Laboratory Investigation (2011) 91, 539–552

Synergistic effect of adipose-derived stem cell therapy and bone marrow progenitor recruitment in ischemic heart

Masaaki Ii[1,2], Miki Horii[2], Ayumi Yokoyama[2], Taro Shoji[3], Yutaka Mifune[3], Atsuhiko Kawamoto[2], Michio Asahi[1] and Takayuki Asahara[2,4]

Human multipotent adipose-derived stem cells (hMADSCs) have recently been isolated featuring extensive expansion capacity *ex vivo*. We tested the hypothesis that hMADSC transplantation might contribute to cardiac functional recovery by its direct or indirect effect on myocardial infarction (MI). Nude rats were either transplanted with hMADSCs or PBS (control) in ischemic myocardium immediately following MI. Echocardiographical assessment of cardiac function after MI with hMADSCs showed significant improvement of each parameter compared to that with PBS. Histological analysis also showed significantly reduced infarct size and increased capillary density in peri-infarct myocardium by hMADSC treatment. However, remarkable transdifferentiation of hMADSCs into cardiac or vascular lineage cells was not observed. Despite the less transdifferentiation capacity, hMADSCs produced robust multiple pro-angiogenic growth factors and chemokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and stromal cell-derived factor-1α (SDF-1α). Specifically, hMADSC-derived SDF-1α had a crucial role for cooperative angiogenesis, with the paracrine effect of hMADSCs and Tie2-positive bone marrow (BM) progenitor recruitment in ischemic myocardium. hMADSCs exhibit a therapeutic effect on cardiac preservation following MI, with the production of VEGF, bFGF, and SDF-1α showing paracrine effects and endogenous BM stem/progenitor recruitment to ischemic myocardium rather than its direct contribution to tissue regeneration.

Laboratory Investigation (2011) **91,** 539–552; doi:10.1038/labinvest.2010.191; published online 6 December 2010

KEYWORDS: angiogenesis; cytokine; endothelial progenitor cell; growth factor; myocardial infarction; stem cell

Cardiovascular disease has been recognized as one of the major causes of death and numerous patients are suffering from heart failure, which is a leading reason for hospitalization in the world. Despite a variety of therapeutic options to prevent progression of heart failure caused by cardiovascular diseases, patients who are at the end stage of the disease can only be treated with invasive cardiac surgery, including heart transplantation. Transplantation of bone marrow (BM)-derived stem/progenitor cells has recently been reported to be effective for myogenesis and vasculogenesis in animal models of myocardial infarction (MI) and early-phase clinical trials[1–3] as a new therapeutic strategy for severe cardiovascular diseases. On the other hand, tissues such as cord blood,[4] placenta,[5] skeletal muscle,[6] skin,[7] and adipose tissue-derived resident stem cells[8,9] have also been shown to have regenerative capacity in certain diseases, including cardiovascular disease animal models. Among the various sources of tissue-derived stem cells, adipose tissue-derived stem cells (ADSCs) are one of the promising candidates as a tool for cell-based regenerative medicine, and the usage of ADSCs are not remote from clinical field because of its feasibility for collecting sufficient number of cells with the methodological simpleness.

Recently, Rodriguez *et al*[10] have isolated multipotent adipose-derived stem cells (hMADSCs) from young human

[1]Group of Translational Stem Cell Research, Department of Pharmacology, Osaka Medical College, Osaka, Japan; [2]Group of Vascular Regeneration Research, Institute of Biomedical Research and Innovation, Kobe, Japan; [3]Department of Orthopedic Surgery, Kobe University Graduate School of Medicine, Kobe, Japan and [4]Department of Regenerative Medicine, Tokai University School of Medicine, Kanagawa, Japan
Correspondence: Dr M Ii, MD, PhD, Group of Translational Stem Cell Research, Department of Pharmacology, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan or T Asahara, MD, PhD, Group of Vascular Regeneration Research, Institute of Biomedical Research and Innovation, 2-2, Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan.
E-mail: masaii@art.osaka-med.ac.jp or asa777@is.icc.u-tokai.ac.jp

Received 10 June 2010; revised 21 August 2010; accepted 7 September 2010

adipose tissue, and shown the differentiation capacity into not only adipose cells, but also osteoblasts, myoblasts, chondroblasts,[11] cardiomyocytes,[12] endothelial cells,[13] and smooth muscle cells[14] *in vitro*. Transplantation of hMADSCs into the mdx mouse, an animal model of Duchenne muscular dystrophy, resulted in substantial expression of human dystrophin in the injected muscle,[15] suggesting that hMADSCs will be an important tool for muscle cell-mediated therapy. On the basis of these evidences, hMADSCs can also be a significant cell source for regeneration therapy in ischemic heart diseases. Indeed, very recent studies have shown the therapeutic efficacy of ADSCs, which were isolated (and cultured) by different methods, in animal MI models.[9,16–18] However, precise mechanistic insights of the favorable effect of ADSCs on cardiac functional recovery following MI have never been shown in the previous reports. We therefore tested the hypothesis that hMADCs had a favorable effect on acute MI exhibiting its capability of differentiation into vascular cells and cardiomyocytes and other biological activities in ischemic myocardium.

In this study, the therapeutic effect of hMADSCs on ischemic myocardium following MI was examined by histological and functional assessments in an immunodeficient rat MI model. We have also revealed the mechanism for cardioprotective effect of hMADSCs on MI by exploring synergistic contribution with endogenous BM-derived stem/progenitor cells to ischemic myocardium as well as for paracrine effect of hMADSCs by *in vitro* study.

MATERIALS AND METHODS

Isolation of hMADSCs

All the hMADSCs used in this study were provided by Stem Cell Sciences KK (SCSKK). hMADSCs were isolated from adipose tissue of young children (less than 7 years old) with the informed consent of the parents as surgical scraps from surgical specimens of various surgeries, as approved by the Centre Hospitalier Universitaire de Nice Review Board as described previously, and the hMADSCs are no longer connected to the identity of the patients and hence not identifiable human materials.[15]

For isolation of hMADSCs, around 200 mg of adipose tissue was dissociated for 5–10 min in DMEM containing antibiotics (100 U/ml of penicillin and 100 g/ml of streptomycin), 2 mg/ml collagenase, and 20 mg/ml bovine serum albumin. The crude stromal–vascular fraction (SVF) was separated from the adipocyte fraction by low-speed centrifugation (200 *g*, 10 min). The adipocyte fraction was discarded and cells from the SVF pellet were seeded onto uncoated tissue culture plates (Falcon) at 1000–3500 cells per cm^2 in low glucose DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and antibiotics as described before. Only the fast-adherent cells (adherent cells within 12 h after plating) were separated from slow-adherent cells (adherent cells between 12 and 72 h after plating), and expanded in the same culture medium as described before. After reaching 70% confluence, cells were dissociated (0.25% trypsin EDTA; Invitrogen) and replated at 1000–3000 cells per cm^2. hMADSCs were cultured and used between the 25th and 30th of passage in this study.

Animals and Experimental Groups

Male athymic rats (F344/N Jcl/rnu/rnu; CLEA Japan) aged 8 weeks were used in this study. Twenty-two rats were assigned into two groups, either the hMADSC-treated group or the PBS-treated group. In the hMADSC-treated group, 5×10^5 of hMADSCs suspended with 100 μl of PBS were injected to the myocardium (four sites in ischemic border zone) immediately after induction of MI ($n = 12$). In the PBS-treated group, 100 μl of PBS were injected to the ischemic myocardium in the same manner as the hMADSC-treated group (control group) ($n = 10$).

Surgical Procedure

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Institute of Biomedical Research and Innovation's and RIKEN Center for Developmental Biology's Institutional Animal Care and Use Committee approved the following research protocol, including all procedures and animal care. Thirty male 8–10 weeks old athymic nude rats (F344/NJcl/rnu/rnu; CLEA Japan) were anesthetized with ketamine and xylazine (60 and 10 mg/kg i.p., respectively). MI was induced by ligating a left anterior descending (LAD) coronary artery as described previously.[19,20] Briefly, after the fourth to fifth intercostals space was opened, the heart was exteriorized, and the pericardium was incised. Thereafter, the heart was held with forceps, and MI was induced by ligating the LAD coronary artery at just the proximal site of the bifurcation of diagonal branch with a 7-0 (for mice)/6-0 (for rats) nylon suture. Sham operation (open chest and passing suture under LAD without ligation) was also performed. The predetermined dose of hMADSCs (hMADSC-treated group) or PBS (PBS-treated group) was transplanted to the myocardium immediately after MI induction. After the cell or PBS injection was completed, thorax was closed and finished surgery.

Physiological Assessment of Left Ventricular Function

Trans-thoracic echocardiography (SONOS 5500, Hewlett-Packard, Andover, MA, USA) was performed 28 days after MI. The percentage of left ventricular fractional shortening (LVFS), ejection fraction (LVEF), diastolic diameter (LVDD), and systolic diameter (LVDS) were measured at the level of mid-papillary muscle. The regional wall motion score (RWMS) was calculated according to the published criteria.[20] Immediately after the final echocardiography on day 28, the mice underwent cardiac catheterization for more invasive and precise assessment of global LV function as described previously.[20] A 2.0 Fr micromanometer-tipped conductance

catheter (SPR 838, Millar Instruments, TX, USA) was inserted via right carotid artery into LV cavity. LV pressure and its derivative (dP/dt) were continuously monitored using a multi-channel recording system (Pressure-Volume Conductance System ARIA and Pressure-Volume Analysis Using P-V Analysis Software (Millar Instruments) and Power Lab DAQ System (ADInstrument, Australia)). $+dP/dt$ and $-dP/dt$ were continuously recorded for 20 min. All data were acquired under stable hemodynamic conditions. All procedures and analyses were performed by an experienced researcher who was blinded to treatment assignments.

Histological Analysis

Hearts were removed from killed animals 28 days after MI and perfused with PBS followed by 4% PFA retrogradely through the right carotid artery. The hearts were fixed for 6 h in 4% PFA and incubated overnight in 15% sucrose solution. The tissues were embedded in OCT compound (Sakura FineTek, Japan) and sectioned at 1 mm for rats and 0.5 mm for mice just below the LAD ligation level at 5-mm thickness as described previously.[19] To measure fibrosis area, Masson's Trichrome staining was performed, and the fibrosis area (blue) was measured and calculated as the percent of the area of fibrosis to entire cross-sectional area of LV by using the NIH Image J 1.42 software and Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) software.

Mouse Bone Marrow Transplantation Model

Twenty male BALB/c AJcl nude mice (CLEA Japan) aged 6–8 weeks were used as recipients for bone marrow transplantation (BMT). Transgenic mice of FVB/N-TgN (Tie-2-LacZ)^{182Sato} (Jackson Laboratories) were used as donors for the BMT. The procedure of BMT was performed as described previously with some modifications.[19] Briefly, The background/recipient mice were lethally irradiated for BM ablation with 9.0 Gy and received 5 million of donor BM mononuclear cells. At 4–6 weeks after BMT, by which time the BM of the recipient mice was reconstituted, MI surgery with hMADSCs (1×10^5/20 μl PBS) or PBS (20 μl) injection was performed. The MI-induced hearts of BMT mice were harvested 24 h after surgery for histological analysis.

Immunohistochemistry

The hearts of MI-induced rats were harvested at pre-determined time point after surgery, and prepared for frozen tissue sectioning after fixation with 4% PFA/PBS. Double fluorescent immunostaining was performed with an antibody against human nuclear/mitochondria antigen (hNA/hMitC; Chemicon) and human-specific FITC-Ulex europaeus lectin type 1 (UEA-1; Vector Laboratories), smooth muscle α-actin (Dako), or cardiac troponin-I (Chemicon) to detect the transdifferentiation of the transplanted hMADSCs into endothelial cells, smooth muscle cells, or cardiomyocytes, respectively. Normal mouse IgG or PBS was served as negative controls. The endothelial cell marker, (biotinylated) isolectin-B4 (ILB4, 1:100; Vector Laboratories), was used for capillary staining with Rhodamine-conjugated streptavidin-biotin binding method in rat hearts. An anti-β-galactosidase (β-gal; Cappel) was used to detect *LacZ* gene expressing BM-derived cells. Nuclei were counterstained with DAPI (Sigma) and sections were mounted in aqueous mounting medium. Images were examined using a fluorescent microscope (Olympus, Japan). The number of ILB4-positive capillaries and β-gal-positive BM cells were counted in bilateral peri-infarct areas in high-power field (HPF) ($\times$ 200), and averaged for the assessment of capillary density and recruited BM cell number, respectively.

Cell Function Assay

The proliferation activity of hMADSCs was examined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, hMADSCs were seeded onto 96-well culture plates at a density of 5×10^3 cells per well and cultured in DMEM containing 10% FBS for 48 h at 37°C in 1, 5, and 20% O_2/5% CO_2. Optical density was measured using a plate reader at 450 nm wavelength.

The migration activity of hMADSCs was evaluated with a modified Boyden's chamber method as described previously.[19] Briefly, hMADSCs (5×10^4 cells per well) were seeded onto upper chambers in 24-well culture plates and lower chambers were filled with DMEM with 20% FBS followed by incubation for 6 h at 37°C in 1, 5, and 20% O_2/5% CO_2. Migrated cells were stained with DAPI and counted in randomly selected four HPFs ($\times$ 200, 0.15 mm^2 per HPF) per chamber under a fluorescent microscope and averaged.

The cell apoptosis assay was examined using a Cell Death Detection Kit (Roche Diagnostics KK, Tokyo, Japan) according to the manufacturer's instructions. Briefly, hMADSCs (2×10^4 cells per well) were cultured with serum-free DMEM medium in four-well glass chamber slide (Nalgene Nunc) for 48 h, and the apoptotic cells were detected as TUNEL-positive cells in randomly selected five HPFs ($\times$ 200). The percentage of apoptotic cells was calculated by dividing TUNEL-positive cells with total cells and averaged for the assessment of antiapoptosis activity.

Quantitative Real-Time RT-PCR

hMADCs were seeded onto six-well culture plate and cultivated for 0 (normoxic condition), 8, 16, and 24 h under hypoxic condition (5% O_2 in CO_2 incubator) in the regular culture medium. Cells were harvested at the pre-determined time point, and RNA was extracted with RNeasy Mini Kit (Qiagen) and cDNA was synthesized using ExScript RT Kit (Takara), and amplification was performed on the Sequence Detection System 7000 (Applied Biosystem) according to the manufacturer's instructions.

Primer sequences and GenBank accession numbers are as follows: vascular endothelial growth factor (VEGF, AB021221)—forward, TCTCCCTGATCGGTGACAGT and reverse, GGGCAGAGCTGAGTGTTAGC; basic fibroblast

growth factor (bFGF, J04513)—forward, CTCAGTCGGAAC AAATTGGAA and reverse, GCCTGTCAGAGCCTGAAGAA; endothelial nitric oxide synthase (eNOS, NM_000603)—forward, AGGTGGTGCCTTCTCACATC and reverse, GAGG GGCCTTCCAGATTAAG; insulin-like growth facor-1 (IGF-1, X00173)—forward, CCATGTCCTCCTCGCATCTC and reverse, CGTGGCAGAGCTGGTGAAG; angiopoietin-1 (Ang-1, D13628)—forward, GTTGGCAAGGTAGCAATACCA and reverse, GCATAGTGGATCAAGTCACCAA; stromal cell-derived factor-1α (SDF-1α, L36033)—forward, TGAGAGCT CGCTTTGAGTGA and reverse, GCCTCCATGGCATACATA GG; and glyceraldehyde-3-phosphate dehydrogenase (GAP DH, AF261085)—forward, CAGCCTCAAGATCATCAGCA and reverse, TGTGGTCATGAGTCCTTCCA. Relative mRNA expression of the target gene was calculated with the comparative C_T method. The amount of the target gene was normalized to the endogenous GAPDH control gene. The experiments were triplicated and averaged.

Human SDF-1 Gene Silencing by siRNA Technique

hMADSCs were transfected with either SDF-1 siRNA plasmid vector or randomly sequenced control siRNA plasmid vector using a specific transfection reagent (Ambion) according to the manufacturer's instructions, and SDF-1 mRNA expression was analyzed by real-time RT-PCR 2, 4, and 7 days after transfection. SDF-1α protein concentration was measured by ELISA for human SDF-1α kit (R&D Systems) according to the manufacturer's instructions in the SDF-1 siRNA-transfected hMADSCs and in the control siRNA-transfected hMADSCs 2, 4, and 7 days after transfection. Intact hMADSCs were used as another control for the assessment of SDF-1 mRNA expression and the protein production. For cell transplantation study, either SDF-1 gene silencing or control hMADSCs were injected to ischemic myocardium 4 days after siRNA plasmid vector transduction immediately after MI induction.

Statistical Analysis

All values were expressed as mean ± s.e.m. Statistical analyses were performed using a Prism^{TM} software program (GraphPad Software, CA, USA). Non-parametric unpaired *t*-test (Mann–Whitney *U*-test) was used for the comparison between two groups, and repeated-measure two-way ANOVA with Bonferroni *post hoc* test was used for the comparison among multiple groups. A value of $P<0.05$ was considered significant.

RESULTS

Preserved LV Function with Reduced Infarct Size and Increased Vascularity by hMADSC Transplantation in Ischemic Myocardium following MI

Three rats in each group died owing to mechanical problems of ventilator or technical errors during surgical procedure.

Echocardiography performed 28 days after cell transplantation showed that the following parameters: LVEF and LVFS were significantly greater and RWMS was significantly smaller in the hMADSC-treated group ($n=9$) than that in the PBS-treated group ($n=7$). (LVEF: 53.6 ± 3.1 *vs* $32.7\pm1.9\%$; LVFS: 24.4 ± 1.7 *vs* $13.4\pm0.8\%$; and RWMS: 8.1 ± 0.6 *vs* 11.7 ± 0.5, $P<0.05$) (Figure 1a–c). Although there were no significant differences in the other echocardiographic parameters: LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD), the hMADSC-treated group exhibited small values compared with the PBS-treated group. (LVEDD: 7.7 ± 0.3 *vs* 7.9 ± 0.4 mm and LVESD: 5.9 ± 0.3 *vs* 6.9 ± 0.4 mm, $P=0.75$ and 0.11) (Figure 1d and e) Overall, the hMADSC-treated group exhibited improved cardiac functional recovery up to not equivalent level, but similar level in sham-operated group (LVEF: $53.9\pm2.5\%$; LVFS: $28.8\pm1.7\%$; RWMS: 6.7 ± 0.3; LVEDD: 7.0 ± 0.2; and LVEDS: 5.0 ± 0.3). In addition, another hemodynamic study further exhibited the favorable effect of hMADSC treatment on cardiac functional recovery (hMADSC *vs* PBS: $+dP/dt$, $12\,977\pm658.5$ *vs* $10\,159\pm350.9$ mm Hg/s, $P<0.01$ and $-dP/dt$, -7678 ± 400.7 *vs* -6148 ± 102.8 mm Hg/s, $P<0.05$) ($n=5$/each group).

Next, we assessed the effect of hMADSC transplantation on MI histologically. MI size was evaluated by calculating the percent fibrosis area out of entire cross-sectional LV area. Fibrosis area was represented by blue in Masson's Trichrome staining. The hMADSC-treated group exhibited significant small infarct size compared with the PBS-treated group (18.1 ± 1.5 *vs* $25.5\pm0.9\%$, $P<0.05$) (Figure 1h and i) Myocardial neovascularization was also evaluated by capillary density measurement in peri-infarct area 28 days after surgery. Capillary density in the hMADSC-treated group was significantly greater than that in the PBS-treated group (254.1 ± 12.2 *vs* 162.1 ± 16.8/HPF, $P<0.05$) (Figure 1j and k) These results suggest that transplantation of hMADSCs preserved global and regional LV function, with the reduction of infarct size promoting neovascularization in ischemic myocardium after MI.

Proliferation, Migration, and Antiapoptosis Activities of hMADSCs under Hypoxic Conditions

To assess transplanted cell functions in ischemic myocardium, hMADSCs were treated with serum-containing culture medium for proliferation assay (10% FBS) and migration assay (20% FBS) and with culture medium without serum for apoptosis assay under hypoxic conditions mimicking *in vivo* settings. Although relative proliferation activity of hMADSCs to that in low serum concentration (0.5%) culture (1.0 ± 0.0) was significantly increased under hypoxic conditions (1% O_2: 1.54 ± 0.03 and 5% O_2: 1.49 ± 0.05 *vs* 20% O_2: 1.27 ± 0.04, $P<0.001$, $n=6$) (Figure 2a), both migration and antiapoptosis activities were significantly reduced under severe (1% O_2) hypoxic condition (migration activity, 1% O_2: 72.3 ± 2.7 *vs* 20% O_2:

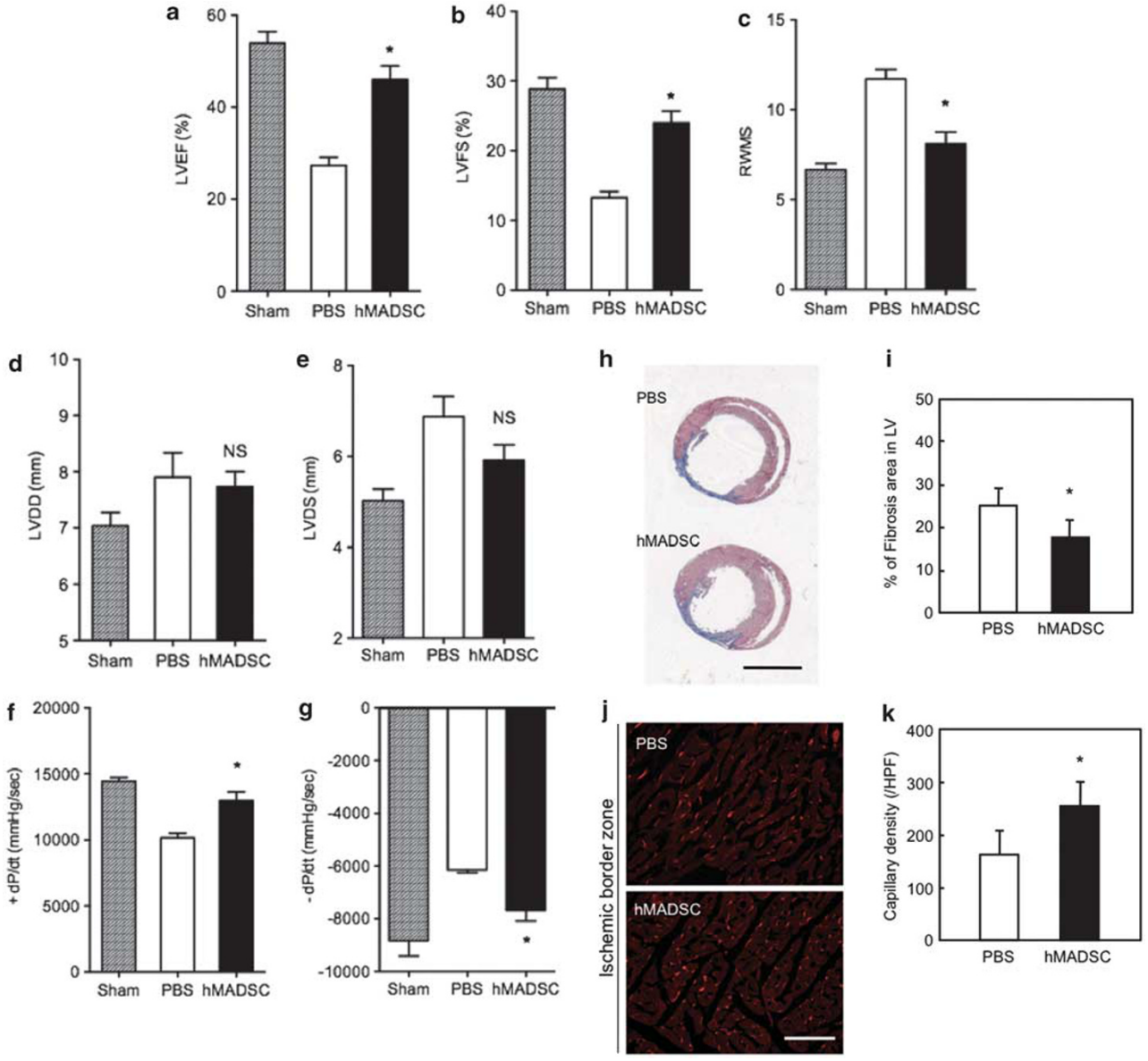


Figure 1 Echocardiographical analysis and hemodynamic study for cardiac function following myocardial infarction (MI) 28 days after surgery. The following parameters: left ventricular ejection fraction (LVEF) (**a**); left ventricular fractional shortening (LVFS) (**b**); regional wall motion score (RWMS) (**c**); left ventricular diastolic diameter (LVDD) (**d**); left ventricular systolic diameter (LVDS) (**e**); $+dP/dt$ (**f**) ($n=5$); and $-dP/dt$ (**g**) ($n=5$) were measured in the phosphate-buffered saline (PBS)-treated group ($n=7$), the human multipotent adipose-derived stem cells (hMADSC)-treated group ($n=9$), and sham-operated group ($n=6$), and averaged. $^*P<0.05$ *vs* PBS. Histological analysis for ischemic heart samples. (**h**) Infarct area in the PBS-treated group and the hMADS-treated group was visualized by Masson's Trichrome staining. Fibrosis area is stained in blue and the intact area is stained in red. Bar = 5 mm. (**i**) The percent of fibrosis area in entire LV cross-section was calculated and averaged in the PBS-treated group ($n=7$) and the hMADSC-treated group ($n=9$). (**j**) Capillaries in the peri-infarct area were visualized by red fluorescent with isolectin-B4 (ILB4) in each group. Bar = 100 μm. (**k**) Capillary densities in both sides of ischemic border zone were calculated and averaged in each group. $^*P<0.05$ *vs* PBS.

94.9 ± 7.7/HPF, $P<0.01$, $n=8$ and 5% O_2: 82.6 ± 3.2 *vs* 20% O_2: 1.27 ± 0.04/HPF, NS, $n=8$) (Figure 2b) (number of TUNEL-positive cells: 1% O_2: 3.5 ± 0.6 *vs* 20% O_2: 1.0 ± 0.3/HPF, $P<0.01$, $n=15$ and 5% O_2: 1.2 ± 0.2 *vs* 20% O_2: 1.0 ± 0.3/HPF, NS, $n=15$) (Figure 2c) These results suggest that cell functions of transplanted hMADSCs in ischemic myocardium might differ depending on the cell distribution in tissue and the grade of tissue ischemia/hypoxia, which may determine transplanted cell survival and the therapeutic efficacy.

hMADSCs Produce Pro-angiogenic Cytokines and Stem/Progenitor Chemokine SDF-1α

The production of cytokines in hMADSCs was evaluated by quantitative real-time RT-PCR under normoxic and hypoxic conditions in the indicated time course mimicking the

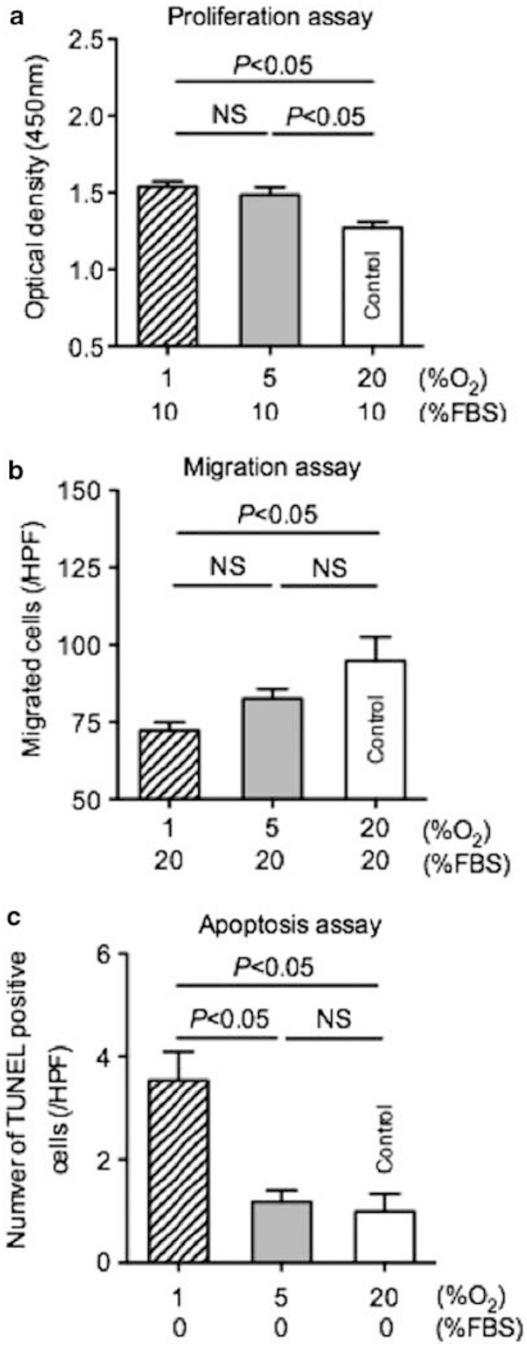


Figure 2 Cell function analyses of human multipotent adipose-derived stem cells (hMADSCs) under normoxic and hypoxic conditions. Proliferation activity was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay after 48 h culture in 10% fetal bovine serum (FBS)-containing medium (**a**), 20% FBS-induced migration activity was assessed by counting migrated cells in a Trans-Well system after 6 h culture (**b**), and cell apoptosis was assessed by calculating the percent of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells after 48 h culture with serum-free medium (**c**). Cells were pre-incubated at 1, 5, and 20% O_2 concentrations for 24 h before assay. All the assays were triplicated and showed similar results in each assay.

condition of myocardial ischemia following MI. For comparison, we used not only human BM-derived mesenchymal stem cells (hBM-MSCs), which show similar cell surface marker-based characteristics to hMADSCs (Supplementary Figure 1), but also CD34-positive cells isolated from G-CSF-mobilized human MNCs, and AC133-positive cells isolated from human umbilical cord blood were used as other stem/progenitor cell controls for this assay. Interestingly, although the mRNA expression levels were decreased in hMADSCs under severe hypoxic conditions (VEGF: 16 and 24 h, 711 ± 83 and 246 ± 102, respectively, and bFGF: 784 ± 55 and 1066 ± 149, respectively), VEGF and bFGF mRNA expressions in hMADSCs under normoxic or mild hypoxic condition were significantly great compared with those of hBM-MSCs (VEGF: hMADSC 0 h, 1348 ± 27 *vs* hBM-MSC 0 h, 808 ± 52, $P<0.05$, $n=3$ and hMADSC 8 h, 1374 ± 86 *vs* hBM-MSC 8 h, 1226 ± 52, $P<0.05$, $n=3$, and bFGF: hMADSC 0 h, 2037 ± 88 *vs* hBM-MSC 0 h, 1113 ± 147, $P<0.05$, $n=3$ and hMADSC 8 h, 2591 ± 151 *vs* hBM-MSC 8 h, 1255 ± 178, $P<0.05$, $n=3$) (Figure 3a and b) and of other stem/progenitor cells (Supplementary Figure IIa and b). On the other hand, hMADSCs did not express significant other angiogenic factors, eNOS mRNA (Figure 3c) and Ang-1 mRNA (Figure 3d), and survival factor, IGF-1 mRNA (Figure 3e) as well as hMSCs differing from other stem/progenitor cells (Supplementary Figure IIc–e), regardless of hypoxic conditions. A notable finding is that hMADSCs exhibited high stem/progenitor chemokine, SDF-1α, mRNA expression with limited downregulation by hypoxic conditions compared with hBM-MSCs (Figure 3f) and other stem/progenitors (Supplementary Figure IIf).

Next, we further confirmed the pro-angiogenic cytokine and stem/progenitor chemokine production from transplanted hMADSCs in ischemic border zone (Figure 4b) 3 days after surgery by double-fluorescent immunostaining. Most of the transplanted hMADSCs detected by hMitC-positive staining (red in Figure 4a, left panels) co-expressed VEGF, bFGF, and SDF-1α (green in Figure 4a, center panels) that could be assessed in the merged images (yellow in Figure 4a, right panels), indicating that transplanted hMADSC secreted these cytokines/chemokine in ischemic myocardium. However, around 50% of the transplanted hNA-positive hMADSCs were positive for TUNEL 3 days after MI, and only a small number of hNA- and UEA-1-double-positive cells could be observed in ischemic myocardium 28 days after MI (Supplementary Figure III).

These results suggest that hMADSCs have a capability of producing pro-angiogenic cytokines such as VEGF and bFGF in acute phase following MI promoting neovascularization, but did not exhibit a massive cell survival in chronic phage following MI in ischemic myocardium. Moreover, high SDF-1α secretion from the transplanted hMADSCs allowed us to focus on endogenous BM-derived stem/progenitor cell recruitment to ischemic myocardium as an additional key player for cardiac regeneration.

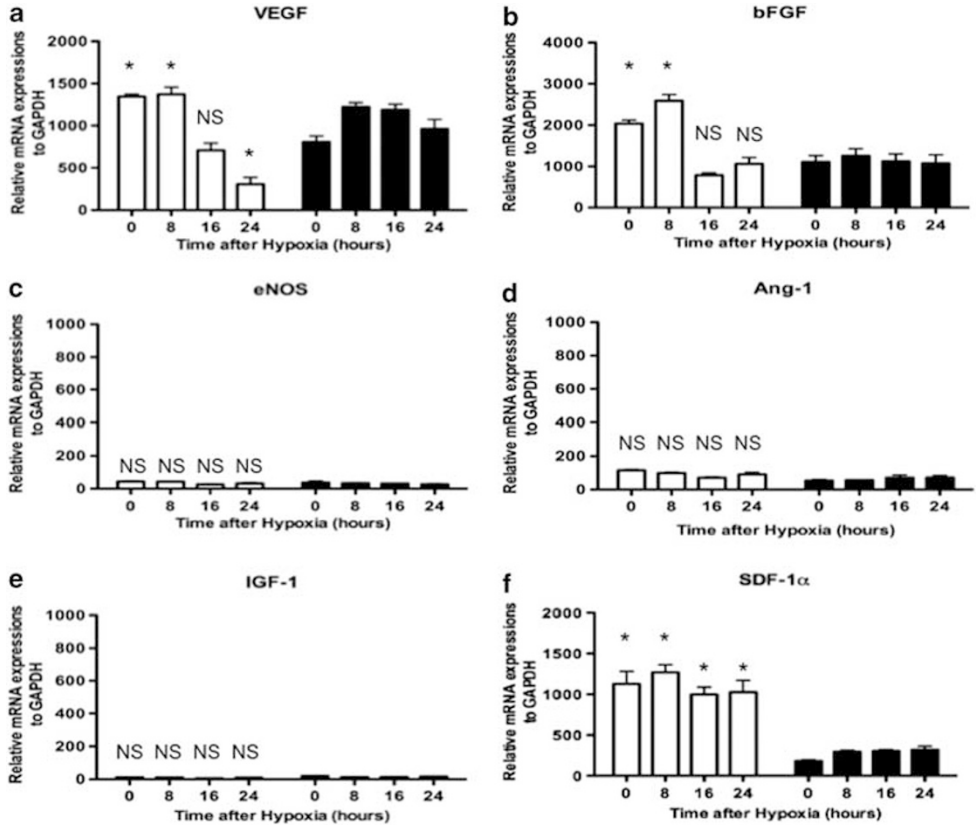


Figure 3 Quantitative real-time reverse transcriptition-polymerase chain reaction (RT-PCR) analyses for cytokine expressions in human multipotent adipose-derived stem cells (hMADSCs) (open bar) *vs* human bone marrow-derived mesenchymal stem cells (hBM-MSCs) (closed bar). Cells were cultured under hypoxic conditions (5% O_2) for 0 (normoxic condition), 8, 16, and 24 h. The mRNA expressions of vascular endothelial growth factor (VEGF) (**a**), basic fibroblast growth factor (bFGF) (**b**), endothelial nitric oxide synthase (eNOS) (**c**), angiopoietin-1 (Ang-1) (**d**), insulin-like growth facor-1 (IGF-1) (**e**), and stromal cell-derived factor-1α (SDF-1α) (**f**) were assessed by amplification of RNA followed by cDNA synthesis with each specific primer. Relative mRNA expressions of the indicated cytokines are normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented in the graph. Nonsignificant (NS) and $^*P<0.05$; hMADCs *vs* hBM-MSCs at each time point after hypoxia. All the assays were triplicated and showed similar results in each assay.

Critical Role of SDF-1/CXCR4 Axis in hMADSC-mediated Endogenous BM-derived Stem/Progenitor Recruitment to Ischemic Myocardium

To assess endogenous BM-derived stem/progenitor recruitment to ischemic myocardium and cardiac apoptosis, hMADSC-transplanted MI heart samples were harvested from the chimera mice with Tie2/LacZ BM 24 h after surgery and analyzed histologically. The Tie2/β-gal-positive cells, namely BM-derived stem/progenitors, were frequently observed in infarct zone (Figure 5b) of the myocardium in hMADSC-transplanted group compared with PBS-injected group (control) (Figure 5a, left panels). In contrast, cardiac apoptosis assessed by TUNEL-positive cell frequency was reduced in hMADSC-transplanted group compared with PBS-injected group (control) (Figure 5a, center panels). Quantitative analysis also exhibited significantly increased number of β-gal-positive stem/progenitors (hMADSC, 29.0 ± 2.0 *vs* PBS, 19.8 ± 0.8/HPF, $P<0.05$, $n=4$) (Figure 5c) and reduced the number of TUNEL-positive cells (hMADSC, 16.8 ± 3.8 *vs* PBS, 61.8 ± 5.9/HPF, $P<0.05$, $n=4$), TUNEL-positive cardiomyocytes (hMADSC, 9.3 ± 1.8 *vs* PBS, 22.5 ± 3.0/HPF, $P<0.05$, $n=4$), and TUNEL-positive fibroblasts (hMADSC, 8.3 ± 1.8 *vs* PBS, 31.3 ± 2.4/HPF, $P<0.05$, $n=4$) in hMADSC-transplanted group than in PBS-injected group (Figure 5d–f).

Finally, we examined the significance of SDF-1α secreted from transplanted hMADSCs in terms of BM stem/progenitor recruitment to ischemic myocardium. *SDF-1* gene was silenced in hMADSCs by siRNA transduction technique and the cells were transplanted to the myocardium immediately after MI induction. The efficacy of SDF-1 gene silencing was evaluated by real-time RT-PCR analysis and ELISA. SDF-1 mRNA expression was significantly reduced in SDF-1 siRNA-transduced hMADSCs by 50% at day 4 and by 60% at day 7 compared with control siRNA-transduced hMADSCs (day 4: 160.5 ± 16 *vs* 295 ± 18, $P<0.05$, $n=3$ and day 7: 84.3 ± 2.5 *vs* 216.6 ± 21.9, $P<0.05$, $n=3$) after siRNA transduction (Figure 6a), and the concentration of SDF-1α protein was also significantly reduced by 30% at day 4 and by 50% at day 7 after siRNA transduction (day 4: 191.1 ± 0.8 *vs* 293.0 ± 18.8, $P<0.05$, $n=3$ and day 7: 264.3 ± 10.9 *vs* 507.7 ± 41.0, $P<0.05$, $n=3$) (Figure 6b). Both SDF-1 mRNA

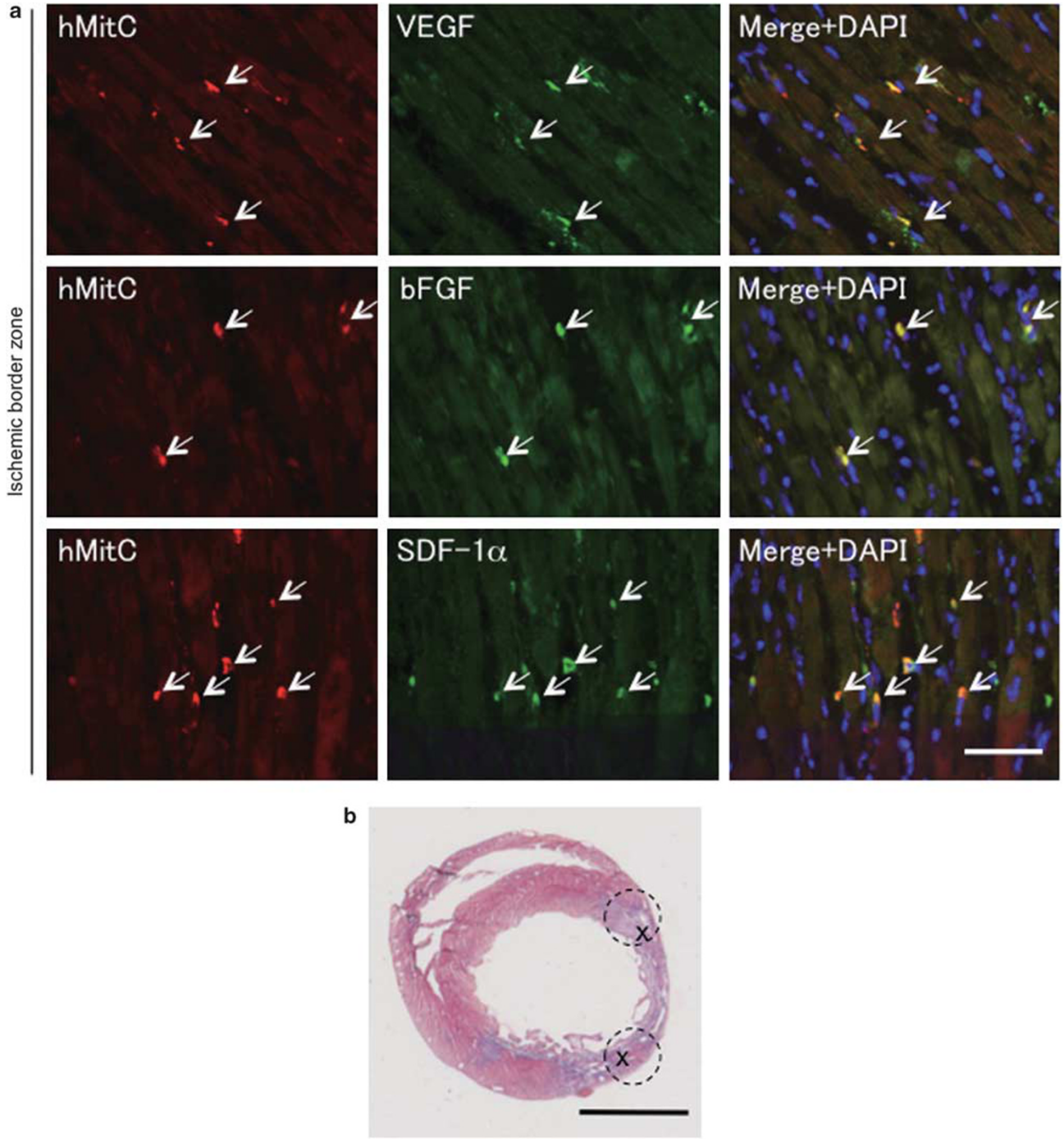


Figure 4 Immunohistochemical analysis for ischemic myocardium following myocardial infarction (MI) with cell transplantation. (**a**) Human multipotent adipose-derived stem cells (hMADSCs) were transplanted to the myocardium immediately after MI and peri-infarct ischemic border zone was examined by double fluorescent immunostaining for human mitochondria (hMitC) and vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or stromal cell-derived factor-1α (SDF-1α) 3 days after surgery. hMitC is indicated in red and VEGF, bFGF, and SDF-1α are indicated in green. Nuclei were counter stained by 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrows indicate double-positive cells in each immunostaining. Bar = 50 μm. (**b**) Masson's Trichrome staining in the hMADSC-treated rat MI heart sample. Ischemic border zone in which the immunostaining was performed is indicated by dotted circles (area) and x (spot in the area). Bar = 5 mm.

expression and SDF-1α protein production in non-transduced hMADSCs (SDF-1 mRNA, day 2: 180.0 ± 15.3, day 4: 263.3 ± 21.9, and day 7: 164.7 ± 13.4 and SDF-1α protein, day 2: 192.7 ± 9.3, day 4: 310.0 ± 15.3, and day 7: 598.0 ± 29.6) exhibited similar levels to those in control siRNA-transduced hMADSCs. We then transplanted either SDF-1 or control siRNA-transduced hMADSCs to ischemic myocardium in chimera mice with Tie2/LacZ BM immediately after MI

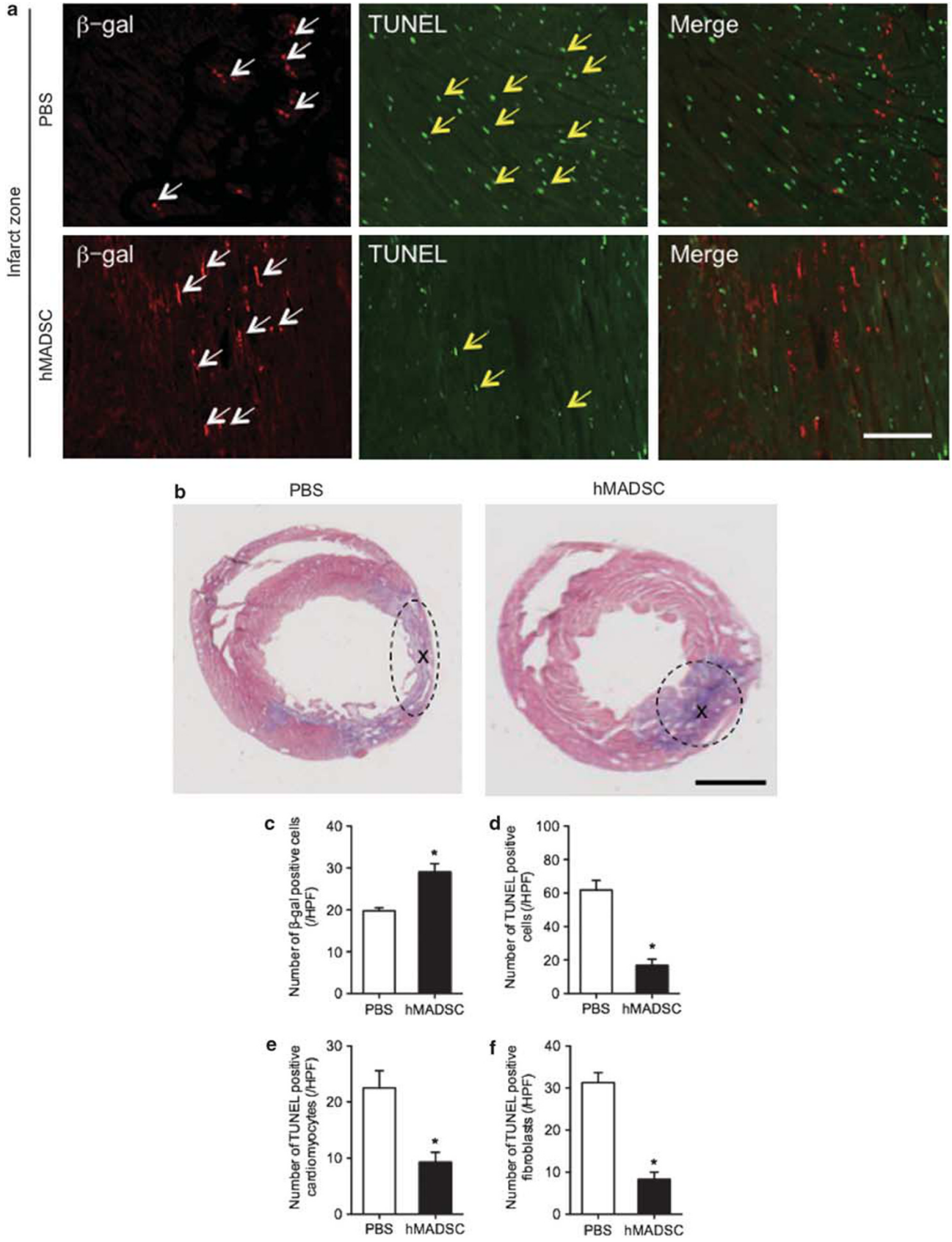


Figure 5 See Caption on next page.

induction, and the heart samples were examined histologically 24 h after surgery. The β-gal-positive stem/progenitors were rarely observed in infarct zone (Figure 6d) of the myocardium in the SDF-1 siRNA group compared with the control group. In contrast, a number of TUNEL-positive cells were observed in the SDF-1 siRNA group (Figure 6c). Quantitative analysis also exhibited significant decreased number of β-gal-positive stem/progenitors (SDF-1 siRNA, 19.3 ± 3.0 *vs* control, 34.0 ± 3.8/HPF, $P<0.05$, $n=4$) (Figure 6e) and increased TUNEL-positive cells (SDF-1 siRNA, 148.5 ± 8.5 *vs* control, 29.0 ± 6.0/HPF, $P<0.05$, $n=4$) (Figure 6f) in the SDF-1 siRNA group compared with control group. However, there was no significant difference of β-gal/TUNEL-double-positive cell number (SDF-1 siRNA, 32.8 ± 4.9 *vs* control, 23.5 ± 4.5/HPF, NS, $n=4$) (Figure6g) between the SDF-1 siRNA group and control group. Consistent with the results of reduced β-gal-positive stem/progenitor recruitment, the percent of fibrosis area in LV was significantly increased in the SDF-1 siRNA group (SDF-1 siRNA, 15.8 ± 1.3 *vs* control, $10.6 \pm 1.0\%$, $P<0.05$, $n=5$) (Figure 6h and i) and capillary density in ischemic border zone was significantly decreased in the SDF-1 siRNA group (SDF-1 siRNA, 242.2 ± 13.3 *vs* control, 311.4 ± 12.3/HPF, $P<0.05$, $n=5$) (Figure 6j and k), suggesting that SDF-1α produced from transplanted hMADSCs have, at least in part, a crucial role for endogenous BM-derived stem/progenitor recruitment to ischemic myocardium with MI size. These findings further suggest that SDF-1α–CXCR4 axis mediates synergistic effect of exogenous transplantation of hMADSCs and endogenous BM-derived stem/progenitor recruitment on tissue preservation in ischemic myocardium. Although the relative contributions of these two mechanisms for myocardial repair cannot be assessed exactly, based on our summarized findings, the extent of both contributions appears to be equivalent (Supplementary Figure V).

DISCUSSION

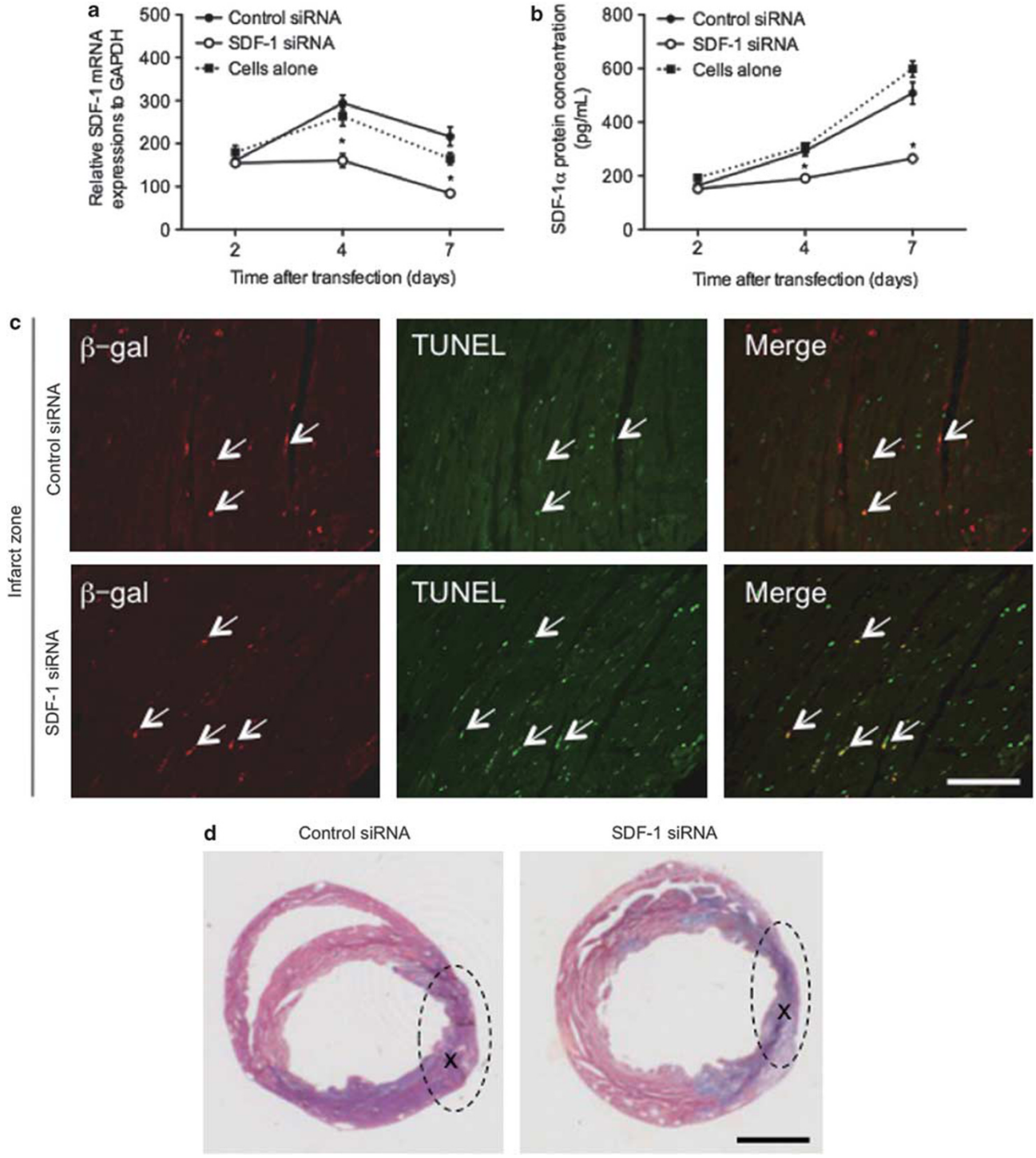
This study provides evidence that adipose tissue-derived stem cells, hMADSCs, have a capability of therapeutic tool for ischemic heart diseases, specifically acute MI by promoting angiogenesis/neovascularization in jeopardized myocardium following ischemic insult. The preserved microcirculation in ischemic myocardium, perhaps by indirect effect of pro-angiogenic cytokine release from the transplanted hMADSCs rather than its direct participation in neovasculature differentiating endothelial cells, contributes to cardiac tissue survival limiting infarct size and therefore results in better cardiac functional recovery. Our *in vitro* data suggest that the transplanted hMADSCs produce at least two major potent angiogenic cytokines, VEGF and bFGF, in ischemic myocardium. These cytokines are known to promote angiogenesis in ischemic tissue and contribute to blood flow recovery in the clinical study[21,22] as well as animal study.[23] On the basis of our *in vitro* data, the cytokine production will be expected to decrease during hypoxic/ischemic condition; however, the initial mRNA expressions of VEGF and bFGF in hMADSCs are quite greater than that in the other stem/progenitors or total MNCs under normoxic condition, suggesting that despite the decline of the cytokines by ischemia, the initial great production or secretion of the cytokines, perhaps, will be sufficient to promote local neovascularization reducing ischemic area in acute phase following MI.

Figure 5 Assessment for endogenous bone marrow (BM) stem/progenitor recruitment and cardiac apoptosis in ischemic myocardium following myocardial infarction (MI) with human multipotent adipose-derived stem cell (hMADSC) transplantation. (**a**) hMADSCs were transplanted to the myocardium immediately after MI and infarct zone was examined by double fluorescent immunostaining for β-galactosidase (β-gal) (red) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (green) in Tie2/LacZ BM transplantation mice 24 h after surgery. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). White arrows indicate β-gal-positive cells and yellow arrows indicate TUNEL-positive cells. Bar = 100 μm. (**b**) Masson's Trichrome staining in the phosphate-buffered saline (PBS)- or the hMADSC-treated mouse MI heart sample. Infarct zone in which the immunostaining was performed is indicated by dotted circles (area) and x (spot in the area). Bar = 1 mm. Recruited β-gal-positive cells (BM-derived Tie2-positive BM stem/progenitors) (**c**) and TUNEL-positive cells (**d**), TUNEL-positive cardiomyocytes (**e**), and TUNEL-positive fibroblasts (**f**) were counted in both sides of ischemic border zone and averaged ($n=4$ in each group).

Figure 6 Effect of stromal cell-derived factor-1 (SDF-1) gene silencing in human multipotent adipose-derived stem cells (hMADSCs) on endogenous bone marrow (BM) stem/progenitor recruitment to ischemic myocardium following myocardial infarction (MI) with hMADSC transplantation. (**a**) hMADSCs were transduced with either SDF-1 small interfering RNA (siRNA) plasmid vector or control siRNA plasmid vector, and SDF-1 mRNA expression was analyzed by real-time reverse transcriptition-polymerase chain reaction (RT-PCR) 2, 4, and 7 days after transduction. Intact hMADSCs were used as another control for the assessment of SDF-1 mRNA expression. *$P<0.05$ *vs* control siRNA. (**b**) SDF-1α protein concentration was measured by enzyme-linked immunosorbent assay (ELISA) in the SDF-1 siRNA-transduced hMADSCs and in the control siRNA-transduced hMADSCs 2, 4, and 7 days after transfection. *$P<0.05$ *vs* control siRNA. All assays were triplicated and showed similar results in each assay. (**c**) Either SDF-1 siRNA- or control siRNA-transduced hMADSCs were transplanted to the myocardium immediately after MI, and ischemic myocardium was examined by fluorescent immunostaining for β-galactosidase (β-gal) (red) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (green) in Tie2/LacZ BM transplantation mice 24 h after surgery. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrows indicate β-gal- and TUNEL-double-positive cells. Bar = 100 μm. (**d**) Masson's Trichrome staining in the control siRNA- or the SDF-1 siRNA-transfected hMADSC-treated mouse MI heart sample. Infarct zone in which the immunostaining was performed is indicated by dotted circles (area) and x (spot in the area). Bar = 1 mm. Recruited BM-derived Tie2/β-gal-positive cells (**e**), TUNEL-positive cells (**f**), β-gal/TUNEL-double-positive cells (**g**) were counted and averaged ($n=3$ in each group). *$P<0.05$ *vs* control siRNA. The percent of fibrosis area (**i**) in entire LV cross-section of Masson's Trichrome staining (**h**) and capillary density (**k**) assessed by immunofluorescent staining with ILB4 (green) (**j**) in ischemic border zone were calculated and averaged 28 days after MI. Bar = 1 mm (**h**) and 100 μm (**j**). *$P<0.05$ *vs* control.

On the other hand, proliferation and antiapoptosis activity of hMADSCs were differentially affected by serum concentration under hypoxic conditions (Figure 2), suggesting that the fate of transplanted hMADSCs might be determined by nutritional circumstances in ischemic myocardium.

Although the transplanted hMADSCs were not frequently observed in the heart samples 28 days after surgery, the survived cells could be differentiated into endothelial cells from the observation in immunohistochemical analyses as the other investigators have also reported the transdifferentiation of adipose-derived stem cells into endothelial cells.[13,24] However, the remote possibility of the transdifferentiation of hMADSCs into cardiomyocytes or smooth muscle cells in ischemic myocardium was noted contrary to



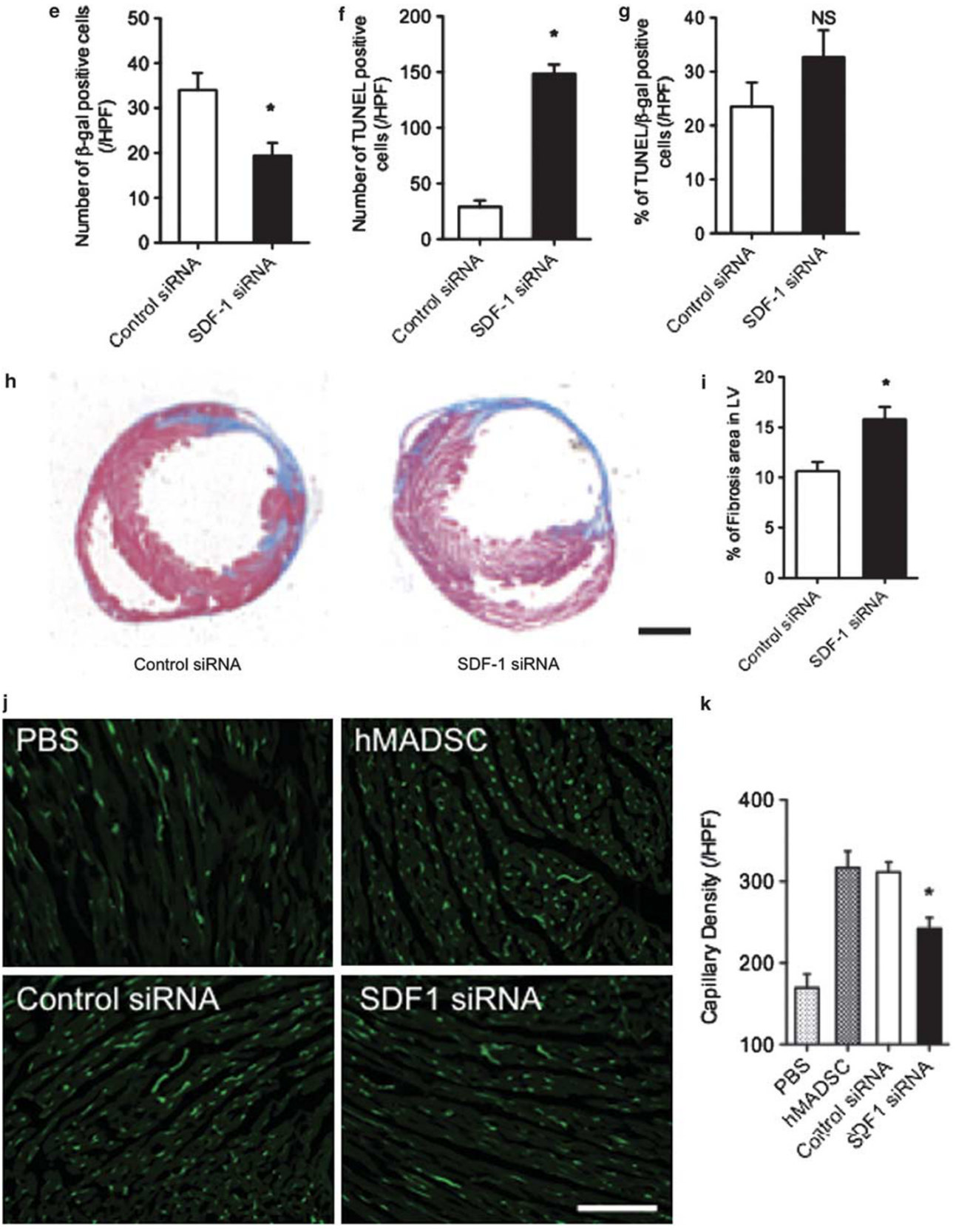


Figure 6 Continued.

recent reports.[10,12,14] Our *in vitro* data exhibited a failure of hMADSC transdifferentiation into cardiovascular cell lineages even under a specific culture condition compared with human cardiac stem cells isolated from patients' heart biopsy sample (Supplementary Figure IV). These data suggest that the transplanted hMADSCs have significant roles as a favorable cytokine donor in ischemic myocardium and have beneficial effects on the cardiac functional recovery following MI.

Apart from the above major effects of hMADSCs on the recovery of myocardium with ischemic injury, notably, the mRNA expression of SDF-1α was upregulated in hMADSCs, and moreover the expression level was not affected by hypoxia differing from that of VEGF and bFGF. SDF-1α is a member of the chemokine CXC subfamily originally isolated from murine BM stromal cells,[25] and hematopoietic stem cell, including endothelial progenitor cells (EPCs)[26] express the receptor CXCR4. SDF-1α and its cognate receptor, CXCR4, are recognized to have key regulatory roles in the trafficking and homing of stem/progenitor cells to the marrow compartment. Also, SDF-1α/CXCR4 interaction induces rapid and effective mobilization of stem/progenitor cells from BM,[27] and is essential for organ system vascularization.[28] On the basis of these evidences, it is speculated that the transplanted hMADSCs could recruit BM-derived stem/progenitors, perhaps EPCs, which is another promising candidate for the angio/vasculogenesis promoter, and might exhibit beneficial effects on ischemic myocardium by the synergistic effect of exogenously transplanted hMADSCs and endogenously recruited BM-derived stem/progenitors (EPCs). Indeed, our SDF-1 gene silencing study exhibited that reduction of SDF-1α production in transplanted hMADSCs resulted in not only less number of endogenous BM-derived stem/progenitor (EPC) recruitment to ischemic myocardium, perhaps, via SDF-1α/CXCR4 axis-dependent mechanism, but also impaired cardiac functional recovery with reduced vascularity and increased scar/fibrosis size in infarct heart following MI (data not shown).

Recently, a number of investigators have isolated pluripotent stem cells from adipose tissue and showed the differentiation into certain lineage cell types, including cardiovascular cells *in vitro*[10,12–14,24] and the favorable effect on MI;[9,16–18] however, little is known regarding the interaction of transplanted donor cells and biological host tissue response, specifically, in the setting of acute MI. In this study, hMADSCs were applied to rat MI model to examine not only the therapeutic effect, but also the potential mechanism, and we have shown the favorable effect of hMADSC transplantation on cardiac functional recovery following MI characterizing the cells with a variety of pro-angiogenic cytokine and stem/progenitor chemokine expressions. Furthermore, we have also revealed the cooperative effect of transplanted hMADSCs with endogenous BM-derived stem/progenitors via an SDF-1α-dependent cell homing mechanism. However, as the hMADSCs are delivered immediately after LAD ligation in this study, whereas in the human clinical setting stem cells are being delivered hours to days after the onset of acute MI, our experimental animal model does not address the more clinically relevant situation. Nevertheless, we believe that our data might give rise to not only a novel mechanistic insight into the research field of ADSC biology, but also a promising candidate for autologous cell transplantation therapy in ischemic heart diseases.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENTS

This work was supported by research funding from Stem Cell Science KK and the Foundation of Biomedical Research and Innovation of Kobe. We would like to thank Stem Cell Sciences KK for providing hMADSCs and the animal facility of RIKEN Center for Developmental Biology for providing the space to perform animal surgery.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Silva GV, Litovsky S, Assad JA, *et al.* Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 2005;111:150–156.
2. Waksman R, Baffour R. Bone marrow and bone marrow derived mononuclear stem cells therapy for the chronically ischemic myocardium. Cardiovasc Radiat Med 2003;4:164–168.
3. Losordo DW, Schatz RA, White CJ, *et al.* Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation 2007;115:3165–3172.
4. Ghodsizad A, Niehaus M, Kogler G, *et al.* Transplanted human cord blood-derived unrestricted somatic stem cells improve left-ventricular function and prevent left-ventricular dilation and scar formation after acute myocardial infarction. Heart 2009;95:27–35.
5. Zhang X, Mitsuru A, Igura K, *et al.* Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering. Biochem Biophys Res Commun 2006;340:944–952.
6. Usas A, Huard J. Muscle-derived stem cells for tissue engineering and regenerative therapy. Biomaterials 2007;28:5401–5406.
7. Marchesi C, Pluderi M, Colleoni F, *et al.* Skin-derived stem cells transplanted into resorbable guides provide functional nerve regeneration after sciatic nerve resection. Glia 2007;55:425–438.
8. Jumabay M, Matsumoto T, Yokoyama S, *et al.* Dedifferentiated fat cells convert to cardiomyocyte phenotype and repair infarcted cardiac tissue in rats. J Mol Cell Cardiol 2009;47:565–575.
9. Mazo M, Planat-Benard V, Abizanda G, *et al.* Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur J Heart Fail 2008;10:454–462.
10. Rodriguez AM, Elabd C, Delteil F, *et al.* Adipocyte differentiation of multipotent cells established from human adipose tissue. Biochem Biophys Res Commun 2004;315:255–263.
11. Rodriguez AM, Elabd C, Amri EZ, *et al.* The human adipose tissue is a source of multipotent stem cells. Biochimie 2005;87:125–128.
12. Strem BM, Zhu M, Alfonso Z, *et al.* Expression of cardiomyocytic markers on adipose tissue-derived cells in a murine model of acute myocardial injury. Cytotherapy 2005;7:282–291.
13. Cao Y, Sun Z, Liao L, *et al.* Human adipose tissue-derived stem cells differentiate into endothelial cells *in vitro* and improve postnatal neovascularization *in vivo*. Biochem Biophys Res Commun 2005;332:370–379.
14. Abderrahim-Ferkoune A, Bezy O, Astri-Roques S, *et al.* Transdifferentiation of preadipose cells into smooth muscle-like cells: role of aortic carboxypeptidase-like protein. Exp Cell Res 2004;293:219–228.

15. Rodriguez AM, Pisani D, Dechesne CA, *et al.* Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J Exp Med 2005;201:1397–1405.
16. Cai L, Johnstone BH, Cook TG, *et al.* IFATS collection: human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. Stem Cells 2009;27:230–237.
17. Bai X, Yan Y, Song YH, *et al.* Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur Heart J 2010;31:489–501.
18. Schenke-Layland K, Strem BM, Jordan MC, *et al.* Adipose tissue-derived cells improve cardiac function following myocardial infarction. J Surg Res 2009;153:217–223.
19. Ii M, Nishimura H, Iwakura A, *et al.* Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via 'imported' nitric oxide synthase activity. Circulation 2005;111:1114–1120.
20. Iwasaki H, Kawamoto A, Ishikawa M, *et al.* Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 2006;113:1311–1325.
21. Losordo DW, Vale PR, Symes JF, *et al.* Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation 1998;98:2800–2804.
22. Laham RJ, Chronos NA, Pike M, *et al.* Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. J Am Coll Cardiol 2000;36:2132–2139.
23. Hughes GC, Biswas SS, Yin B, *et al.* Therapeutic angiogenesis in chronically ischemic porcine myocardium: comparative effects of bFGF and VEGF. Ann Thorac Surg 2004;77:812–818.
24. Martinez-Estrada OM, Munoz-Santos Y, Julve J, *et al.* Human adipose tissue as a source of Flk-1+ cells: new method of differentiation and expansion. Cardiovasc Res 2005;65:328–333.
25. Tashiro K, Tada H, Heilker R, *et al.* Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. Science 1993;261:600–603.
26. Asahara T, Murohara T, Sullivan A, *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275: 964–967.
27. Liles WC, Broxmeyer HE, Rodger E, *et al.* Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood 2003;102:2728–2730.
28. Tachibana K, Hirota S, Iizasa H, *et al.* The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 1998;393:591–594.