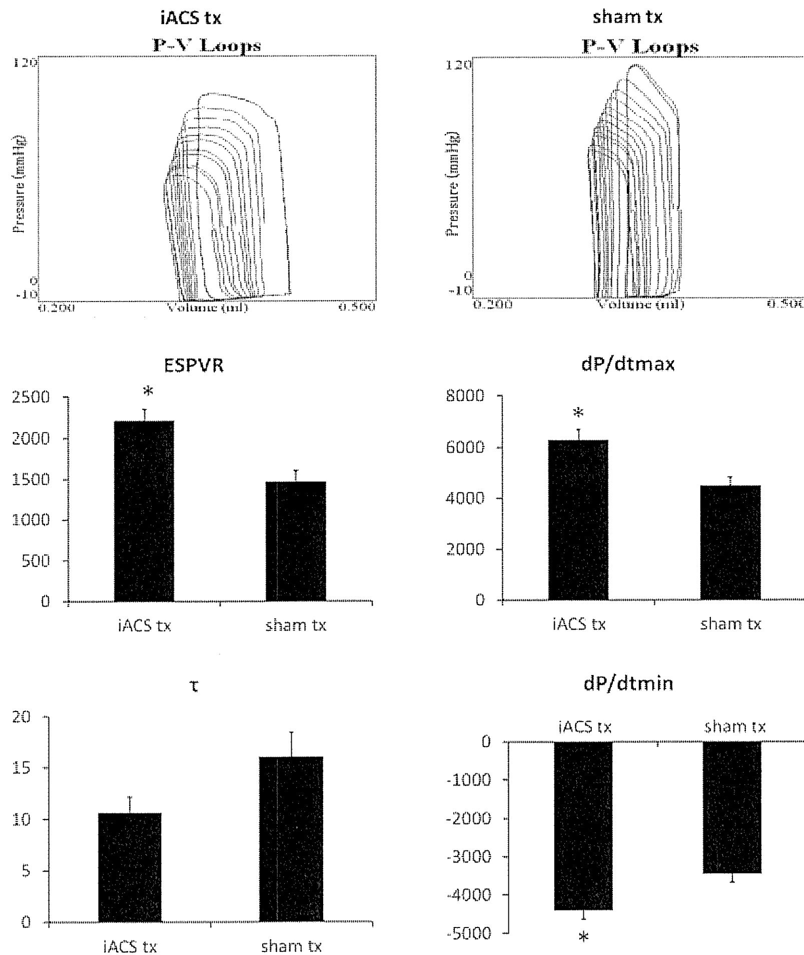
Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

Supplementary figure 1



Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012

Supplementary figure 2



Downloaded from http://circ.ahajournals.org/ at Hyogo College of Medicine on January 29, 2012